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Viral Infection and Immune Response in Early Life and the Influences of Maternal Immunity

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Abstract

Limited capacity in early life to defeat viral infections is initially compensated by the transmission of maternal immunity. Establishing the point in time at which the number of maternal antibodies have decreased to a nonprotective level is critical since the immune system of the progeny only gradually attains its capacity for a functional immune defense. In our search for means to provide immune protection during this period, we have to take into consideration factors such as the maternal influences including antibodies and the immaturity of the offspring's immune system.

In the first part of this thesis, the importance of maternal immunity was assessed in breeding herds suffering from enzootic viral infections. In one herd infected with mouse hepatitis virus (MHV), the consequences of the lack of maternal antibodies were demonstrated. Mice nursed by immunoglobulin (Ig)-deficient dams showed growth retardation and high mortality, and polymerase chain reaction (PCR) revealed MHV nucleic acid in lung cells. No signs of a viral infection were seen in Ig-deficient mice nursed by immune dams. In the second breeding herd, the transmission pattern and kinetics of a parainfluenza type 3 virus (PIV 3) infection were mapped in guinea pig offspring. The knowledge gained in this study provides essential information for the design of an eradication model based on controlled breeding.

Immunization by infection or vaccination during the neonatal period is generally dominated by a T helper 2 (Th2) response and a T helper 1 (Th1) deficiency, with an impaired capacity to defeat viral infections as a consequence. In the second part of this thesis, the conditions for a functionally balanced immune response were explored in an experimental BALB/c mouse model. Sendai virus (SV) envelope proteins in three formulas differing in terms of their adjuvant activity were used. It was shown that the Th1-adjuvanted SV immunostimulating complex (ISCOM) has the ability to prime neonatal mice for a Th1 memory effector function and that neonatal immunization to a large extent influences postnatal immunity. Importantly, later immunizations with antigen supplemented with a potent adjuvant can modify a neonatally polarized immune response. It was also shown that it is possible to prime for a Th1 type of immunity in early life despite the presence of maternal antibodies. It is noteworthy that the Th1/Th2 balance in the dams influenced the postnatal development of immune response in their offspring.

Key words: mouse hepatitis virus (MHV), parainfluenza type 3 virus (PIV 3), Sendai virus maternal immunity, neonatal immunization, immunomodulation, Th1/Th2, adjuvants.

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To my family

Contents

Abbreviations

Ab	antibody
APC	antigen presenting cell
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
Fas-L	Fas ligand
FcR	Fc cell surface receptor
FcγR	Fc receptor for IgG
FcRn	neonatal Fc receptor for IgG
IDO	indoleamine 2,3-dioxygenase
Ig	immunoglobulin
IFN-γ	interferon- γ
IL	interleukin
IL-12R	IL-12 receptor
ISCOM	immunostimulating complex
LGL cell	large granular lymphocytic cell
MBP	mannose-binding protein
MHC	major histocompatibility complex
MHV	mouse hepatitis virus
NK cell	natural killer cell
NO	nitric oxide
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PIV 3	parainfluenza type 3 virus
PRR	pattern recognition receptor
rIFN-γ	recombinant interferon-γ
rIL-12	recombinant interleukin-12
RT-PCR	reverse transcription-polymerase chain reaction
sIg	surface immunoglobulin
SV	Sendai virus
Tc	cytotoxic T cell
TcR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
Th1	T helper cell type 1
Th2	T helper cell type 2
TNF	tumor necrosis factor

Introduction

Birth involves a dramatic transition from a protected intrauterine existence to an exposed life outside the mother's body. During the first period of life, the offspring is protected against environmental microorganisms by passively transferred maternal immunity. With time, however, the maternal immunity gradually vanishes and the offspring is susceptible to the prevailing infections of the population or herd. While the immune system of the offspring matures, the neonate may face dangerous infections. Malnutrition and stress may further undermine the capacity of the young to defeat invading pathogens.

Large-scale transportation, settlement, and time spent in breeding and fattening units are practices of our time that increase the risks of spread of pathogens among individuals of different origin. Immature young individuals lacking a protective maternal immunity against environmental pathogens are most liable to catch a threatening infection. There are therefore several reasons for inducing protective immunity in early life to prevent infectious diseases. The prevalence of maternal antibodies and the immaturity of the immune system are, however, interfering components that have to be considered. Moreover, epidemiological studies indicate that natural infections in early life can have nonspecific beneficial effects on the nature of the immune response to unrelated antigens. For example, in humans, recovery from natural measles infection reduces the incidence of atopy and allergy to half that seen in vaccinated children, suggesting a systemic and nonspecific switch to T helper 1 (Th1) activity [Shaheen et al. 1996]. If a vaccine prevents infection by using a T helper 2 (Th2)-mediated, antibody-dependent mechanism, it may deprive the immune system of the learning experience it would have derived from clearing established infection by the more natural Th1 pathway. Besides preventing specific infection, vaccines should therefore provide an educational stimulus to the immune system [Graham & Stanford 1998].

Part of this thesis deals with the critical importance of maternal antibody protection against viral infections during the neonatal period and the possibilities of, and limitations to, immunizing during early life. The focus will be on the educational capacities of different viral antigen-presenting systems. Part of the thesis also deals with the mother's influence on the development of CD4+ T cells in the young to a Th1 or Th2 pathway. The model systems include natural coronavirus infection in mice and natural infection of guinea pig herds with parainfluenza type 3 virus (PIV 3), as well as an induced immunity in mice by the use of Sendai virus (SV) envelope proteins.

General Background

Herd infections and maternal immunity

During its early life, the neonate is protected from environmental pathogens by passively transferred antibodies from the mother. This is applicable especially to "closed" populations with a settled balance between circulating pathogens and hosts.

Global farming practices, with densely populated areas and large-scale handling and transportation of animals have increased the risks of transmission of microorganisms including viruses to unprotected individuals. Today, young animals from different herds are brought to large units for fattening up, with increased exposure under stress to infections including new pathogens. Under such conditions, the possibilities of preventing transmission of pathogens are limited. The spread of infectious diseases can be controlled by providing the population with adequate protection, including vaccination, adequate breeding programs and housing.

