

**"Immunogenicity of hantavirus Dobrava  
nucleocapsid protein derivatives in mice"**

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**Diplombiologin Astrid Geldmacher**

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Präsident der Humboldt-Universität zu Berlin

Prof. Dr. Jürgen Mlynek

Dekan der Mathematisch-Naturwissenschaftliche Fakultät I

Prof. Thomas Buckhout, PhD

Gutachter:

1. Prof. Dr. Richard Lucius
2. Prof. Dr. Detlev H. Krüger
3. Prof. Dr. Paul Pumpens

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## Zusammenfassung

Das in Europa vorkommende Dobravavirus (DOBV) wird durch zwei unterschiedliche Nagetierwirte, die Gelbhalsmaus *Apodemus flavicollis* und die Brandmaus *A. agrarius*, übertragen. DOBV kann bei humanen Infektionen zum Auslösen eines "Hämorrhagischen Fiebers mit renalem Syndrom" (HFRS) unterschiedlicher Schweregrade führen. Wie alle Hantaviren ist das DOBV ein umhülltes Virus, das in seiner Hülle die Glykoproteine G1 und G2 trägt. Im Inneren der Viruspartikel befinden sich die drei mit Nukleokapsid (N) Protein assoziierten Negativstrang-RNA Genomsegmente, sowie die RNA-abhängige RNA-Polymerase.

Das N Protein von Hantaviren ist stark immunogen, sowohl in natürlich vorkommenden Infektionen von Menschen als auch in natürlichen und experimentellen Infektionen von Nagetieren. Des Weiteren rufen Impfungen von Nagetieren mit N Protein eine starke N-spezifische Immunantwort hervor. Eine Impfung mit rekombinanten N Protein Derivativen schützt in Nagetiermodellen vor einer Hantavirusinfektion. Dies konnte unter anderem für chimaere Hepatitis B Virus (HBV) Corepartikel und das komplette rekombinante N (rN) Protein gezeigt werden.

In der vorliegenden Arbeit wurde die Immunogenität von zwei auf dem DOBV N Protein basierende Protein Derivativen in Mäusen getestet. Zum einen wurden in *E. coli* exprimierte chimaere HBV Corepartikel verwendet, die von verkürztem Core-(HBcd)-Protein gebildet wurden, das die 120 amino-terminalen Aminosäuren (AS) des DOBV N Proteins trugen (HBcdDOB120). Das zweite Protein, komplettes DOBV rN Protein (429 AS), wurde in Hefen exprimiert. Anschließend wurden BALB/c (H2-d) und C57BL/6 (H2-b) Mäuse dreimal subkutan mit 50 µg HBcdDOB120 oder DOBV rN Protein in komplettem Freund's, inkomplettem Freund's und anschließend ohne Adjuvants immunisiert. Für die Immunisierungen wurde ein Schema verwendet, mit dem bereits das Potential verschiedener Hantavirus Impfstoffkandidaten im Nagetiermodell getestet wurde. Vor jeder Impfung, sowie zwei Wochen und 29 Wochen nach der dritten Impfung wurde der N-spezifische Antikörpertiter im Serum bestimmt.

Sowohl BALB/c, als auch C57BL/6 Mäuse entwickelten eine starke N-spezifische Antikörperantwort nach Impfung mit sowohl HBcdDOB120, als auch nach Impfung

mit DOBV rN-Protein, mit maximalen Titern von über 1:1.000.000. Die Antikörperantwort war langanhaltend und N-spezifische Titer waren 29 nach der dritten Impfung mit HBcdDOB120 und DOBV rN Protein immer noch höher als 1:35.000 in allen Mäusen. Beide Proteine induzierten Antikörper, die eine starke Kreuzreaktivität gegenüber den rN Proteinen der Hantaviren Puumala, Hantaan, Andes und Sin Nombre aufwiesen.

HBcdDOB120 und DOBV rN-Protein induzierten in BALB/c und C57BL/6 Mäusen N-spezifische Antikörper aller Subklassen (IgG1, IgG2a, IgG2b und IgG3), was auf eine gemischte Th1/Th2 Antwort schließen lässt. Ebenfalls auf eine gemischte Th1/Th2 Immunantwort deuteten die N-spezifischen IFN- $\gamma$  und IL-4 sekretierenden Lymphozyten von HBcdDOB120 oder DOBV rN Protein immunisierten Tieren nach *in vivo* Restimulierung. Die Frequenz der durch die Immunisierungen induzierte N-spezifischen Lymphozyten war allerdings gering.

Auch in Mäusen, die hohe HBc-spezifische Antikörpertiter aufwiesen konnte eine starke N-spezifischen Immunantwort mittels Impfung mit HBcdDOB120 induziert werden. Das heißt, auf chimären Core Partikel basierende Impfstoffe sollten selbst in anti-HBc-positiven Individuen nach einer HBV Infektion wirksam sein.

HBcdDOB120 und Hefe-exprimiertes DOBV rN Protein stellen vielversprechende Vakzinekandidaten dar, die auf ihre Protektivität hin getestet werden sollten, sobald ein DOBV Infektionsmodell verfügbar ist. Da HBcdDOB120 sowie DOBV rN Protein eine starke Antikörperantwort und nur eine schwache T-Zellantwort induzieren sollte zusätzlich die Rolle von N-spezifischen Antikörpern im Schutz gegen die Virusinfektion weiter charakterisiert werden.

## Summary

In Europe, the human pathogenic Dobrava virus (DOBV) is carried by the yellow-necked mouse *Apodemus flavicollis* and the striped field mouse *A. agrarius* and causes "haemorrhagic fever with renal syndrome" of different severity in humans. Like other hantaviruses, DOBV is an enveloped virus with the glycoproteins G1 and G2 embedded in the envelope. Inside the virions are the RNA-dependent RNA-polymerase and the three negative-strand RNA segments which are associated with the nucleocapsid (N) protein.

The N protein is very immunogenic in natural infections of humans and in natural as well as experimental infections of rodents. Even immunisations of rodents with N protein induces a strong N-specific immune response. Moreover, immunisation with N protein derivatives could protect rodents from a hantavirus infection. This was shown for several derivatives, including chimeric hepatitis B virus core (HBc) particles and entire recombinant N (rN) protein.

In this study, the immunogenicity of the two following derivatives based on the DOBV N protein was tested in mice. Chimeric HBV core particles, consisting of truncated HBc (HBcd) particles carrying the amino-terminal 120 amino acids (aa) of the DOBV N protein (HBcdDOB120) were expressed in *E. coli*. The second derivative, the entire DOBV rN protein (429 aa) was expressed in the yeast *Saccharomyces cerevisiae*. Hence BALB/c (H2-d) and C57BL/6 (H2-b) mice were immunised subcutaneously three times with 50 µg HBcdDOB120 or DOBV rN protein in complete Freund's, incomplete Freund's and without adjuvant, respectively. The immunisations were thereby identical to the immunisation scheme used previously in a hantavirus challenge model. Before each immunisation as well as two and 29 weeks after the last immunisation N-specific antibody titers in the serum were determined.

Mice of both strains elicited strong N-specific antibody responses after HBcdDOB120 as well as after DOBV rN protein immunisation, with endpoint titers as high as 1:1,000,000. The antibody response was long-lived and N-specific titers were above 1:35,000 in all mice 29 weeks after the third immunisation with either derivative. Both derivatives induced antibodies that were highly cross-reactive to the rN proteins of the hantaviruses Puumala, Hantaan, Andes and Sin Nombre.

HBcdDOB120 and DOBV rN protein induced in BALB/c and C57BL/6 mice N-specific antibodies of all IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) suggesting a mixed Th1/Th2 immune response. In the same line, IFN- $\gamma$  and IL-4 was secreted by N-specific lymphocytes from mice immunised with HBcdDOB120 or DOBV rN protein after *in vitro* restimulation which also indicated a mixed Th1/Th2 response. However, the frequency of N-specific lymphocytes that were induced by HBcdDOB120 and DOBV rN protein seemed to be low.

In mice that exhibited a high HBc-specific antibody titer HBcdDOB120 induced a strong N-specific immune response. Therefore, vaccines based on chimeric HBcd particles will probably be effective even in anti-HBc positive individuals after HBV infection.

HBcdDOB120 and yeast-expressed DOBV rN protein represent a promising vaccine candidate that should be tested for their protective potential in an DOBV challenge model as soon as one gets available. Additionally, as protection might be partially based on N-specific antibodies, their role in protecting against a hantavirus infection should be characterised further.

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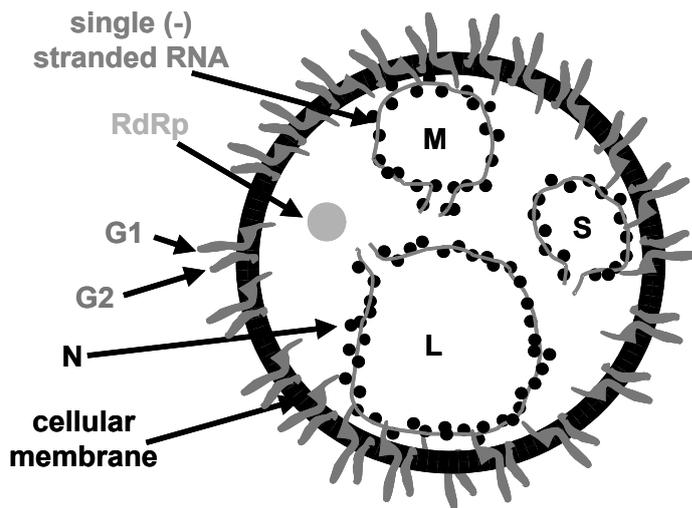
# 1 Introduction

## 1.1 Structure of hantaviruses

Hantaviruses are spherical, enveloped RNA viruses with a diameter of 78-210 nm and belong to the family of *Bunyaviridae* [reviewed in Schmaljohn, 96]. Their genome consists of three segments of negative-sense, single-stranded RNA that code for three proteins. The large (L) segment encodes the RNA-dependent RNA polymerase (RdRp) and the small (S) segment the nucleocapsid (N) protein. The medium (M) segment codes for the glycoprotein precursor which is cleaved by a cellular protease into the two glycoproteins G1 and G2.