Like the farm animals that are stabled together, laboratory animals caged with animals from other breeding units may be at increased risk of transmission of microorganisms. Since infection of laboratory animals is not only a question of disease, and related suffering from disease symptoms, but also a question of experimental interference, the introduction of pathogens, including viruses, has to be excluded.

In a laboratory breeding unit, if a viral infection does occur, vaccination is generally not applicable and virus transmission has to be stopped by other measures. Within a closed breeding unit, the adaptation of the virus to the host population leads to an endemic situation. Since the viral infection is maintained by a continuous presence of susceptible individuals, mostly young animals whose maternally derived immune protection has decreased to a level of providing no protection, a temporary break in breeding might put a stop to the virus transmission.

The immune system and viral infections

The capacity to recognize infectious agents and respond with appropriate defensive measures is one of the fundamentals of the survival of the individual and the species.

The host defense against pathogens ranges from what are in terms of evolutionary stages "early" *constitutive* or *inducible* mechanisms of the *innate*

system and largely determines whether the adaptive immune response will be dominated by a cellular responses including macrophage activation (a Th1 influence) or by antibody production (a Th2 influence). What is clear is that the interaction between pathogens and the APCs influences the overall balance of cytokines present during the initial proliferative phase of the T cell activation and thus determines whether Th1 or Th2 cells develop preferentially.

The effector and regulatory functions of T cells are guided by cytokines. Interferon-γ, the important cytokine produced by Th1 cells as well as cytotoxic T cells, can block viral replication or even eliminate viruses from infected cells without killing the cells. Interferon-γ also activates the macrophages to produce IL-12, which in turn activates Th1 cells to produce IFN-γ. Thus, an autocrine Th1 cycle is established, which may lead to development of inflammatory reactions that are too strong. Downregulation of the Th1 autocrine cycle and its products is exerted by Th2 cytokines, mainly IL-10 and IL-4. An autocrine Th2 cycle also exists, mainly sustained by IL-4. This cycle is downregulated by IFN-γ produced by Th1 cells. A balanced immune response may, therefore, be obtained when both Th1 and Th2 cell cycles are induced.

Immune interaction between the mother and her offspring

Tolerance during pregnancy

The pregnancy is a period of coexistence between a mother and her immunologically dissimilar offspring. During the gestation period, the fetus is provided with nutrients and shelter, including protection against invading microorganisms. In performance of this task, the immune system of the mother has to remain tolerant to the fetus and yet be able to defend herself and her offspring against foreign pathogens. In the hemochorial placentation seen in humans, and species such as rats and mice, the fetal trophoblast cells are quite close to the maternal leucocytes in blood and tissues. Shortly after blastocyst implantation, the decidua of the mother is invaded by monocytes and uterine NK cells of the maternal innate immune system [Adkins 1999; Hunt et al., 2000; Sacks et al., 1999], an event which is thought to be linked to species with hemochorial placentas [Sacks et al., 1999]. This milieu of local inflammation at the maternal-fetal interface, i.e., in the decidua of the mother and the trophoblast cells of the fetus, plays a pivotal role and may shape the context in which maternal T cells encounter fetal alloantigen [Mellor & Munn 2000]. This unique immune relation between the mother and her fetus includes several interacting mechanisms.

other cell surface molecules such as CD3 and MHC. In addition, co-stimulating factors, such as CD80, CD86, and CD40 of APCs are required. The binding of these cell-surface molecules to their corresponding ligands CD28, CTLA-4, and CD40 ligand (CD40L) of the lymphocytes constitutes the go-ahead signal to the expansion of T cell clones specific to the pathogens involved. Thus, the innate immunity influences the initiation and type of adaptive immune response by regulating the T cell costimulatory activity and antigen presentation [Janeway et al., 1999].

The adaptive immune system

The two major classes of lymphocytes, the B and T lymphocytes, carry antigen-specific receptors on their cell surfaces. As described above the T lymphocytes express a receptor, TcR, that is associated with an accessory molecule, CD3, which serves as a signal transducer for the TcR [Springer 1990].

The B lymphocyte has a specific antigen-binding receptor, surface immunoglobulin (sIg), which, in contrast to the TcRs, interacts directly with viral proteins or virions. Besides the sIg receptors, the B lymphocyte also carries receptors for complement and Fc receptors for immunoglobulin (Ig), factors which all interact in the initiation of the Ab response.

The T lymphocytes are functionally classified into two subsets: *Th lymphocytes* and *cytotoxic T (Tc) lymphocytes* or *killer cells*. The *Th lymphocytes* carry a cell surface molecule CD4 [Davies & Björkman 1988; Springer 1990] which interacts with TcR when the latter specifically recognizes viral peptides presented by MHC class II molecules of APCs. The *Tc lymphocytes* carry the CD8 cell surface molecule which together with the TcR binds to the MHC I molecule when viral peptides are presented on the cell surface in association with MHC class I molecules [Murphy et al, 1999].

Viruses or viral proteins that are taken up via the endosomal pathway of APCs are degraded by proteolytic enzymes active at the low pH of prelysosomes. The antigens are processed to peptides in the range of 15–25 amino acids and are taken up by the MHC class II molecules for further presentation to lymphocytes. During the antigen-processing stage, the APCs migrate to the T cell zones of the peripheral lymphoid organ, where the processed peptides are presented by the MHC class II molecule to recirculating naive CD4 T lymphocyte clones.

The moment when the CD4 T lymphocytes encounter their specific antigen in the form of the peptide-MHC complex, the T cells are activated to proliferate and differentiate into two classes of effector T cells, the Th1 and Th2 cells, or to memory T cells. It should be noted that the go-ahead signal of the co-stimulation, as discussed above, is required. The step at which naive CD4 T cells become either Th1 or Th2 cells is guided by the cytokine profile of the innate immune

recognition by cytotoxic T lymphocytes makes the infected cells susceptible to attack by NK cells [Biron & Brossay 2001].

The *complement system* is an early actor of the innate immune system against viral infections. This system is present in systemic fluids but not on mucosal surfaces. It is ready to immediately target and eliminate virus particles and interact with the surface of virus-infected cells to mark them for destruction by other branches of the immune system [Lachmann & Davies 1997].