In contrast to other genera of *Bunyaviridae*, nonstructural proteins have not been described for hantaviruses. However, almost all hantaviruses associated with rodents from the subfamilies Arvicolinae and Sigmodontinae have a second open reading frame (ORF-2) on the S segment [Ulrich, 02]. Murinae associated hantaviruses do not have a second ORF on the S segment. If this second ORF of the Arvicolinae and Sigmodontinae associated hantaviruses, which encodes for a putative 60 – 90 amino acid (aa) long protein, is expressed in infected cells remains to be elucidated. Presence or absence of this second ORF on the S segment does not seem connected to the virulence of the viruses to humans, but might be relevant for the adaptation of the hantavirus to its rodent hosts.

Maturation of the majority of viruses from genera in the family of *Bunyaviridae* occurs intracellularly by budding into the Golgi cisternae [Kuismanen, 85; Ellis, 88; Hobman, 93; Rwambo, 96; Jantti, 97]. Budding virus particles were found in the Golgi compartment in endothelial cells of patients with an epidemic haemorrhagic fever in China that was most probably caused by members of the *Bunyaviridae* family [Wang, 97]. Therefore, maturation of the hantavirus virions was thought to take place mainly at the Golgi compartment. In contrast, Sin Nombre virus (SNV) and Black Creek Canal virus, both members of the New World hantaviruses, have been found to bud predominantly at the plasma membrane [Goldsmith, 95; Ravkov, 97]. These controversial findings show that further investigations are needed to precisely identify the site and mechanism of budding for the different hantaviruses [Spiropoulou, 01].



**FIGURE 1: Schematic drawing of a hantavirus particle.** Hantaviruses are enveloped negative-strand RNA viruses. The virus particle consists of an RNA-dependent RNA-polymerase (RdRp), two glycoproteins (G1 and G2) and the nucleocapsid (N) protein encoded by the three RNA segment, the large (L), the medium (M) and the small (S) segment, respectively. The RNA segments are associated with the N protein.

## 1.2 Geographic distribution and natural hosts of hantaviruses

Hantaviruses, in contrast to the other, arthropod borne genera of the *Bunyaviridae*, are transmitted by rodents. In these rodents, their natural hosts, they establish a persistent infection without causing disease [Meyer, 00; Plyusnin, 01a; Plyusnin, 01b]. Hantaviruses show a strong host specificity and interspecific spill over seems to be a rare event. So far about 25 hantavirus species have been identified that are associated with different rodent species (for a selection see Tab. 1).

The transmission of hantaviruses from rodents to humans is thought to occur mainly through aerosols of infected animal excreta, i.e. saliva, urine and faeces. In contrast to the Old World hantaviruses, there are indications for person to person transmission during an ANDV outbreak in Argentina [Padula, 98] and a series of cases in Buenos Aires [Pinna, 04].

## 1.3 Diseases caused by hantaviruses

Hantaviruses cause two diseases in humans. Haemorrhagic fever with renal syndrome (HFRS), with a case fatality rate of up to 15 % is caused by Old World hantaviruses. With a lower frequency than HFRS worldwide, hantavirus cardiopulmonary syndrome (HCPS), with a case fatality rate of up to 40 % is

caused by New World hantaviruses [for reviews see Schmaljohn, 97; Krüger, 01; Ulrich, 02].

In 1978 it was proven that the hantavirus prototype HTNV was the causative agent of KHF, a severe form of HFRS [Lee, 78c]. The virus had been isolated from the lungs of an *A. agrarius coreae* captured in the rural endemic areas of Korean haemorrhagic fever (KHF) cases in 1976 [Lee, 78a]. Much earlier however, a war nephritits clinically very similar to the milder form of HFRS occurring in Scandinavia (nephropathia epidemica, NE, see Tab. 1) had been reported among British soldiers stationed in Flanders during World War I [reviewed in Lee, 82a].

**TABLE 1: Natural reservoir and geographical distribution of selected hantaviruses and their associated diseases.** [Krüger, 01; For a more complete summary of hantaviruses see Hooper, 01c; Khaiboullina, 02]

<b>virus</b>	<b>human disease</b>	<b>rodent host (subfamily / species)</b>	<b>distribution</b>	<b>reference</b>
		<u><i>Murinae</i></u>		
Hantaan (HTNV)	HFRS	<i>Apodemus agrarius</i> (striped field mouse)	Asia	[Lee, 78b]
Dobrava (DOBV-Af)	HFRS	<i>Apodemus flavicollis</i> (yellow-necked mouse)	Europe	[Avsic-Zupanc, 92a] [Klempa, 03]
(DOBV-Aa)		<i>Apodemus agrarius</i> (striped field mouse)		
Seoul (SEOV)	HFRS	<i>Rattus</i> species (rats)	Asia / worldwide <sup>a</sup>	[Lee, 82b]
		<u><i>Arvicolinae</i></u>		
Puumala (PUUV)	NE	<i>Clethrionomys glareolus</i> (bank vole)	Europe	[Brummer-Korvenkontio, 80]
Tula (TULV)	-	<i>Microtus</i> species (common voles)	Europe	[Plyusnin, 94] [Sibold, 95]
		<u><i>Sigmodontinae</i></u>		
Sin Nombre (SNV)	HCPS	<i>Peromyscus maniculatus</i> (deer mouse)	North America	[Nichol, 93]
Andes (ANDV)	HCPS	<i>Oligoryzomys</i> species (rice rats)	Argentina, Chile	[Levis, 97]

HFRS haemorrhagic fever with renal syndrome; NE nephropathia epidemica; HCPS hantavirus cardiopulmonary syndrome

a SEOV has mostly been found in Asia, but occurs world wide

In Europe mainly two hantaviruses have been found to cause HFRS of different severity in humans [Mustonen, 98b; Sibold, 99a; Plyusnin, 01a]. PUUV is known to cause NE [Brummer-Korvenkontio, 82], with a case fatality rate of up to 0.1 % [Lähdevirta, 82]. In south-east Europe DOBV carried by the yellow-necked field mouse *A. flavicollis* (DOBV-Af) is responsible for clinically severe HFRS cases with a case fatality rate of up to 12 % [Avsic-Zupanc, 92b; Avsic-Zupanc, 95a; Papa, 01]. Recently, DOBV-Af-like strains (Saaremaa and DOBV-Aa) have been found in the striped field mouse *A. agrarius* [Nemirov, 99; Sibold, 01a; Klempa, 03]. It has been proposed that mild clinical courses of DOBV infections in central and eastern Europe might be due to infections by those virus strains [Schütt, 01; Plyusnin, 01a; Golovljova, 02; Ulrich, 02; Klempa, 04a; Klempa, 04b].

The clinical features of HFRS are fever, headache, back and abdominal pain, drop in blood pressure, hypotension, and in severe cases haemorrhages, renal failure, shock and cardiovascular collapse. Some of these symptoms are thought to be caused by an increased capillary permeability and vascular leakage, a characteristic phenomenon of HFRS [Kanerva, 98b].

The reasons for the differences in severity of disease and case fatality rate caused by the different hantaviruses are not clear, but seem to be determined by virus- and host-specific factors. A major virulence factor is represented by the G1 protein, as a change of HTNV virulence was accompanied by a change of one amino acid in the G1 protein [Isegawa, 94; Ebihara, 00]. In another study, indications were found that a mutation in the noncoding region of the S segment might be responsible for the infectivity of PUUV in bank voles and cell culture [Lundkvist, 97b]. On the other hand, it has been shown that the human HLA alleles B8, DR3 and DQ2 are associated with a more severe outcome of PUUV infection, whereas HLA allele B27 is associated with a milder outcome of PUUV infection in humans [Mustonen, 98a; Vapalahti, 01].

#### **1.4 Treatment of hantavirus infections**

Hantaviruses can cause severe infections, which in some cases can result in a lethal outcome. Except for treatment with ribavirin, curing hantavirus infections is restricted to a treatment of the symptoms caused by the infection. Ribavirin, which

is used against a wide range of RNA viruses can help against hantavirus infections. There seems to be no benefit of a ribavirin treatment for patients infected with New World hantaviruses [Mertz, 04]. For Old World hantaviruses however, it has been shown that treatment with ribavirin decreases virus titres and increases surviving probabilities in suckling mice infected with HTNV [Huggins, 86]. Ribavirin is a guanosine analogue and its incorporation into mRNA is followed by a stop in transcription. Recently, Severson and colleagues have found a higher mutation rate in the S segment mRNA in HTNV infected VeroE6 cells in the presence of ribavirin. They consider this "error catastrophe" as the reason for the antiviral property of ribavirin [Severson, 03]. *In vivo* there might be an additional antiviral effect of ribavirin as it has been shown that ribavirin alters the T cell balance to the T helper 1 (Th1) subset in hepatitis B virus (HBV)- and hepatitis C virus (HCV)-specific immune response [Hultgren, 98]. If this alteration in T cell balance maybe relevant for ribavirin treatment of hantavirus infections remains to be elucidated.

## **1.5 Vaccine development**

### **1.5.1 Whole virus vaccines**

Because of the therapeutical limitations of infections, a prophylactic vaccine for hantavirus infections is needed. Several killed whole virus vaccines generated in mouse brains or cell culture are commercially produced and licensed for human use in Asia [reviewed by Krüger, 01]. Hantavax<sup>TM</sup> is a formalin-inactivated HTNV vaccine grown in suckling mouse brains and supplemented with alum gel as adjuvant [Lee, 99]. Hantavax<sup>TM</sup> seems to be efficient in preventing HFRS: In Korea, the number of hospitalised HFRS cases have dropped by half since Hantavax<sup>TM</sup> became available, from 1234 cases in 1991 to 687 cases in 1996. In Yugoslavia 2000 people became vaccinated with placebo or Hantavax<sup>TM</sup>. In the placebo group five cases of HFRS occurred while in the Hantavax<sup>TM</sup> group no cases of HFRS occurred [both trials reviewed in Lee, 99]. In Asia bivalent HTNV/SEOV vaccines have been developed [Krüger, 01; reviewed by Hooper, 01d]. However, the whole virus vaccines are not licensed outside Asia. There are certain disadvantages of whole virus vaccines. (i) handling of the hantaviruses requires level three safety facilities which complicates the production of a vaccine.

(ii) Inactivation of the virus used for the vaccine could be inefficient which makes it obligatory to test each vaccine lot for infectivity. The need for biosafety level precautions and the danger due to ineffective inactivation can be circumvented by producing a subunit vaccine by recombinant technology.

### **1.5.2 Recombinant proteins as potential hantavirus vaccines**

As has been outlined above, a recombinant subunit vaccine against hantaviruses is needed. Even though many antiviral subunit vaccines in clinical trial, so far there are only two on the market. One is the recombinant HBV surface antigen (HBsAg) expressed in yeast [McAleer, 92]. The fact that HBs particles can protect against HBV has been found out by immunisations with non-recombinant HBs particles purified from hyperimmune serum. The other is a non-recombinant influenza subunit vaccine [Fluad<sup>TM</sup>, Chiron, Minutello, 99].

Previously, hantavirus vaccine candidates have been generated on the basis of recombinant technologies, e.g. naked DNA vaccines, recombinant vaccinia and related poxviruses, and recombinant proteins expressed in transgenic plants, *E. coli*, yeast and insect or mammalian cells. Taken together, several of these recombinant vaccines based on N or the glycoproteins are able to induce protective immune responses in rodent animal models [Krüger, 01; for reviews see Hooper, 01e].

### **1.5.3 Recombinant virus-like particles**

Non-infectious virus-like particles (VLPs) can be generated by heterologous expression of viral structural proteins and their spontaneous self-assembly. A variety of viral proteins have been used for the development of VLPs [for review see Pumpen, 03]. Bacteriophage coat proteins have been found to have a very limited insertion capacity for foreign protein segments [Pushko, 93; Voronkova, 02]. In contrast, bluetongue virus NS1 tubules, parvovirus B19 and yeast retrotransposon Ty-derived VLPs have been found to tolerate extended insertions of up to 100 - 200 foreign aa [Miyano-hara, 86; Adams, 87; Mikhailov, 96].

The surface and the core antigen of the HBV have been used since the 1970s as carrier proteins for the generation of chimeric VLPs. The core protein of HBV

(HBc) expressed in bacteria forms shells resembling those in HBV-infected liver cells [Cohen, 82]. Due to its advantageous features HBc has been extensively exploited as a carrier for foreign epitopes [for reviews see Ulrich, 98b; Pumpens, 01]. Indeed, on the basis of HBV core (HBc), highly promising vaccine candidates have been generated for influenza [Neiryneck, 99] and malaria [Sällberg, 02].

In vaccine development, HBc provides several advantages as a carrier for foreign epitopes: (i) The carboxy-terminal region of HBc responsible for nucleic acid binding can be deleted without disturbing the formation of HBc particles [Borisova, 89; Gallina, 89]. Particles formed by carboxy-terminally truncated HBc protein contain only traces of RNA [Birnbaum, 90; Ulrich, 93]. (ii) The structure of HBc particles has been resolved by cryoelectron microscopy and X-ray crystallography. In line with epitope mapping data the major immunodominant region (MIR) has been identified as the surface-exposed tip of spikes on the surface of HBc particles [Salfeld, 89; Böttcher, 97; Wynne, 99]. (iii) The MIR has been shown to be dispensable for particle assembly [Schödel, 92]. (iv) As expected for a highly repetitive antigen, HBc particles are highly immunogenic and improve the immunogenicity of *per se* low immunogenic foreign peptides presented on their surface [Clarke, 87; Francis, 90]. (v) In comparison to other VLP carriers, HBc has a favourable property in terms of vaccine development; it is not only a T cell dependent but also T cell independent antigen [Milich, 86b]. This T cell independence can be transferred to foreign segments presented on HBc particles [Fehr, 98].

In previous experiments, three potential insertion sites for foreign protein segments into HBc have been used: the amino-terminus, the MIR and different carboxy-terminal positions [for reviews see Ulrich, 98b; Pumpens, 01]. According to the three-dimensional structure [Böttcher, 97; Wynne, 99], epitope mapping data [Salfeld, 89] and empirical insertion data, the MIR represents the most preferential insertion site for foreign sequences [Schödel, 92; Borisova, 96; Lachmann, 99]. Therefore the MIR of the HBc protein has been chosen in this study as the place to insert parts of the DOBV N protein (see chapter 1.8).

#### **1.5.4 The need of adjuvants in subunit vaccines**

One of the problems of generating a protein subunit vaccine, e.g. based on VLPs is that proteins by themselves have a rather low immunogenicity. Therefore, adjuvants are needed to supplement the proteins to induce a strong protein-specific immune response. Adjuvants can either enhance or modify the immune response. Alternatively, they can also act as a depot so that the protein is released over a long period of time and thereby continuously stimulating a protein-specific immune response. Until recently only alum (aluminium hydroxide or aluminium phosphate) has been used as an adjuvant in vaccines for human use. In the year 2000 an influenza vaccine (Fluad™, Chiron) was introduced to the market in which MF59, a water in oil emulsion, is used as adjuvant [Podda, 01]. The development of new adjuvants helping in inducing a strong protective immune response is a crucial step in vaccine development. Ongoing studies investigate other adjuvants containing saponins, or small unmethylated DNA oligonucleotides, (CpG dinucleotides) to be used in human vaccines. Another approach is to use recombinant cytokines as adjuvants in subunit vaccines.

Complete Freund's adjuvant (CFA) due to its high content of mycobacterial cell wall components is a useful adjuvant in research only, but will not be certified for human use. But it represents a useful adjuvant to investigate if a protein is able to induce an immune response in animal models. In this study recombinant proteins were applied with CFA and incomplete Freund's adjuvant (IFA) to enable comparisons to the results of protection studies in an animal model [Lundkvist, 96; Dargeviciute, 02; de Carvalho Nicacio, 02].

#### **1.5.5 Hantavirus proteins suitable as a subunit vaccine**

An efficient hantavirus vaccine should protect against infections by all members of the genus *Hantavirus*. To develop a broadly protective vaccine against hantaviruses two alternative approaches could be followed: (i) generation of a bi- or multivalent vaccine consisting of antigens from different hantavirus species or (ii) identification of antigen(s)/epitope(s) providing cross-protection against a broad range of different hantaviruses.

So far, nothing is known about the potential of the RdRp as a vaccine. In the same

line, research about RdRp is rather limited and until recently it had not been heterologously expressed as an entire protein [Jonsson, 01]. In a recent study a part of a recombinant RdRp expressed in *E. coli* has been shown to be immunogenic in rabbits [Kukkonen, 04]. Compared to the other proteins, the RdRp of different hantaviruses have the highest aa identity; there is a minimum of 70 % aa identity between the RdRp of the hantaviruses [Tab. 2 and Kanerva, 98a]. Therefore, one can expect a high cross-reactivity of the immune response induced by RdRp. This cross-reactivity would be highly favourable for vaccine development. However, further investigations are needed to characterise the potential of RdRp as a vaccine.

**TABLE 2: Amino-acid identities (in %) in the glycoprotein precursor protein and the RNA-dependent RNA polymerase of different hantaviruses after alignment by Clustal Method.**

		glycoprotein precursor							
		DOBV Slovenia Slovakia		HTNV 76-118	PUUV Vranica Sotkamo Kazan			ANDV AH1	SNV 3H226
RdRp	DOBV	Slovenia							
		Slovakia		65	48			48	50
	HTNV	76-118			49			49	49
	PUUV	Vranica						55	58
		Sotkamo Kazan		69					
	ANDV	AH1		68			77		64
SNV	3H226		69			78	87		

Glycoprotein precursor sequences: DOBV-Slovakia (Genbank accession number AY168578), HTNV 76-118 (M14627), PUUV-Vranica/Hällnäs (U14136), ANDV (AF324901), SNV (L37903). RNA-dependent RNA polymerase (RdRp) sequences: HTNV 76-118 (D25531), PUUV-Sotkamo (Z66548), ANDV (AF291704), SNV (L37902). Grey areas: no sequence data available for the glycoprotein precursor or the polymerase of the respective hantavirus.

In contrast to the RdRp, the glycoproteins are well known to have a potential as a vaccine. The proteins G1 and G2 can be expressed in baby hamster kidney (BHK) cells with the help of a alphavirus replicon [Kallio-Kokko, 01b] or by vaccinia virus (VACV) vectored expression [Schmaljohn, 90e]. Passive transfer of serum from SEOV infected rats protected rats from a subsequent SEOV challenge [Zhang, 89]. As the serum had high neutralising antibody titres, protectivity was thought to be mediated by G1/G2-specific antibodies. The specificity of the transferred antibodies, however, was not determined. Immunisation with a VACV vector expressing G1 and G2, as well as passive transfer of G1/G2-specific monoclonal antibodies protected hamsters [Schmaljohn, 90d] and suckling mice [Arikawa, 92]

from a HTNV challenge.

The glycoprotein precursors of different hantaviruses have the lowest aa identity among the hantavirus proteins. There is an aa identity of 50 % to 70 % of the glycoprotein precursors [Tab. 2 and Kanerva, 98d]. Due to these large aa differences in the glycoproteins of different hantavirus strains it is improbable that a subunit vaccine based on the glycoprotein of one hantavirus strain will protect against heterologous hantaviruses [Schmaljohn, 90c; Ruo, 91]. In line, serum from SEOV infected rats protects newborn rats against SEOV but not HTNV [Zhang, 89]. As a neutralisation titre of 1:640 against SEOV was found in the passively transferred sera, protection was thought to be provided by G1/2 specific antibodies. Thus, G1/G2 specific antibodies might not be very cross-protective.

It is thought that the G1/G2-specific immune response protecting against hantaviruses is based primarily on antibodies. However, the role of G1/G2-specific cellular immune response in protection against hantavirus infection has not been investigated so far.

**TABLE 3: Amino acid (aa) identities (in %) of the entire nucleocapsid proteins and their amino terminal 120 aa of different hantavirus strains used in this study.** The aa identities were determined by alignment using the Clustal method.

		N protein, aa 1-428 (ANDV, SNV), 1-429 (DOBV, HTNV), 1-433 (PUUV)							
		DOBV		HTNV	PUUV			ANDV	SNV
		Slovenia	Slovakia	Fojnica	Vranica	Sotkamo	Kazan	AH1	3H226
N protein, aa 1 - 120	DOBV	Slovenia	98	83	60	61	60	64	62
		Slovakia	98	83	60	61	60	63	61
	HTNV	Fojnica	84	84	60	61	61	65	62
		Vranica	51	51	52	96	96	73	69
	PUUV	Sotkamo	50	50	51	96	97	72	70
		Kazan	52	52	53	96	98	73	71
		ANDV	AH1	60	60	59	73	73	74
	SNV	3H226	55	55	55	71	72	72	90

Sequences: DOBV-Slovenia, DOBV-Slovakia, HTNV 76-118, PUUV-Vranica/Hällnäs, PUUV-Sotkamo, PUUV-Kazan [For all sequences see Razanskiene, 04]; ANDV-AH1 [Lopez, 97a] and SNV-3H226 [Hjelle, 94b].