Cytokines and *chemokines* are essential mediators and regulators for the development of immune responses and inflammation. Examples of early regulatory proinflammatory cytokines are tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6. These cytokines, which are important for initiation of the response, are assisted by cytokines such as IL-12, IL-15, and IL-18 for the up-regulation of a Th1-type response, while IL-4, IL-10, and also prostaglandin E_2 (PGE_2) and transforming growth factor β (TGFβ) have properties which help guide towards a Th2 type of response. It should be noted that some cytokines of the innate system are also produced by the adaptive system. Interleukin-4 and IL-10, for example, are produced both by various cells of the innate system and by Th2 lymphocytes of the adaptive system. The activation of macrophages and DCs by infectious agents leads to secretion of IL-12, which subsequently induces interferon (IFN)-γ production of NK cells. Interferon-γ in turn acts on monocytes to augment IL-12 secretion and produce nitric oxide (NO), which eradicates infectious microbes [Ohteki & Koyasu 2001]. Interleukin-12, via the activity of IFN-γ, and TNF-α mediate a potent antiviral activity including a capacity to directly suppress virus replication [Ramshaw et al., 1997].

Protective mechanisms of the innate system also include the production of *IFNs* of the type I of the IFN-α/β superfamily constituting an initial response to many viral infections of mammalian host cells. These antiviral proteins block the spread to uninfected cells and activate NK cells to selectively kill virus-infected cells.

Interaction between the innate and the adaptive immune responses

Following an infection with a virus, viral proteins or antigens are processed and displayed on the cell surface of APCs as bimolecular complexes of peptides and MHC molecules, to be presented to T cell receptors (TcRs) on specific T lymphocyte clones. The APCs include members of both the innate system, i.e., DCs and macrophages, and the adaptive immune system, i.e., the B cells.

The pathogen derived antigens processed by the APCs of the innate immune system are transported by MHC class 2 molecules to the surface of the APCs and presented to TcRs of selected clones of T cells of the acquired (i.e., adaptive) immune system. The antigen presentation is guided by the TcR together with

immunity to the "late" *adaptive immune system* that is specifically induced in response to microbes including viral antigens [Medzhitov & Janeway 2000].

Aspects of the innate immunity

The mechanisms protecting multicellular organisms against invaders that appeared early in the evolution constitute the so-called "innate" or "natural" immunity. The *constitutive* type of the innate immunity includes the barrier functions of the body's surfaces, antimicrobial peptides produced by surface epithelial cells, and antibacterial enzymes present in bodily fluids such as lysozyme. The *inducible* part of the innate immune system is provoked when molecular patterns present on microbial pathogens, the pathogen-associated molecular patterns (PAMPs), are recognized by cellular receptors of the innate immune system called "pattern recognition receptors (PRRs)" [Janeway 1989; Medzhitov & Janeway 1997]. Pattern recognition receptors are also secreted by the cells into the bodily fluids in a soluble form. The soluble mannose-binding protein (MBP) and the cell-bound mannose receptor are examples of PRRs. The cell-bound mannose receptor is the most commonly described PRR. It is found on resident macrophages and dendritic cells (DCs), and has a unique capacity to bind and mediate internalization both by endocytosis and by phagocytosis. The opsonin activity of the soluble MBP gives this protein a role as a broad spectrum antibody and a promoter of antigen uptake by antigen-presenting cells (APCs). The recognition of PAMPs by the receptors of certain cell populations e.g. APCs and also epithelial cells allows the innate immune system to distinguish pathogen-associated nonself-structures from self-molecules; however, this is probably not the only strategy of innate immune recognition. Other strategies used by the innate immune system include recognition of altered self and the recognition of the absence of self, as in the case of major histocompatibility complex (MHC) class I recognition by natural killer (NK) cell receptors [Lanier 1998; Long 1998].

The cells mainly involved in the innate immune system derive from a myeloid progenitor and include granulocytes, macrophages, DCs, and mast cells. The neutrophil (a granulocyte) and the macrophage are the two major *phagocyte* populations of the immune system and play an important role in innate immunity. The macrophages occur throughout the tissues of the body, while the neutrophils are abundant in the blood but absent from the other tissues. The neutrophils are the most numerous component of the innate immune response.

The *NK cells* are specialized non-T, non-B lymphoid cells that can contribute to the innate defense against viral infections by their rapid response to early events following challenge. Although the NK cells lack antigen-specific receptors they have a capacity to detect and attack certain virus-infected cells: the downregulation of surface MHC class I expression by many viruses to escape

Protective strategies of the fetal trophoblast cells to escape rejection

To escape maternal immune rejection, the antigen presentation of fetal trophoblast cells differs from that of a tissue allograft inasmuch as the MHC class I expression is drastically limited. This means that the MHC I antigens are not expressed on the cell surface of the labyrinth trophoblast cells [Bulmer & Johnson 1985; Chatterjee-Hasrouni & Lala 1979, 1981 1982; Head 1991; Philpott et al., 1988; Redman et al., 1984], and the MHC class II molecules are not at all expressed by cells of fetal origin at the fetal-maternal [Chatterjee-Hasrouni & Lala 1981; Bulmer & Johnson 1985; Hunt 1992; Mattson 1998; Ober 1998; Rodrigues et al., 1997].

The expression of Fas ligand (Fas-L) in trophoblast cells has been suggested to contribute to nonrejection of trophoblasts. A Fas-dependent deletion mediated by the trophoblast cells induces apoptosis of Fas-positive maternal immune cells at the maternal-fetal interface [Jiang & Vacchio 1998; Sakata et al., 1998; Weetman 1999]. Another protective mechanism of the trophoblast to prevent rejection includes the expression of the complement regulatory proteins CD46, CD55, and CD59 [Holmes & Simpson 1992; Weetman 1999;].

Blastocyst implantation and maternal hormonal influences

Already at ovulation, the uterus is prepared for blastocyst implantation through changes in the metabolism, and the hormone and cytokine balance. These changes are fundamental to the development of maternal immune tolerance to the fetus.