As the N proteins of different hantaviruses are more closely related to each other than the glycoproteins [Tab. 2 and 3, and see Kanerva, 98c] it has since long been speculated that a vaccine based on N protein might be more cross-protective than a vaccine based on G1 and G2 [Asada, 89]. The N protein of hantaviruses is highly immunogenic. Natural hantavirus infections of rodents and humans result in

the induction of strong N-specific antibody and T cell responses [reviewed in Khaiboullina, 02]. In addition, immunisation of rodents with recombinant N (rN) protein induced N-specific B and T cell responses [Lundkvist, 97c; Ulrich, 98a; de Carvalho Nicacio, 01f; Dargeviciute, 02; de Carvalho Nicacio, 02]. In rodent animal models a protective immune response has been mediated by immunisation with vaccinia-vectored N-encoding vaccines [Schmaljohn, 90b; Xu, 92], *E. coli*-expressed chimeric HBc particles carrying amino-terminal parts of N protein [Ulrich, 98a] as well as with rN proteins expressed in *E. coli* [Lundkvist, 96; de Carvalho Nicacio, 02], yeast *Saccharomyces cerevisiae* [Dargeviciute, 02] or insect cells [Schmaljohn, 90a; Lundkvist, 96; Schmaljohn, 99]. However, in a hamster challenge model the protection mediated by a SEOV N-encoding DNA vaccine was found to be low [Kamrud, 99].

## **1.6 Animal models for hantavirus research**

As described above, in nature hantaviruses can persistently infect their natural host without showing signs of disease. Although groups have tried to infect various rodents with hantaviruses [Asada, 88a], there are only a few natural hantavirus rodent hosts that have been bred in captivity to investigate hantavirus infection and challenge. Bank voles can be infected experimentally with PUUV [Lundkvist, 96], rats with HTNV [Lee, 81] and deer mice with SNV [Botten, 00]. Moreover, PUUV and HTNV as well as SEOV can infect Syrian hamsters [Chu, 95; Hooper, 99; Hooper, 01a]. Additionally to hamsters, HTNV can infect Mongolian gerbils (*Meriones unguiculatus*) in the laboratory [Xu, 92]. However, the New World hantaviruses ANDV and Maporal virus are the only ones that cause a lethal infection resembling HCPS in Syrian hamsters [Hooper, 01b; Milazzo, 02a].

PUUV, which readily infects its natural host, the bank vole *C. glareolus* could not infect adult immunocompetent laboratory mice like BALB/c mice [Klingström, 02a]. Comparably, BALB/c and ICR have been reported to be susceptible to only transient infection with HTNV, with virus titres in the infected mice lasting for a maximum of five days [Asada, 87d] or ten days [Kariwa, 95] post infection. In contrast to the only transient infection of laboratory mice, it has recently been described that immunocompetent adult BALB/c, C57BL/6 and SJL/J mice were

susceptible to HTNV [Wichmann, 02a]. C57BL/6 and BALB/c mice have even been reported to die eight to eleven days post infection [Wichmann, 02b]. These experiments were conducted with the same HTNV strain (76-118) used in studies where the virus could only transiently infect laboratory mice (see above). Thus the peculiarities of the HTNV used by Wichmann et al. have to be determined. DOBV infection experiments with BALB/c and NMRI mice resulted in almost all mice in the induction of DOBV N-specific antibodies. However, only some animals had S segment RNA and none of them had N-antigen in their lungs [Klingström, 02b]. Similar findings were obtained recently for DOBV infection of C57BL/6 mice, where infected mice developed G1/2-specific antibody response but N antigen and S segment RNA could not be detected in the lung [Klingström, 04]. Hence, additional investigations are needed to prove if DOBV can reproducibly infect laboratory mouse strains and if infection is transient or long lasting.

Athymic nude mice, which have only very limited amounts of T cells, died from an HTNV infection [Asada, 87c]. In the same line, SCID mice which lack the recombinant VDJ region, leading into T cell and B cell deficiencies, died from HTNV and SEOV infections [Yoshimatsu, 97]. Besides the adult rodent models, intracerebral or subcutaneous injection with HTNV [Nakamura, 85b; Yoshimatsu, 93] and injection with DOBV [Klingström, 03] has been shown to be lethal for suckling mice.

Besides rodents, nonhuman primates can be experimentally infected with hantaviruses. PUUV and ANDV can both infect cynomolgous macaques leading to symptoms similar to those seen in human HFRS and HCPS patients, respectively [McElroy, 02; Klingström, 02c].

## **1.7 Nucleocapsid protein specific immune response**

### **1.7.1 Antibody response**

The N protein is the major antigenic target in the early IgG response of NE patients [Lundkvist, 93b] whereby after disease, more and more G1/2-specific antibodies can be found [Lundkvist, 93b; Vapalahti, 95a]. N-specific antibodies were present already at the onset of disease, while G1/2-specific IgG antibodies were present in only 2 % of the acute sera compared to 87 % of old immune sera from NE patients

[Kallio-Kokko, 01a]. Human sera contained IgG1 and IgG3 in acute sera and IgG1 and IgG4 in the sera 2 years after infection against all three structural proteins N, G1 and G2 [Lundkvist, 93a].

In rodents, the N-specific antibody response has been extensively studied, both after hantavirus infection as well as after immunisation with N protein constructs. Experimental PUUV infection as well as immunisation with PUUV rN protein induced a strong N-specific antibody response in bank voles [Lundkvist, 96; Dargeviciute, 02; de Carvalho Nicacio, 02] and laboratory mice [de Carvalho Nicacio, 01e].

The immunodominant B cell epitope region has been located at the amino terminus of the N protein. Human IgG response in sera of HFRS and HCPS patients was mostly directed to the 119 amino terminal amino acids of hantavirus rN protein [Jenison, 94a; Lundkvist, 95; Vapalahti, 95b; Elgh, 96a; Gött, 97; Milazzo, 02b]. In sera from rodent, like PUUV infected bank voles as well as most monoclonal antibodies derived from PUUV infected animals reacted mostly with amino-terminal peptides of the N protein [Lundkvist, 96; Lundkvist, 02]. Sera of deer mice infected with SNV also reacted more strongly to the N-terminal aa 17-58 compared to the remaining portion of the protein [Yamada, 95].

There are several indications that N-specific antibodies may play a role in protecting against hantavirus infection. N-specific antibodies have been demonstrated to provide protection against a hantavirus infection in cell culture, in the suckling mouse model as well as in adult bank voles [Yoshimatsu, 93; Yoshimatsu, 96e; Lundkvist, 02]. The immunological mechanisms behind the protectivity induced by N-specific antibodies remains to be clarified. Antibody-dependent cytotoxicity (ADCC) has been discussed as one of the mechanisms by which the antibodies can confer protection by binding to infected cells and marking them for destruction [de Carvalho Nicacio, 01d]. An inhibition of transcription has been discussed as another mechanism by which N-specific antibodies can confer protection by binding to N protein, which is bound to the viral RNA and which subsequently inhibits transcription [Yoshimatsu, 96d].

### 1.7.2 Cellular immune response

In patients infected with HTNV and PUUV, the CD8<sup>+</sup> cellular immune response was found to be at least partly directed against the N protein [Van Epps, 99; Van Epps, 02a; Terajima, 02a]. In PUUV infected NE patients G1/2-specific CD8<sup>+</sup> cells have been detected [Terajima, 02b]. PUUV induced CD8<sup>+</sup> memory cells in NE patients [Van Epps, 02b]. Moreover a milder NE course could be connected to HLA B27 [Mustonen, 98a]. Thus, cellular immune responses are likely beneficial to humans in terms of recovering from hantavirus disease.

There is an ongoing controversy if the cellular immune response measured in acutely ill patients is connected to immunopathogenesis or protective immune response. It has been suggested that the N-specific CD4<sup>+</sup> and CD8<sup>+</sup> cells from SNV infected patients might be involved in immunopathogenesis [Ennis, 97]. In line with these findings, numerous cells producing cytokines as IL-1, IL-6, TNF- $\alpha$ , IFN- $\gamma$ , IL-2, IL-4, and TNF- $\beta$  were detected by immunohistochemical staining in autopsy tissues from HCPS patients [Morii, 99]. In another study the ratio of activated to non-activated lymphocytes was higher in acute HFRS patients than in convalescent patients [Huang, 94]. Whether these cytokine producing cells or the activated lymphocytes helped clearing the virus infection or were involved in immunopathology has yet to be elucidated.

Hantavirus specific T cell responses have been investigated in various rodent studies. Cytotoxic splenocytes from HTNV infected mice have been found to lyse HTNV and SEOV infected macrophages [Asada, 88b]. In another study, BALB/c mice infected with HTNV developed IFN- $\gamma$  secreting CD8<sup>+</sup> cells as well as HTNV-specific cytotoxic T cells (CTLs) [Araki, 03]. HTNV-specific CD4<sup>+</sup> and CD8<sup>+</sup> cells induced in mice after HTNV infection can protect mice against a HTNV challenge as shown in adoptive transfer experiments [Asada, 87b]. Most important in protection were CD5<sup>+</sup> positive lymphocytes (T cells and subsets of B cells) as protection dropped most when these cells were lysed before transferring spleen cells from HTNV immunised mice into naive mice. As in the investigations outlined above, it remains to be investigated to which of the hantaviral proteins the protective immune response was directed against.

For the first time cytotoxic T cells with a proven N-specificity have been generated

in C57BL/6 mice by infecting them with HTNV or immunising them with HTNV N protein-derived peptides [Park, 00]. In other studies, PUUV N-specific proliferation was found in splenocytes of PUUV rN protein immunised BALB/c [de Carvalho Nicacio, 01c] as well as of bank voles immunised with rN proteins of DOBV, ANDV or TOPV [de Carvalho Nicacio, 02].

Taken together, it is not clear what kind of N-specific immune response is needed to protect against hantaviruses, but it can be deduced from the studies mentioned above, that N-specific antibodies as well as T cells can play a role in protecting against a hantavirus infection.

### **1.8 Objectives of the study**

DOBV carried by the yellow necked field mouse *A. flavicollis* is a highly virulent virus responsible for clinically severe HFRS cases with a high case fatality rate [up to 12 %, Avsic-Zupanc, 99] and able to kill suckling mice [Klingström, 03]. The main objective of this study was to compare the immunogenicity of entire rN protein of DOBV (strain Slovenia) to the immunogenicity of HBc particles harbouring 120 amino-terminal aa of DOBV (strain Slovenia) N protein (HBcdDOB120). To allow comparison to earlier studies, the immunisation scheme used in this study has previously been used in challenge experiments [Lundkvist, 96].

As humoral, as well as cellular immune responses can be involved in protection against hantaviruses, these investigations included the characterisation of humoral and cellular immunity after immunisation with DOBV rN or HBcdDOB120 protein. The characterisation of humoral immunity should be obtained by analysis of the antibodies against homologous DOBV rN protein and heterologous rN proteins. The estimation of N-specific IgG subclasses should allow a first idea about the cytokine milieu created by lymphocytes involved in the N-specific immune response. In addition, the proliferation of N-specific lymphocytes and their secreted cytokines were analysed to characterise the N-specific T cell response after immunisation with the two proteins.

A potential problem for the use of chimeric VLPs based on HBc might be a pre-existing immunity due to HBV infection. Therefore, an additional aim of the study

was to investigate whether a pre-existing anti-HBV core immunity influences the subsequent immune response against a foreign protein sequence presented on HBc particles in mice.

## **2 Material and methods**

### **2.1 Protein derivatives for immunisation**

#### **2.1.1 Expression and purification of VLPs**

The generation of the expression plasmids for the carboxy-terminally deleted HBc protein (aa 1-144, HBcd) and chimeric HBc protein carrying 120 aa of the N protein of the hantavirus DOBV at aa 78 of HBcd (HBcdDOB120) have been described previously [Borisova, 88; Geldmacher, 04k].

Purified HBcd and HBcdDOB120 were kindly provided by Dr. Galina Borisova (Biomedical Research and Study Centre, Riga, Latvia). The expression in *E. coli* and purification of the HBcd particles have been described previously [Geldmacher, 04j]. Briefly, cells of *E. coli* strain K802 were transformed with plasmids encoding the respective core proteins. After sedimentation the cells were lysed and soluble proteins extracted. Core proteins were precipitated and loaded onto a saccharose gradient or a sepharose CL4B column. Fractions containing the core proteins were identified by SDS-PAGE and Western blot analysis (see chapter 2.2.1), concentrated and stored in glycerol at  $-20^{\circ}\text{C}$  until further use. Prior to immunisation, the particles were diluted in PBS.

The formation of particles of HBcd and HBcdDOB120 particles was kindly proven by negative staining electron microscopy by Dr. Hans R. Gelderblom [Robert Koch-Institut, Berlin, Germany, see Geldmacher, 04i].

#### **2.1.2 Expression and purification of full-length rN protein**

The vectors for the yeast expression of the rN proteins of the hantaviruses DOBV, strains Slovenia [DOBV-Slo; Avsic-Zupanc, 95b] and Slovakia [DOBV/Esl/862Aa/98; DOBV-Slk; Sibold, 01b], HTNV, strain Fojnica [HTNV-Foj; Sibold, 99b], PUU, strains Vranica/Hällnäs [PUUV-Vra; Reip, 95], Kazan [PUUV-Kaz; Lundkvist, 97a]) and Sotkamo [PUUV-Sot; Vapalahti, 92] have been generated and generously provided by Ausra Razanskiene [Dargeviciute, 02;

Razanskiene et al., 2004]. The yeast expression plasmids for the rN proteins of the New World hantaviruses ANDV, strain AH1 [Lopez, 97b] and SNV, strain 3H226 [Hjelle, 94a] were generated and kindly provided by Jonas Schmidt (Schmidt et al., submitted).

The expression and purification of the rN proteins was performed according to protocols previously described [Dargeviciute, 02; Razanskiene et al., 2004]. Briefly, pFX7-derived expression plasmids encoding a fusion of an amino-terminal hexahistidine (His)-tag and the hantavirus N proteins under the control of a galactose inducible yeast promoter were transformed into the yeast *S. cerevisiae* wild-type strain FH4C. The synthesis of rN proteins was induced by addition of galactose and proteins were purified under denaturing conditions via their His-tag according to the protocol of the manufacturer (Qiagen). Proteins were characterised in SDS polyacrylamid gel and Western blot (chapter 2.2.1). Hamster polyomavirus (HaPyV) VP1 protein expressed in yeast, kindly provided by Dr. Alma Gedvilaite [see Gedvilaite, 04], served as a negative control in Western Blot. For immunisations DOBV rN protein was dialysed against phosphate buffered saline (PBS) and subsequently lyophilised.

## **2.2 Characterisation of the recombinant protein derivatives**

### **2.2.1 SDS-PAGE and Western Blot**

Protein samples were separated by electrophoresis in 12.5 or 15 % SDS polyacrylamide gels. Protein bands were stained by Coomassie blue. For Western blot, proteins were transferred to cellulose nitrate membrane by semi-dry blotting. After transfer, cellulose membranes were blocked with 5 % dry milk / PBS containing 0.1 % Tween (PBS/T) for one hour and incubated 16 to 18 h in PBS/T dilutions of the mAbs 1C12, 4C3 [Lundkvist, 91; diluted 1:1,000], mouse anti-DOBV rN serum (1:2,000 in PBS/T) or polyclonal rabbit serum raised against HBc/GFP particles [Kratz, 99; diluted 1:5,000 in PBS/T]. Thereafter, filters were incubated with the respective horse radish peroxidase (HRP)-conjugated anti-mouse IgG (1:3000, Sigma-Aldrich) or anti-rabbit IgG (1:6,000, Sigma-Aldrich) in PBS/T for 2 hours. The peroxidase staining was performed by adding 4-chloro-1-naphthol (Sigma-Aldrich) supplemented with H<sub>2</sub>O<sub>2</sub>.

## **2.2.2 Determination of protein concentration**

To determine the concentration of purified proteins, protein samples were mixed with Bradford reagent (0.01 % Coomassie brilliant blue G250, 8.5 % phosphoric acid, 5 % ethanol). After 10 to 30 min the OD<sub>595nm</sub> values were measured. To estimate the protein concentration, a standard curve with two-fold dilutions of bovine serum albumin (BSA, Sigma-Aldrich) in PBS ranging from 16 - 250 µg/ml was generated. In addition, the estimated protein concentrations of the different rN proteins were compared to each other in SDS polyacrylamid gel by Coomassie blue staining and adjusted accordingly.

## **2.3 Immunisation of mice**

### **2.3.1 Mice strains**

To analyse the immunogenicity of HBcdDOB120 and DOBV rN protein inbred mice strains of two different haplotypes, H-2d (BALB/c) and H2-b (C57BL/6), were used for analysis. Groups of five female mice each were immunised at sex to ten weeks of age. All mice were obtained from the "Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin" in Berlin and held in the Max-Planck-Institute for Infectious Biology, Berlin. Permission of all animal experiments were obtained from the "Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit" in accordance to the German laws (§ 8 Abs. 1 des Tierschutzgesetzes).

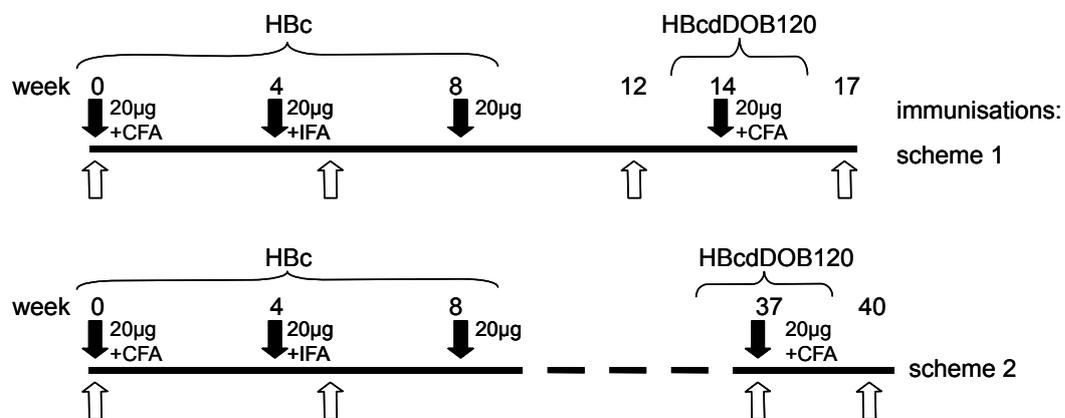
### **2.3.2 Immunisation of mice to investigate the influence of HBc-specific preexisting immunity**

The effect of a preexisting immunity against the carrier protein HBc on the development of anti-N antibodies after immunisation with HBcdDOB120 should be addressed. Thus BALB/c and C57BL/6 mice were immunised according to two different protocols, a "short-term" (scheme 1, Fig. 2A) and "long-term" (scheme 2, Fig. 2A) protocol. In general, mice were first immunised with HBc and then, after animals had developed an antibody titre against HBc as evidenced by ELISA (see chapter 2.4.1), mice were immunised with HBcdDOB120.

In the first set of immunisations, four BALB/c and four C57BL/6 mice per group were immunised subcutaneously (sc) three times with 20 µg each of full length

HBc in complete Freund's adjuvants (CFA, Sigma-Aldrich), incomplete Freund's adjuvants (IFA, Sigma-Aldrich) and without adjuvants. Six weeks after the last immunisation the mice had developed a high antibody titre against HBc as evidenced by ELISA (see chapter 2.4.1). In the "short term" experiment the immunisation with HBcdDOB120 (20  $\mu$ g in CFA) was performed six weeks after the last immunisation with HBc (scheme 1, Fig. 2A). In the "long term" experiments, the immunisation with HBcdDOB120 (20  $\mu$ g in CFA) was given five months after the last immunisation with HBc (scheme 2, Fig. 2A). Blood was collected three weeks after the final immunisation.

### A pre-existing immunity



### B immunogenicity of HBcdDOB120 and DOBV rN



**FIGURE 2: Immunisation schemes for BALB/c and C57BL/6 mice.** To address the question of the influence of HBc-specific immunity to the response to the DOBV rN protein, mice were immunised with HBc and subsequently with HBcdDOB120 (A, schemes 1 and 2). Immunisations of mice with HBcdDOB120 and DOBV rN protein were done to compare the immune responses of mice after immunisation with the two proteins in antibody response and cellular response (B, scheme 3). Immunisation scheme 3 has previously been used in a hantavirus challenge model in bank voles (Lundkvist et al 1996). Solid arrows illustrate the time points of immunisations, while open arrows show the time points of bleeding of the animals. The grey arrow indicate the time point where animals were sacrificed and proliferation and cytokine assays performed after immunisation with a sub-immunogenic dose (2  $\mu$ g) of DOBV rN protein. For details of the immunisations see text (scheme 1 and 2, chapter 2.3.2; scheme 3, chapter 2.3.3) .