The antigen presentation by uterine cells is under hormonal control and varies, depending on the stage of the estrous cycle [Prabhala & Wira 1995]. The unique mixture of hormones and cytokines seen at the time of blastocyst implantation strongly influences the nature of the immune response during pregnancy [Piccinni et al., 1995; Prabhala & Wira 1995; Rukavina & Podack 2000]. To what extent the local uterine T cell function and the Th1/Th2 cytokine environment are influenced by hormones in humans is not yet clear [Dealtry et al., 2000]. However, it is well known that a sufficiently high level of progesterone production is critical to the maintenance of pregnancy. Studies in mice have shown that both progesterone and PGE_2 suppress the T cell differentiation into Th1 and also have the capacity to act directly upon the T cells to switch their cytokine production towards the Th2 type [Kraan et al., 1995; Miyarua & Iwata 2002; Piccinni et al., 1995]. Prostaglandin E_2 strongly induces IL-10 production by macrophages [Kraan et al., 1995;], while progesterone enhances the induction of IL-10-producing Th2 cells [Miyarua & Iwata 2002]. Interleukin-10 in turn inhibits production of IL-12 and MHC class II antigen expression of macrophages, monocytes, and DCs [Moore et al., 2001]. Interleukin-10 treatment of DCs may also induce antigen-specific anergy of CD4+ cells. Thus, the

existence of a complex network of regulatory pathways influenced by steroid activity contributes to the explanation of fetal allograft survival [Piccinni et al., 1995].

Microenvironment of the uterus

Other mechanisms that contribute to the specific immunity of pregnancy include the expression of indoleamine 2,3-dioxygenase (IDO). This enzyme catabolizes tryptophan and thus provides suppression of maternal allospecific T cell activation [Miyarua & Iwata 2002]. The interaction of the cell surface molecule Fas (CD95) and Fas-L leads to apoptosis of cells that express Fas [Sakata et al., 1998]. While Fas is expressed on different types of cells, Fas-L is expressed on cells of immune-privileged tissues, such as cells of the cornea [Strelein 1995]. Fas ligand is also expressed on fetal trophoblast cells. The interaction between the trophoblast Fas-L molecules and the Fas molecule of male antigen-specific CD8+ T cells leads to the deletion of the latter.

The T helper 2-type cytokine pattern during pregnancy

The most striking feature of the antigen-specific tolerance during pregnancy is the avoidance of cell-mediated immunity against fetal alloantigen [Lin et al., 1993]. At the time of implantation, the innate immune response is activated, as indicated by the influx of high numbers of maternal macrophages, NK cells, and large granular lymphocytic (LGL) cells at the maternal-fetal interface [Bonney & Matzinger 1997; Dealtry et al., 2000]. The composition of hormones and cytokines and the nature of the fetal "cell invasion" determine the innate immune reactions of the mother during pregnancy and, in turn, the context in which maternal T cells respond to fetal alloantigens [Mellor & Munn 2000; Prabhala & Wira 1995; Rukavina & Podack 2000]. In mice and humans, a shift in cytokines occurs during pregnancy, from pro-inflammatory cytokines leading to Th1-type inflammatory response to anti-inflammatory and antibody-stimulating Th2 cytokines [Dealtry et al., 2000]. The prevailing cytokine environment at the time of T helper 0 (Th0) cell stimulation is therefore characterized by Th2-driving cytokines. There are divergent hypotheses on whether the shift from Th1-type to Th2-type cytokines is confined to a local maternal response or whether it also influences the systemic maternal immune response [Guilbert 1996; Raghupathy 1997; Wegman et al., 1993]. In any case, a local Th2-type environment of the placenta is essential to regulate the function of locally activated cells of the innate immunity and any infiltrating lymphocyte [Dealtry et al., 2000].

Transmission of maternal immune protection

The immunity of the mother is transferred to the fetus and newborn through the transmission of her antibodies. The time of transmission varies from species to

species. Human offspring are born with immunoglobulin G (IgG) obtained via the placenta, whereas in mice and rats, most of the IgG is obtained postnatally from colostrum and milk [Brambell 1970].

The antibodies interact with cells of the immune system via the Fc of the antibody and the Fc cell surface receptor (FcR). For each antibody, there is a Fc receptor. For instance, IgG binds to the Fc receptor FcγR. The transfer of maternal IgG via intestinal cells of neonatal mice and rats is mediated by the Fc receptor FcRn. The neutralizing maternal antibodies may not always eliminate an infectious agent but they do attenuate infection during the initial period of life. Thus, they contribute to optimizing conditions for natural immunization in early life [Zinkernagel 2001].

The neonatal immunity

The immune system of the newborn is functionally different from that of the adult, with greater susceptibility to intracellular pathogens and poor response to vaccine antigens [Goriely et al., 2001]. The Th2 bias of the neonatal immune response was first described in the early 1990s by Streilein and colleagues [Powell & Streilein 1990]. Since that time, several studies have delineated the neonate's immunity and its characteristic features.

The neonatal allogeneic response

There are numerous reports on how the neonate's immunity differs from that of an adult by its Th2-biased immune response. The patterns of certain viral infections in early life strongly suggest that impaired T cell response limits viral clearance *in vivo* [Kovarik & Siegrist 1998a)]. Response to vaccination in newborn mice has clearly shown to be Th2-skewed under conditions which induce Th1-type response in adult animals [Bot et al., 1997a) b); Bot et al., 1998; Goriely et al., 2001; Martinez et al., 1997]. It has also been reported that animals initially immunized as neonates show a Th2-dominated memory response [Adkins & Du 1998].

Since the early 1950s, it has been known that neonates are prone to tolerization [Billingham et al., 1953]. Later, this induction of tolerance in early life was shown to be associated with the development of alloantigen- or peptide antigen-specific Th2 cells [Forsthuber et al., 1996; Schurmans et al., 1990; Singh et al., 1996]. Zaghuani et al. describe the long-term effect of neonatal immunization, seen as a Th2-polarized response in adults originally tolerized to peptide antigens as neonates [Min et al., 2000a), b); Min et al., 1998].

Animals immunized as neonates are therefore impaired in their capacity to generate Th1 memory effector function following reimmunization. Moreover, the Th2-biased neonatal immune response does not only persist but it also influences

the outcome of vaccination in adult life [Barrios et al., 1996; Bot et al., 1997a), b); Adkins & Du 1998]. Adkins et al. report regional differences with regard to the attenuated memory effector function in neonatal mice. The primary splenic responses of neonates are exclusively Th2-like, whereas neonatal lymph node T cells mount mature mixed Th1/Th2-like primary responses [Adkins et al., 2000; Adkins et al., 2001].