### **2.3.3 Immunisation of mice with HBcdDOB120 and DOBV rN protein**

To investigate the immunogenicity of HBcdDOB120 and DOBV rN protein, groups of five BALB/c and five C57BL/6 mice were immunised according to a scheme which was previously used in a PUUV challenge model [Lundkvist, 96]. Briefly, mice were immunised sc three times with 50 µg of HBcdDOB120 or DOBV-Slo rN protein at intervals of three weeks (scheme 3, Fig. 2B). Mice were immunised with the proteins in PBS with CFA, IFA and without adjuvant, respectively. For negative control, mice were immunised three times with HBcd or PBS in the same adjuvants, respectively.

To assess the N-specific cellular immune response, BALB/c and C57BL/6 mice used for the characterisation of the antibody response were injected with 2 µg of DOBV rN protein seven to eight months after the last immunisation with HBcdDOB120 or DOBV rN protein, respectively (scheme 3, Fig 2B). Four days after immunisation, mice were sacrificed and single cell suspensions were prepared from a pool of inguinal, axial and brachial lymph nodes (see chapter 2.4.3).

### **2.3.4 Bleeding and storage of blood**

Blood was collected by bleeding of the tail vein. After the blood clotting it was centrifuged and sera were aliquoted and stored at -20 °C. Once sera were thawed for use in ELISA, they were subsequently stored at 4 °C.

## **2.4 Characterisation of the immune response of mice**

### **2.4.1 ELISA**

Elisa was performed as described previously [Geldmacher, 04a]. Briefly, rN proteins [10 µg/ml] in coating buffer (40 mM Na<sub>2</sub>CO<sub>3</sub>, 60 mM NaHCO<sub>3</sub>, pH 9.8) were coated onto Maxisorb Plates (Nunc) overnight at 4°C. Coated plates were blocked with 150 µl / well of blockingbuffer (1 % BSA, 0.01 % Tween-20, PBS) for

30 min at 37 °C. The sera were then diluted in 100 µl / well at a minimum dilution of 1:50 in ELISA-buffer (0.5 % BSA, 0.01 % Tween-20, PBS), titrated three-fold and incubated for 1 h at 37°C. Specific antibody binding was detected by incubation with HRP-labelled rabbit anti-mouse-IgG in ELISA-buffer (1:8,000, Sigma-Aldrich) for 1 h at 37°C. Assays were developed with o-phenylenediamine (Sigma-Aldrich) in 0.05 M phospho-citrate buffer (Sigma-Aldrich) supplemented with 1.5 ‰ H<sub>2</sub>O<sub>2</sub> and stopped with 50 µl 0.6 M H<sub>2</sub>SO<sub>4</sub> after 20 min at room temperature. The optical density (OD) was read at 492 nm with a reference wavelength of 620 nm.

The titres of antibodies of the different IgG subclasses were determined similarly to total IgG as follows. Coating, blocking and serum dilution were done as described above for the IgG ELISA. After incubation of sera, goat anti-IgG1, anti-IgG2a, anti-IgG2b and anti-IgG3 antibodies (1:1,000, Sigma-Aldrich) were added to the plates for one hour. Finally, binding of anti-IgG subclass antibodies was detected by HRP-labelled anti-goat IgG and immuno-staining as described above.

The endpoint titre was defined as the serum dilution where the OD is three times the background OD. The background OD is the OD that is measured in highly diluted sera and does not decrease with further dilution. In our experiments the background OD varied between 0.02 and 0.1. The OD due to unspecific binding was subtracted from each OD of the respective serum dilution. Unspecific binding is the binding to N of antibodies in the negative control sera of the respective dilution.

To rule out that the IgG subclass titres differed due to a difference in affinity of IgG subclass specific antibodies, ELISAs with coating of plates with threefold dilutions of recombinant IgG1, IgG2a, IgG2b and IgG3 were performed (Sigma Aldrich), starting with concentrations of 100 – 300 ng/ml. After blocking, the adding of anti-IgG1, -IgG2a, -IgG2b or -IgG3 was done according to the IgG subclass ELISA described above. It turned out that the anti-IgG1 antibody gave a three times lower OD as the anti-IgG subclass antibody that gave the highest OD (anti-IgG3) at the same IgG-subclass concentration. Thus the mistake in the IgG subclass distribution in the mouse sera due to the difference in affinity of the anti-IgG subclass antibodies used in the ELISA is maximal three fold and – as log scale is

used in the figure – does not have a big impact on the ELISA results.

#### **2.4.2 Immunofluorescence assay (IFA)**

VeroE6 cells infected with DOBV (strain Slovenia), HTNV (strain 76-118) or PUUV (strain Sotkamo) were seeded in 8 – 12-well coverslips and grown overnight. The next day, cells were washed with PBS, fixed with acetone/methanol (1:2) and a 1:1,000, 1:5,000 or 1:10,000 dilution of serum pools of mice immunised three times with HBcdDOB120 or DOBV rN protein (scheme 3, Fig. 2) in PBS/10% rabbit serum was added. For visualisation, rabbit anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated secondary antibody was used.

#### **2.4.3 Preparation of single cell suspensions from lymph nodes**

.For the investigation of the lymphocyte proliferation, inguinal, axillary and brachial lymph nodes of single mice were pooled and mashed through a 70 µm cell strainer (FALCON). Cells were pelleted by centrifugation (530 x g, 5 min, 4 °C) and taken up in RPMI medium (Biochrome), 5 % heat-inactivated foetal calf serum (FCS, Biochrome), 2 mM L-glutamine, 100U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol and 25 mM HEPES. The concentration of living cells was determined by trypan blue dye staining. A minimum of 100 live cells were counted with a Bürker chamber and cells were adjusted to the concentration needed (see chapter 2.4.4).

#### **2.4.4 Proliferation and cytokine assays for the determination of N-specific lymphocytes**

To analyse the proliferation of N-specific lymphocytes, lymph node cells of mice immunised with HBcdDOB120 or DOBV rN proteins (scheme 3, Fig 2B) were restimulated *in vitro* with DOBV rN protein. Doublets of 600,000 cells were seeded in 200 µl medium / well of a 96-well round bottom plate. Cells were restimulated at 37 °C, 5 % CO<sub>2</sub> for 3 days with concentrations of 0, 0.03, 0.16, 0.80, 4.00 and 20.00 µg/ml DOBV-Slo rN protein. As a positive control cells were treated with 4 µg/ml concanavalin A (Con A, Sigma-Aldrich). In addition, cells from animals

immunised with DOBV rN protein were restimulated with rG2 to assess the impact that the His tag or potential yeast contamination might have on the proliferation.

After 24 hours of restimulation 30 µl per well supernatant was removed for IL-2 testing and BrdU was added to the cells according to the BrdU proliferation test protocol (Roche). After 48 hours of restimulation 50 µl per well supernatant was removed for the IL-4 and IFN- $\gamma$  testing and 44,000 of the 600,000 cells per well were transferred to a flat-bottom microtitre plate. After centrifugation (530 x g, 5 min, 4 °C) of the plate the medium was removed. The rest of the cells were restored in the incubator. After another 72 hours, 44,000 cells were removed for the proliferation test and the supernatant was saved for IL-4 and IFN- $\gamma$  tests. The BrdU ELISA was performed according to the BrdU Proliferation ELISA protocol (Roche). The stimulation indices (SI) was calculated as the ratio of the OD value obtained by the respective antigen concentration to the OD value obtained by medium only.

As outlined above supernatants were collected from lymph node cells after 24 h restimulation for IL-2 testing and after 48 h and 72 h restimulation for IL-4 and IFN- $\gamma$  testing. IL-2, IL-4 and IFN- $\gamma$  was quantified in the supernatant according to the manufacturers protocols (R&D) for a sandwich ELISA. Detection limits of the tests were between 7 and 30 pg/ml.

## **2.5 Data analysis**

Antibody endpoint titres of each mouse as determined by ELISA was transformed to  $_{10}\log$  and average and standard deviation determined for each immunisation group (HBcdDOB120 and DOBV rN protein). By means of the proliferation assay, average SI values (see 2.4.4) and standard deviations were determined for each mouse and each restimulation antigen concentration from duplets. This average SI was then averaged again to calculate the average of each of the immunisation groups for each restimulating antigen concentration. Similarly, cytokine concentrations in the supernatant were determined for each mouse and each restimulating antigen concentration and averages and standard deviations were calculated for each immunisation group. All data analysis were done for BALB/c and C57BL/6 mice separately. Where appropriate, data were analysed for

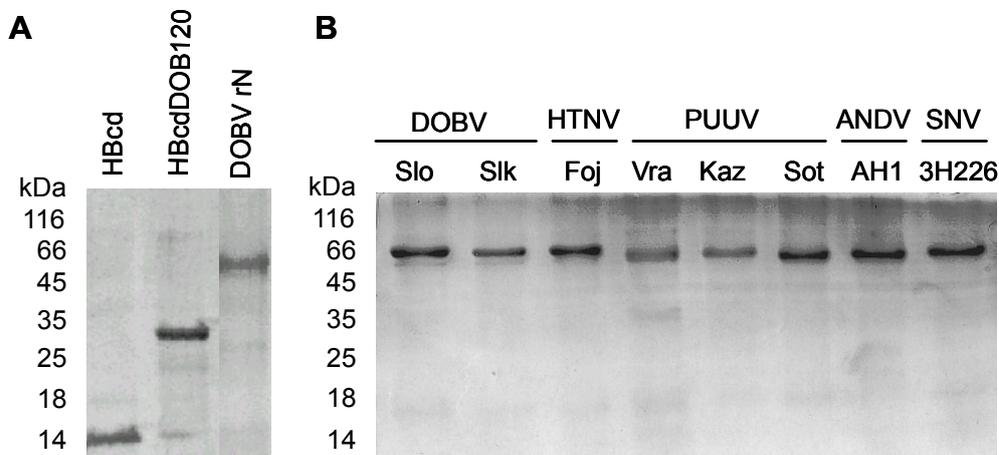
significant differences using a Mann-Whittney U Test (see respective results paragraphs).