How to circumvent the basic T helper 2 bias

Cell-mediated immunity is important in recovery from viral infections and humoral responses are pivotal for protection against viral challenge [Arulanandam 2000]. Thus, there is a need for a strong cell-mediated immune response to clear viral infections, not least in neonatal life. However, the protective value of the Th2 response has to be realized. Induction of successful, balanced responses by immunizations in early life with the use of Th1-driving adjuvants, such as CRL8491 [Barrios et al., 1996; Donckier et al., 1998; Forsthuber, 1996], low-antigen doses, or DNA vaccines has been reported [Bot et al., 1997a) b); Bot 1998; Kovarik & Siegrist. 1998 b); Martinez et al., 1997; Sarzotti et al., 1997; Wang et al., 1997;].

Dendritic cells and their function in early life

Antigen-presenting cells and NK cells determine the functional outcome of most responses to pathogens [Ohteki & Koyasu 2001]. The DCs play an essential role as APCs for the priming of naive T cells and for their differentiation into Th1 cells through the synthesis of IL-12 [Macatonia 1995]. Goriely et al. report that the DCs of human newborns are profoundly deficient in the synthesis of the bioactive form of IL-12, thus contributing to a deficient production of IFN-γ. This group also showed that IFN-γ restores the capacity of neonatal DCs to produce bioactive IL-12 [Goriely 2001].

Interleukin 12, a crucial component of host defense against viral infections

Bioactive IL-12 (p75) is a heterodimeric cytokine composed of two subunits, p35 and p40, encoded by different genes. Both p35 and p40 must be expressed in the same cell to generate the bioactive form of the cytokine [Goriely 2001]. Interleukin-12 is mainly produced by DCs, macrophages, and neutrophils. The major cellular targets of IL-12 are T, NK, and B cells. Interleukin-12 induces IFN-γ secretion, promotes growth of activated T and NK cells, modulates immunoglobulin E (IgE) synthesis, and induces commitment from Th0 to the Th1 phenotype [Lamont & Adorini 1996]. In the mouse, IL-12-induced IFN-γ production is required to maintain the expression of the high-affinity IL-12 receptor (IL-12R) on primed cells and is, therefore, essential not only for the development but also for the maintenance of Th1 effectors [Szabo et al.,

1995]. Since only Th1 cells express the β2 chain of the IL-12R, this would account for the incapability of Th2 cells to respond to IL-12. The consequence of this difference between Th1 and Th2 cells was demonstrated in an experiment on TcR-transgenic T cells, which showed that Th1 can revert to Th2 cells. By contrast, Th2 cannot revert to Th1 as a result of the early commitment of Th2 cells to a stable phenotype [Szabo et al., 1995].

The production of IL-12 by DCs, triggered by exposure to microbial products or interaction with activated T cells, plays a critical role in the induction of Th1 responses [Goriely 2001; Macatonia et al., 1995; Trinchieri 1995]. Goriely et al. found that neonatal DCs are profoundly deficient in the synthesis of the bioactive dimeric form of IL-12 (p75) [Goriely 2001]. By using a real-time PCR technique, they showed a major defect in the expression of the IL-12 (p35) gene in neonatal DCs, whereas the expression of the IL-12 (p40) gene was similar to that of adult DCs. When rIFN-g was added to lipopolysaccharide (LPS)-stimulated newborn DCs, both their expression of IL-12 (p35) and their synthesis of IL-12 (p70) were restored to adult levels. It was also shown that neonatal DCs are less efficient than are adult DCs in inducing IFN-γ production of allogenic adult CD4+ T cells, a defect that could be restored by addition of rIL-12.

CD40 ligand expression in neonatal life

The mechanisms of suppressed autoimmunity involve IL-4-producing lymph node T cells and a failure of splenic T cells to proliferate or produce IFN-γ in response to antigens, a mechanism termed "IFN-γ/IL-12-dependent anergy" [Min et al., 2001].

The interaction between CD40 and CD40L drives IL-12 production by APCs and promotes differentiation to Th1 during priming of T cell responses [Campbell et al., 1996; Min et al., 2001; Stuber et al., 1996]. In the neonatal stage, T cells display a quantitative defect in the expression of CD40L [Flamand et al., 1998; Min et al., 2001]. Depending on the type of APCs, the insufficient CD40L expression on the lymphocyte population may cause minimal IL-12 exposure leading to a deviated response at reexposure to the antigen later in life. Min et al. provide evidence that IFN-γ-dependent T cell anergy arises as a consequence of defective expression of CD40L on the lymphocytes and that an expression of functional IL-12R for IL-12 is required to promote the progress of T cells from proliferation to IFN-γ production [Min et al., 2001].

Why is there a T helper 2 bias in neonatal life?

As has been mentioned, the neonatal susceptibility to tolerance induction has been well documented since the early 1950s, when Medawar and colleagues [Billingham et al., 1953] showed that rodents injected at birth with allogeneic cells were able to accept transplants from the cell donor in adult life. Today, we

know that tolerance in the murine neonate is associated with the development of alloantigen-specific Th2 cells [Adkins 1999; Forsthuber et al., 1996; Schurmans et al., 1990; Singh et al., 1996] but also, that neonates have a potential for mounting effective adult-like T cell responses when the conditions during antigenic priming are optimized [Forthuber et al., 1996; Garcia et al., 2000 Ridge 1996; Sarzotti, et al., 1996]. However, the clear Th2 bias in neonates is likely to be important during the neonatal period of life. Adkins et al. point to the fact that the vast majority of T cells are newly generated and encounter many peripheral antigens not present in the thymus. Tolerance to these self-antigens must therefore first be established during the neonatal period [Adkins et al., 2001].

The present study

Aims of this work

The objective of the present work was to establish how the mother's immunity influences the susceptibility of her offspring to viral infections during the maturing of their immune system.

In the second part of the present work, the prerequisites to protective immunizations in early life are explored.

More specifically, the studies aimed to
- explore the pattern of an endemic viral infection in breeding herds and the importance of maternal antibody protection
- investigate the possibilities of inducing a Th1/Th2-balanced immune profile by neonatal immunization
- investigate how neonatal immunization influences the immune response at a secondary immunization in adult age
- investigate the influences of maternal immunity on neonatal immunization.

Comments on experimental design and methods

Materials and methods are described in the papers included in the thesis. For detailed information, I refer to the "Materials and Methods" section in each paper.

The significance of maternally derived immunity for early life protection was explored in herds of laboratory rodents with an ongoing virus infection (Studies I and II).

Study I was initiated when health investigations revealed infection with MHV as a probable cause of growth retardation and mortality in neonatal mice. To analyze the protective role of a specific antibody, the breeding of B cell-deficient

C57BL/6 mice was established by transferring embryos into pseudopregnant dams kept in an MHV-free environment. The health status of neonatal mice raised in the MHV-free environment was thereafter compared with that of neonatal mice raised in an MHV-infected environment. The prevalence of the virus was explored by use of a RT-PCR for the envelope gene E1 of MHV, whereas antibodies against MHV were detected by enzyme-linked immunosorbent assay (ELISA).

To explore protective effects of maternal antibodies in neonatal mice exposed to MHV, cross-fostering experiments were conducted using foster dams of four different strains: B cell-deficient, MHC class II-deficient, β_2-microglobulin-deficient, and normal C57BL/6 mice.

In Study II, transmission rate, course, and kinetics of an ongoing PIV 3 infection in breeding herds of guinea pigs were explored. In the first instance, virus isolation was the method of choice to demonstrate the patterns of virus transmission. Thus, we tried to isolate the virus on primary cell cultures prepared from lungs of guinea pig embryos. We also used PCR-based analyses; however, all virus isolation failed. The failure could be attributed to practical conditions, such as the lengthy period of time between autopsy and virus isolation and/or the presence of virus-neutralizing antibodies at virus isolation. The failure may also be ascribed to a very short period of virus prevalence after infection, as has been demonstrated in an experimental virus infection study, in which PIV 3 could be isolated only on one occasion, namely, on postinfection day 4 [Graziano et al., 1989]. Contributing to the difficulty in isolating the virus was also the fact that the time at which the individual young pig is susceptible to an infection varies according to both initial (maternally derived) antibody levels and herd immunity, which makes it difficult to determine the exact time at which the pig will be susceptible to a viral infection. As an alternative to virus isolation, we based our conclusions regarding the time of viral infection on the time of an increase in the antibody level in serum samples. Increased antibody levels after a period of declining antibody titers after birth was taken to indicate viral infection. The risks of virus transmission were evaluated at an age at which guinea pigs are usually delivered to experimental units. The seroconversion was estimated after caging guinea pigs from a PIV 3-positive breeding unit with PIV 3-free guinea pigs.

In Studies III and IV, the development of Th1 and Th2-type immune responses was analyzed. The Th1 type of response was represented by the IFN-γ production estimated after *in vitro* restimulation of spleen cells collected at different time points after immunization. The Th2 type of response was estimated by measuring IL-5 production. One and the same preparation of SV envelope proteins was used for different antigen presentation systems to avoid variations due to the antigen.

From birth to about 28 days of age, the production in baby mice of nonsensitized mothers of IFN-γ gradually increases to levels equivalent to levels

in adults [Herz et al., 2000]. Thus, the immunity in young mice undergoes a natural shift of the Th1/Th2 balance towards Th1. Since there is some uncertainty concerning the time after birth at which this occurs, the effects of different antigen-presenting systems on the outcome of neonatal immunization were compared after a secondary immunization at adult age.

In Study IV, the influence of maternal immunity was evaluated in offspring born to mice immunized with one of three antigen presentation systems: (1) immunostimulating complexes (ISCOMs) containing SV envelope proteins (SV-ISCOMs), with capacity to drive a Th1 response (IFN-γ); (2) $Al(OH)_3$-adjuvanted micelles of SV envelope proteins (SV-$Al(OH)_3$) for the induction of a Th2-stamped immune response; or (3) micelles of SV envelope proteins (SV-MICs), without any adjuvant.

If the Th2-driving adjuvant $Al(OH)_3$ is used in neonatal immunization, the natural Th2 bias is further enhanced. To avoid experimental readouts within a Th2-biased immune system, ISCOMs containing SV envelope proteins were used for the neonatal immunizations in Study IV to obtain a Th1/Th2-balanced immune response.

Results and discussion

The herd immunity reflects the equilibrium between susceptible and immune individuals in a certain population [Zinkernagel 2001]. This balance is maintained by the mothers whose immunological experience is transmitted to the next generation. Thus, the newborns of the herd can be protected in early life, during which time their own immune system matures.

Endemic viral infections and the protective significance of maternal antibodies (Papers I and II)

In Study I, the critical importance of maternal antibodies to protecting the newborn against disease was demonstrated in mice exposed to an infection which eventually was diagnosed to be caused by MHV. In a laboratory animal unit of mice, offspring born to Ig-deficient dams suffered from growth retardation and high mortality when kept under conventional conditions. By contrast, no disease symptoms were seen in offspring born to immune-competent mothers. It was also noted that the occurrence or absence of disease symptoms did not depend on whether the offspring were immune-competent or suffered from an Ig deficiency. These observations strongly suggest that maternal antibodies transferred from the immune-competent mothers protected their offspring against disease.

Health monitoring of the mice revealed a herd infection with MHV. Mouse hepatitis virus is a corona virus in mice, which is quite often seen in breeding herds of laboratory mice. In general, the course and severity of an infection with MHV varies from strain to strain of mice and/or viruses and is also dependent on the immune status of the individual animal. However, as a result of the herd immunity, the symptoms are often subclinical without any clear signs of an ongoing herd infection.

A colony of Ig-deficient control mice reared under specific pathogen-free (SPF) conditions was established by embryo transfer to a SPF unit. The embryos were taken from mothers of the infected herd. No MHV antibodies were detected in immune-competent mice raised in the SPF unit. The lack of disease symptoms in newborns born to the Ig-deficient dams in this SPF breeding unit confirmed that the infection had not been transferred by the embryos and also, that the offspring of the conventional unit probably were infected by MHV when nursed by Ig-deficient dams.

Polymerase chain reaction analyses revealed that MHV was prevalent in the lungs of neonates nursed by Ig-deficient dams in the conventional unit. On the other hand, lung tissues from control neonates fostered by immune dams in the same environment were MHV-negative strongly suggesting that maternal immunity protected the offspring.