### 3 Results

#### 3.1 HBcdDOB120 and DOBV rN were expressed in *E. coli* and *S. cerevisiae*, respectively

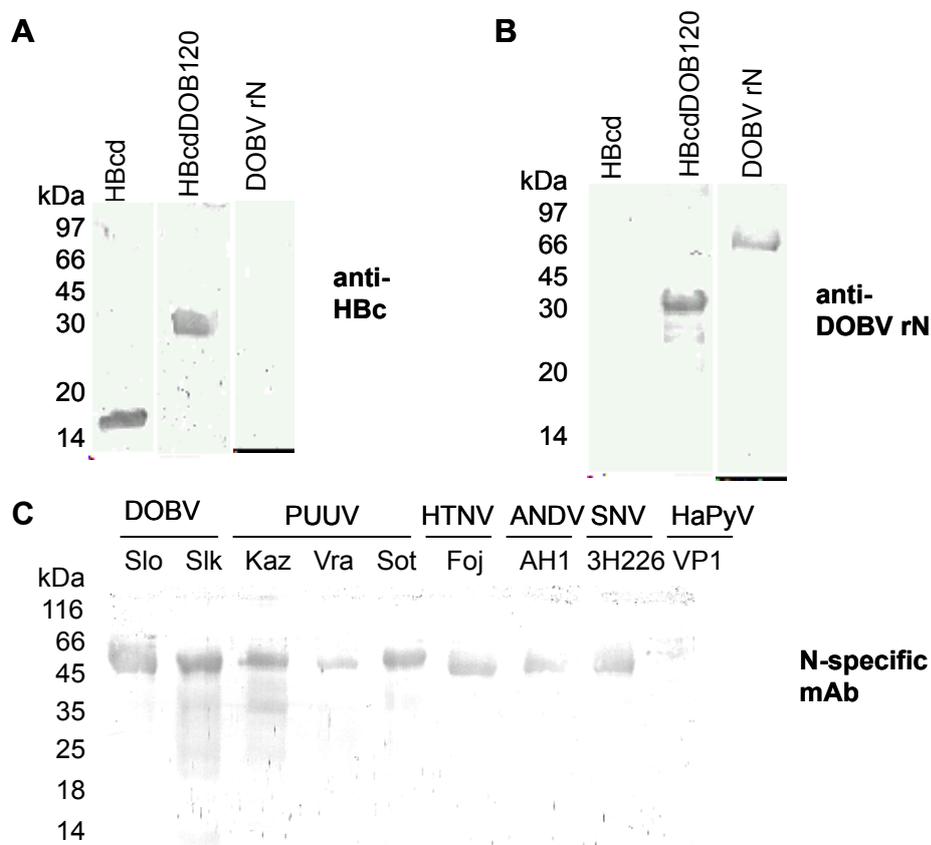
HBcdDOB120 can be expressed in *E. coli* and purified according to protocols described previously [Borisova, 88; Geldmacher, 04h]. The HBcdDOB120 protein is pure as proven by Coomassie blue staining (Fig. 3A), and was recognised by HBc-specific as well as N-specific antibodies in Western Blot (Fig. 4A,B).

The HBcd protein is only recognised by the HBc-specific antibodies. The purified HBcd and HBcdDOB120 proteins self-assembles into particles, as shown by electron microscopy [data not shown, see Geldmacher, 04g]. The 120 amino-terminal aa is at least partly exposed on the outside of the particles, as shown by immuno electron microscopy [data not shown, see Geldmacher, 04f].



**FIGURE 3 Coomassie blue stained SDS polyacrylamid gels of the antigens HBcdDOB120 (A) and rN protein of Dobrava virus (DOBV) strain Slovenia (Slo) (B) used in the immunisation experiments.** In addition to the proteins used for immunisations the following were analysed: HBcd, which was used as a negative control in the immunisations with HBcdDOB120 (A), as well as the proteins that were used for the detection of cross-reactive antibodies against the N protein of the following hantaviruses: DOBV strain Slovakia (Slk), Hantaan (HTNV) strain Fojnica (Foj), Puumala (PUUV) strains Vranica/Hällnäs (Vra), Kazan (Kaz) and Sotkamo (Sot), Andes (ANDV) strain AH1 and Sin Nombre (SNV) strain 3H226.

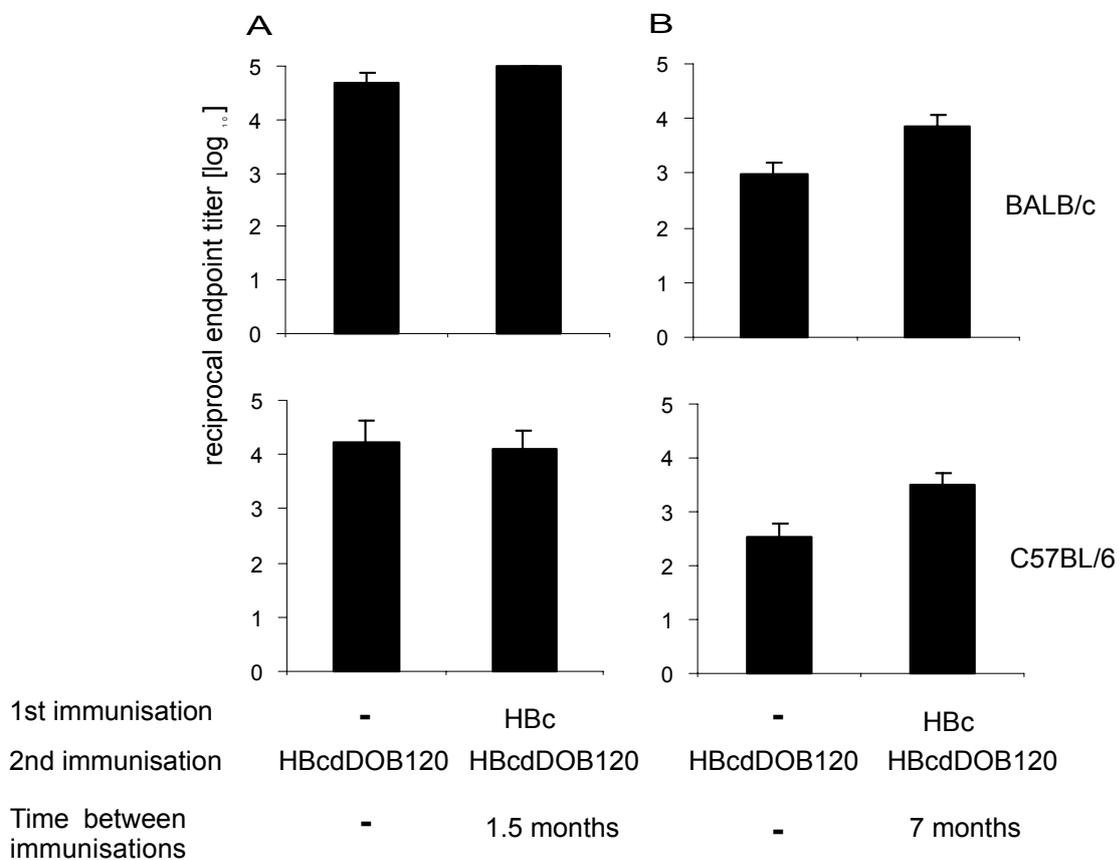
The entire rN proteins of DOBV-Slo, DOBV-Slk, HTNV-Foj, PUUV-Vra, PUUV-Sot, PUUV-Kaz, AND-AH1 and SNV-3H226 could be expressed to high level in the yeast *S. cerevisiae*. They were purified by nickel chelate affinity chromatography by means of the His-tag as proven by Coomassie blue staining of SDS polyacrylamid gel (Fig. 3B). All the proteins, but not the negative control, the hamster polyomavirus (HaPyV) VP1 protein were recognised by an N-specific mAb (Fig. 4C).



**FIGURE 4** Western Blot analysis of the antigens HBcdDOB120 (A, B) and rN protein of Dobrava virus (DOBV) strain Slovenia (Slo) (C) used in the immunisation experiments. Detection of proteins were conducted using polyclonal HBc-specific serum (A), polyclonal hantavirus N-specific serum (B) or the N-specific mAb 1C12 (C). Additionally to the immunising antigens HBcd (A, B) is shown, which was used as a negative control in the immunisations with HBcdDOB120, as well as the proteins that were used for the detection of cross-reactive antibodies against the N protein (C) of the following hantaviruses: DOBV strain Slovakia (Slk), Hantaan (HTNV) strain Fojnica (Foj), Puumala (PUUV) strains Vranica/Hällnäs (Vra), Kazan (Kaz) and Sotkamo (Sot), Andes (ANDV) strain AH1 and Sin Nombre (SNV) strain 3H26.

### 3.2 Preexisting antibodies to HBc did not abrogate the antibody response to DOBV rN protein after immunisation with HBcdDOB120

To investigate if an earlier acquired immune response to the carrier protein HBc influences the N-specific immune response after subsequent HBcdDOB120 immunisation, mice with a high HBc-specific antibody titre were immunised with HBcdDOB120 six weeks after the last HBc immunisation (scheme 1, Fig. 2A). For comparison naive mice were immunised with HBcdDOB120 according to the same scheme.



**FIGURE 5: DOBV N-specific antibody response in BALB/c (above) and C57BL/6 (below) mice with HBc-specific immunity and naive mice after immunisation with HBcdDOB120.** In a first set of immunisations groups of four BALB/c and four C57BL/6 mice were immunised with full-length HBc which resulted in a HBc-specific antibody titre of at least 1:100,000 one months after HBc vaccination (scheme 1, Fig. 2A) or 1:1,000 seven months after HBc vaccination (scheme 2, Fig. 2A). In a second set of immunisation a group of these mice as well as a group of naive mice were immunised once with HBcdDOB120 in complete Freund's adjuvant. Groups of mice were immunised with HBcdDOB120 either 1.5 months (A) (scheme 1, Fig. 2A) or seven months (B) (scheme 2, Fig. 2A) after the last immunisation with HBc. Shown are the means of the reciprocal DOBV rN-specific endpoint titres of four mice with the respective standard deviation. The mean titre of the mice immunised according to the seven months scheme (B) is based on only three mice. Titres did not exhibit significant differences when tested by Mann Whittney U Test ( $p > 0.05$ ).

BALB/c and C57BL/6 mice with a mean HBc-specific antibody titre of 1:250,000 had the same DOBV N-specific antibody titre after immunisation with HBcdDOB120 as mice not possessing an HBc-specific antibody titre before HBcdDOB120 immunisation (Fig. 5A). The preexisting HBc-specific as well as the induced N-specific antibodies were of all IgG subclasses (data not shown). Similarly, BALB/c mice pre-immunised with carboxy-terminally truncated HBcd developed higher DOBV N-specific titres after HBcdDOB120 immunisation than mice that were not preimmunised with HBcd (data not shown, Geldmacher et al. unpublished data).

In another experiment groups of mice immunised with HBc were left for seven months before immunising them with HBcdDOB120 (scheme 2, Fig. 2A), to see if a presumable memory immune response to HBc would influence the immune response to DOBV rN protein.