The significance of maternal antibody protection against infection was further demonstrated when milk containing high titers of MHV antibodies provided for 8 days or more completely prevented Ig-deficient neonates in the conventional unit from developing a wasting syndrome.

Study II was initiated after unexplained experimental variations were seen in airway responsiveness during respiratory experiments in guinea pigs. Serological investigations revealed antibodies to PIV 3 as a common finding in the breeding colonies of origin and a survey was initiated to investigate the course and transmission rate of the suspected infection in selected herds. No clinical signs of an ongoing infection could be demonstrated in any of the herds in question although it was obvious that a subclinical disorder caused experimental disturbances when the guinea pigs were transferred and used for animal experimentation.

A similar respiratory syndrome, called "shipping fever", is well known in cattle and sheep and occurs as a consequence of stress-inducing factors, such as transportation, often in combination with PIV 3. It was, therefore, assumed that the experimental disturbances were due to an acute infection with PIV 3 in combination with stressful conditions during transportation and experimentation.

Guinea pigs, like humans and rabbits, are born with maternally derived IgG which initially protects them against environmental pathogens. Since virus isolation efforts failed (see "Comments on Experimental Design and Methods"), an alternative approach was to explore the approximate duration of maternal protection against infection. The time of infection was estimated by analyzing the course and kinetics of antibody titers against PIV 3. Blood samples for serum antibody titers were collected from guinea pigs stabled with their original PIV 3-positive breeding colony and also, from a group of guinea pigs relocated and separately housed from 2 weeks of age to 13 weeks of age. The guinea pigs remaining in the breeding colony as well as those removed and housed separately showed declining serum antibody titers for about 1 month after birth; thereafter, the titers were stable until about 8 weeks postnatally. Five weeks later, the mean antibody titer of the guinea pigs remaining in the breeding colony had increased to a significantly higher level than that of the relocated and separately housed guinea pigs. Since the mean antibody titer of the isolated group continued to decrease until the end of the experiment, we concluded that young guinea pigs born to PIV 3-positive mothers are protected against infection with PIV 3 during their first 14 days of life by maternal antibodies. The increasing antibody titers recorded after 8 weeks of age indicated that an infection had occurred between about 2 weeks and 8 weeks after birth. The period of infection was confirmed when sentinel animals were housed together with 2–3-week-old guinea pigs from PIV 3-positive breeding units. Six weeks later, seroconversion was demonstrated in seven out of 14 sentinels. Sentinel animals brought to the PIV 3-positive animals 18 weeks after the first lot of sentinel animals did not seroconvert. Virus persistence or a prolonged virus carrier status could therefore not be demonstrated. The notion that PIV 3 serum antibodies from guinea pigs separated at an early age from their PIV 3-positive breeding colony had declined to undetectable levels and the apparent absence of chronic carriers indicate a way of eradicating PIV 3 from the herd by putting a temporary stop to breeding.

Both Studies I and II demonstrate that the maternal antibodies initially protect the offspring from environmental viral infections. However, later on, the maternally derived antibodies decline to non-protective levels and the active immunity of the maturing young comes into use to defeat viral pathogens.

The prevalence of maternal antibodies provides the young individual with gradual exposure to the infectious agents of the herd. Thus, the lowered dose exposure adjusted to a maturing immune system counteracts the risks of being stricken with infectious disease or high-dose tolerance and may also promote the development of a balanced T cell response. However, when new infections are introduced into the herd or when the young individual by transfer is exposed to infections of other populations, there is no such protection. In addition, circumstances such as malnutrition, stress, etc. contribute to reducing the limited capacity of the young animal to defeat an infectious disease. Consequently, it is desirable to induce active immunity in individuals with an immature immune

system to avoid harmful effects of pathogens. This might require immunization of young individuals prone to a Th2-biased response, often in the presence of maternal antibodies. Therefore, immunization in early life is also a question of paving the way for a balanced immune response.

In two studies (Papers III and IV), we explored the prerequisites to inducing protective immunity in early life. In the first of these studies, the neonates used were born to non-immunized mothers, whereas in the second study, the neonates were born to immunized mothers.

Both T helper 1 and T helper 2 immune responses can be induced in neonates (Paper III)

Newborn, ≤ 48-hour-old mice were immunized once with SV envelope proteins formulated into ISCOMs (SV-ISCOMs), unadjuvanted micelles (SV-MICs), or micelles adjuvanted with Al $(OH)_3$ (SV-$Al(OH)_3$). The T cell responses to the three SV formulations were measured at different intervals after priming as spleen cell production of the cytokines IL-5 and IFN-γ at restimulation *in vitro* with SV-MICs (see Fig. 1, Paper III). In addition, sera from all mice were tested for the presence of SV-specific antibodies. The results showed that SV-ISCOMs induced the highest IFN-γ production, as measured at 3 and 7 weeks after priming, whereas the highest concentrations of IL-5 were found in the culture fluid of spleen cells from mice immunized with SV-$Al(OH)_3$. Seven to 8 months after priming, the highest amounts of both IL-5 and IFN-γ were seen in mice immunized with SV-$Al(OH)_3$. However, at every time point of measurement, the ratio of IFN-γ/IL-5 spleen cell cytokine production was higher after immunization with SV-ISCOMs than was the IFN-γ/IL-5 ratio seen after immunization with the Th2-driving SV-$AL(OH)_3$.

Only the SV-$Al(OH)_3$ formula induced detectable serum antibody responses in the 2-day-old mice after one immunization, with production of IgG1 but no detectable IgG2a, recorded as long as mice were kept alive, i.e., up to 8 months. By contrast, SV-ISCOMs and SV-MICs did not induce any detectable primary antibody responses. Interferon-γ is known to promote the production of IgG2a. The absence of IgG2a antibodies in SV-$Al(OH)_3$- immunized mice in spite of high production of IFN-γ seen 7–8 months after priming indicates that the subclass pattern was incompletely settled during the first week of life.

Modulation of a neonatally induced immune response by immunization in adult life (Paper III)

It has been reported that reexposure to antigen after neonatal priming generally results in a Th2-dominated immune response, regardless of whether the priming induces a Th1 or a Th2 type of response in the neonate [Adkins & Du 1998;

Barrios et al., 1996]. To explore the extent to which a second immunization at adult age may modulate a neonatally biased immune response, groups of mice immunized as neonates were reimmunized as adults, i.e., at 6 weeks of age.

The Th2-induced immune response after neonatal immunization with SV-$Al(OH)_3$ was modulated towards Th1 after adult boost with SV-ISCOMs. It was also shown that an immunization at adult age with $Al(OH)_3$ modulated a neonatally induced Th1-inclined immune response towards a Th2 response (see Table 1, Paper III).

In conclusion, although the primary response in early life is marked by a Th1 or a Th2 profile, it can be modulated by a later immunization.

The T helper 1-T helper 2 balance changes with age (Paper III)

Herz et al. have shown that in baby mice of nonsensitized mothers, the T cell production of IFN-γ gradually increases from birth until about 28 days of age [Herz et al., 2000]. In our study, an increase in the serum antibody IgG2a/IgG1 ratios was seen in mice primed as neonates and reimmunized at adult age (i.e., at 6 weeks) when the SV-specific antibody titers at 8 weeks of age were compared with those at 6–8 months of age (see Table 2, Paper III). Therefore, it is clear that the combination of increasing age and a reimmunization with a Th1-driving formulation at adult age counteracted the Th2 bias induced in early life. However, despite a general age-related increase in the IgG2a/IgG1 ratio, it should be noted that the IgG2a/IgG1 ratio in mice after neonatal priming with SV-ISCOMs remained significantly and substantially higher than did the IgG2a/IgG1 ratio seen after priming with SV-$Al(OH)_3$.

Prevalence of maternal antibodies and immunization in early life (Paper IV)

Under natural circumstances, the neonate is supplied with maternal antibodies specified and profiled by the environment. With the experiences gained in Study III regarding neonatal immunizations, we expanded the experiments with neonatal immunizations to include offspring with maternally derived antibodies. In the first part of the study, adult female mice were immunized with one of the three different presentation systems of SV used in Study III, i.e., SV-ISCOMs, SV-MICs, and $Al(OH)_3$-adjuvanted SV-MICs. Their offspring were all immunized once as neonates (i.e., at $\leq$ 48 hours) with the Th1-driving SV-ISCOMs. The results challenge the present view, that a mother's immunity does not exert influence on the T cell responses of her offspring when the offspring are exposed to active immunization [Siegrist et al., 1998]. The *in vitro* analyses of offspring spleen cell production of IFN-γ and IL-5 performed 8 weeks after neonatal immunization with SV-ISCOMs showed a significantly lower IFN-γ/IL-

5 ratio in mice born to mothers immunized with SV-$Al(OH)_3$ than in mice born to nonimmunized mothers (see Fig. 1, Paper IV). We also found a clear-cut correlation between the IgG2a/IgG1 ratios in the mothers, which varied according to the different antigen presentation systems used for their immunizations, and the IFN-g/IL-5 ratios in their 8-week-old offspring that had been primed as neonates with SV-ISCOMs ($r=0.997$; $P<0.05$).

In the second part of Study IV, we compared the influences of maternal immunity on neonatal immunization, on immunization at adult age, i.e., at 6 weeks of age, and on a combined immunization, with priming at neonatal age and a booster given at 6 weeks of age.

Adult female mice were immunized twice with SV-$Al(OH)_3$ before mating. The offspring were immunized once, either as neonates with SV-ISCOMs or as adults with SV-MICs, or twice, first at neonatal age with SV-ISCOMs and then at 6 weeks of age with SV-MICs. The maternal antibodies partially suppressed the IgG2a antibody responses of the offspring, regardless of whether they had been immunized once (as neonates or as adults) or twice (first as newborns and again, at 6 weeks of age). However, in spite of maternal suppression, we found that the neonatal immunization primed for a SV-specific IgG2a antibody response revealed as a booster effect after the second immunization at 6 weeks of age indicating an effect of the Th1 driving adjuvant.

A different pattern of the maternal influence was seen when the T cell responses of the offspring were analyzed. No inhibition by, or significant suppressive effect of, the maternal immunity was seen on the T cell response (IFN-γ and IL-5) when the offspring were immunized at 6 weeks. On the other hand, in offspring immunized as neonates, the maternal influence of SV-$Al(OH)_3$-immunized (Th2-biased) mothers exerted significant suppression on the IFN-γ levels but not on the IL-5 levels. Consequently, the immune status of the mother is of importance to the early development of the Th1-Th2 profile in the offspring.

Concluding remarks

- Supply of maternal antibodies was an imperative for normal development and survival of mice raised in an MHV-infected environment. The importance of protection by specific antibodies was demonstrated both in immune competent offspring and in offspring incapable of producing immunoglobulins.

- The experiments clarified that the susceptibility of neonatal mice to MHV infection is not related to endogenous production of immunoglobulins and that specific immunity of the dams was crucial for the offspring's survival.

- Maternally derived antibodies protected young guinea pigs born to PIV 3 positive mothers against infection with PIV 3 during their first two weeks of life.

- The guinea pig offspring became infected with PIV 3 during a period ranging from about 2 weeks of age up to 8 weeks after birth, creating problems since this period coincides with the period during which guinea pigs usually are delivered for animal research.

- Virus persistence or a prolonged virus carrier status could not be demonstrated. This finding points out a possible way to eradicate a PIV 3 herd infection by a cessation breeding policy.

- The ISCOM can be used as adjuvant for neonatal immunization of mice to evoke an adult-like Th1 type response, while $Al(OH)_3$ in early life enhance the Th2 biased immune response typical of neonates.

- A neonatally induced immune response in mice is conserved and more difficult to modify than an immune response induced in adult life. However, it was possible to modify a neonatally induced immune response in mice by adjuvanted antigen at re-immunization in adult life.

- An age-related drift towards a more Th1 marked immune response was seen in mice with increasing age regardless of a Th1/Th2 induced immune status.

- Maternal antibodies suppressed or blocked the antibody levels of her offspring regardless the latter were immunized at neonatal or adult age as shown in several studies.

  However, the immunity of mothers elicited by the Th2 driving adjuvant $Al(OH)_3$ did not exert detectable influence on the T cell response of the offspring when the latter were immunized as adults. When the offspring born to mice immunized with $Al(OH)_3$ adjuvanted formulation were immunized as neonates the maternal influences evoked significant suppression of the spleen cell IFN-γ production.

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