BALB/c and C57BL/6 mice with an HBc-specific antibody titre of 1:1,000 seven months after the HBc immunisation developed a one order of magnitude higher DOBV N-specific antibody titre after immunisation with HBcdDOB120 compared to mice that did not have any HBc-specific antibody titre before HBcdDOB120 immunisation (Fig. 5B). However, the DOBV N-specific antibody titres of mice with and without a preexisting HBcd-specific titre were not significantly different from each other ( $p>0.05$ ). As observed for the sera of mice immunised according to the "short term" scheme (scheme 1, Fig. 2), HBc-specific and N-specific antibodies were of all IgG subclasses (data not shown).

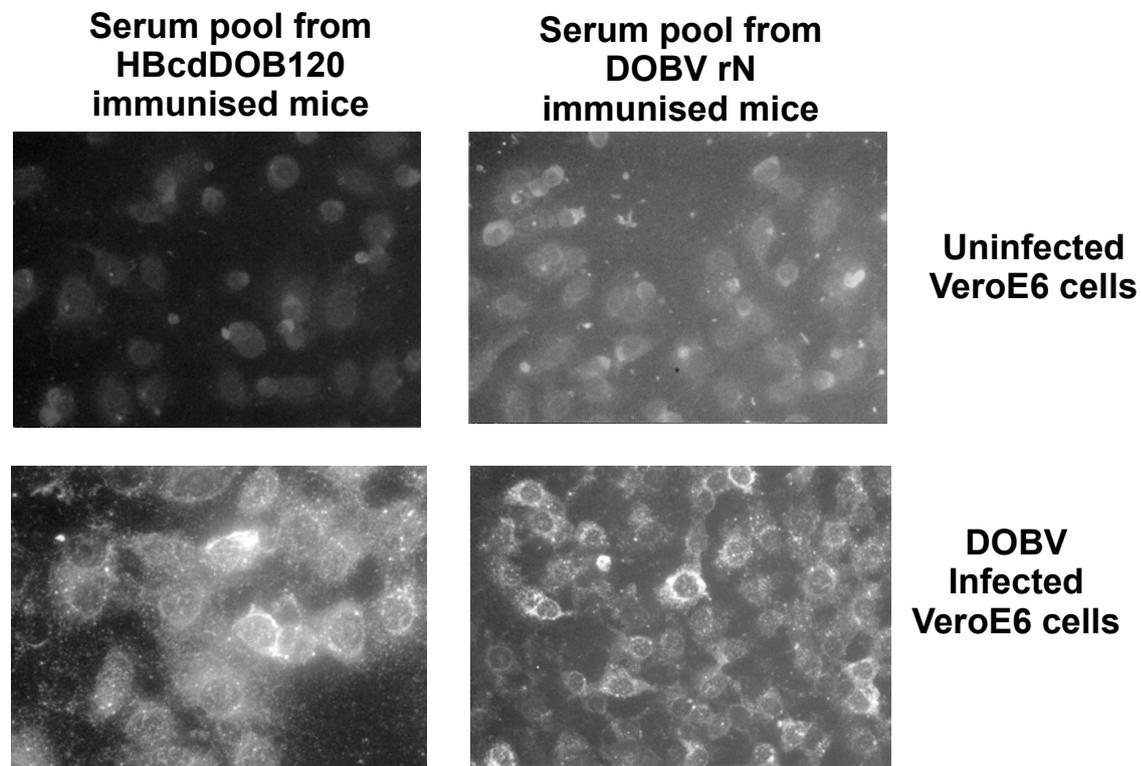
### **3.3 HBcdDOB120 and DOBV rN induced antibodies that reacted to virus infected cells**

Serum pools of mice immunised with HBcdDOB120 particles or DOBV rN protein were tested for their reactivity with the natively folded N protein in hantavirus-infected cells.

A serum pool of mice immunised with HBcdDOB120 particles reacted with natively

folded N protein in DOBV infected cells, but not with uninfected VeroE6 cells (Fig. 6). In addition, the serum pool also reacted with HTNV and PUUV infected cells (data not shown). Sera of mice immunised with HBcd did not react with DOBV infected cells (data not shown).

A serum pool of mice immunised with DOBV rN also reacted with DOBV infected cells, but not with uninfected VeroE6 cells (Fig. 6). The pool of control sera of mice immunised with PBS did not react with DOBV infected cells (data not shown).



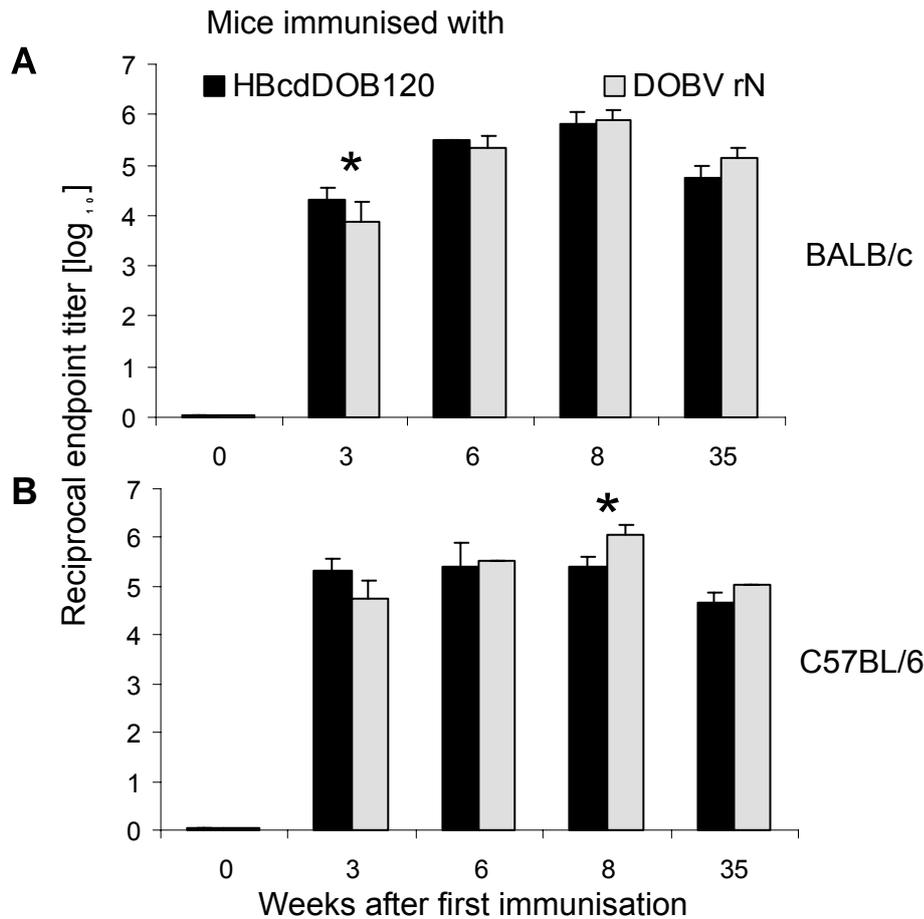
**FIGURE 6: Reactivity of serum pools from mice immunised with HBcdDOB120 and DOBV rN protein with DOBV infected VeroE6 cells.** Sera from five BALB/c mice taken three weeks after the third immunisation (scheme 3, Fig. 2B) were pooled and diluted 1:1,000. Shown is the immunofluorescence of DOBV infected and non-infected cells incubated with serum pools induced by immunisations with HBcdDOB120 and DOBV rN protein detected by a FITC-conjugated anti-mouse antibody at a 40-fold magnification.

### **3.4 HBcdDOB120 and DOBV rN induced a strong and long lasting antibody response**

To compare the immunogenicity of HBcdDOB120 and DOBV rN protein by immunising BALB/c and C57BL/6 mice three times with HBcdDOB120 or DOBV rN protein (scheme 3, Fig. 2B). Individual sera taken after each immunisation were tested in ELISA with the homologous rN protein (DOBV-Slo).

Immunisation of BALB/c and C57BL/6 mice with DOBV rN protein resulted in high titres of homologous N-specific antibody titres. The first immunisation induced N-specific antibody titres of 1:8,000 in BALB/c (Fig. 7A) and 1:60,000 in C57BL/6 (Fig. 7B). In BALB/c mice this titre was significantly higher than in mice immunised with HBcdDOB120 ( $p = 0.032$ ).

BALB/c and C57BL/6 mice immunised with chimeric HBcdDOB120 particles both developed high antibody titres against DOBV rN protein. Three weeks after the first immunisation N-specific antibody titres were as high as 1:20,000 in BALB/c (Fig. 7A) and 1:200,000 in C57BL/6 mice (Fig. 7B). The antibody titre had its peak after the third immunisation with titres of 1:650,000 and 1:250,000, respectively. Eight months after the last immunisation, antibody titres had dropped to 1:55,000 in BALB/c (Fig. 7A) and 1:50,000 in C57BL/6 mice (Fig. 7B). No DOBV rN-specific antibodies were found in the serum pool of control mice immunised with HBcd (data not shown).



**FIGURE 7: Kinetics of DOBV N-specific antibody response.** BALB/c (A) and C57BL/6 (B) mice were immunised according to scheme 3 (Fig. 2B) three times with 50  $\mu$ g of HBcdDOB120 or DOBV rN protein in complete Freund's adjuvant (week 0), incomplete Freund's adjuvant (week 3) or without adjuvant (week 6), respectively. N-specific antibody endpoint titres (three times the background) were determined in ELISA for each mouse separately. Shown are the means of reciprocal endpoint titres of 5 mice and the respective standard deviations. Stars indicate significant differences in  $\log_{10}$  titres between mice immunised with HBcdDOB120 compared to mice immunised with DOBV rN protein as determined by Mann Whitney U Test ( $p < 0.05$ ).

Like in HBcdDOB120 immunised mice the highest antibody titre was found after the third immunisation with titres of 1:800,000 and 1:1,000,000, respectively. At this time point significantly higher titres were found in mice immunised with DOBV rN protein compared to HBcdDOB120. Eight months after the last DOBV rN protein immunisation, antibody titres had dropped to 1:150,000 in BALB/c (Fig. 7A) and 1:100,000 in C57BL/6 mice (Fig. 7B). Serum pools from sera of animals immunised with DOBV-Slo rN protein did not develop any titre against yeast-expressed DOBV-Slo G2, used as a negative control (data not shown). In addition, no DOBV rN-specific antibodies were found in the serum pool of control mice

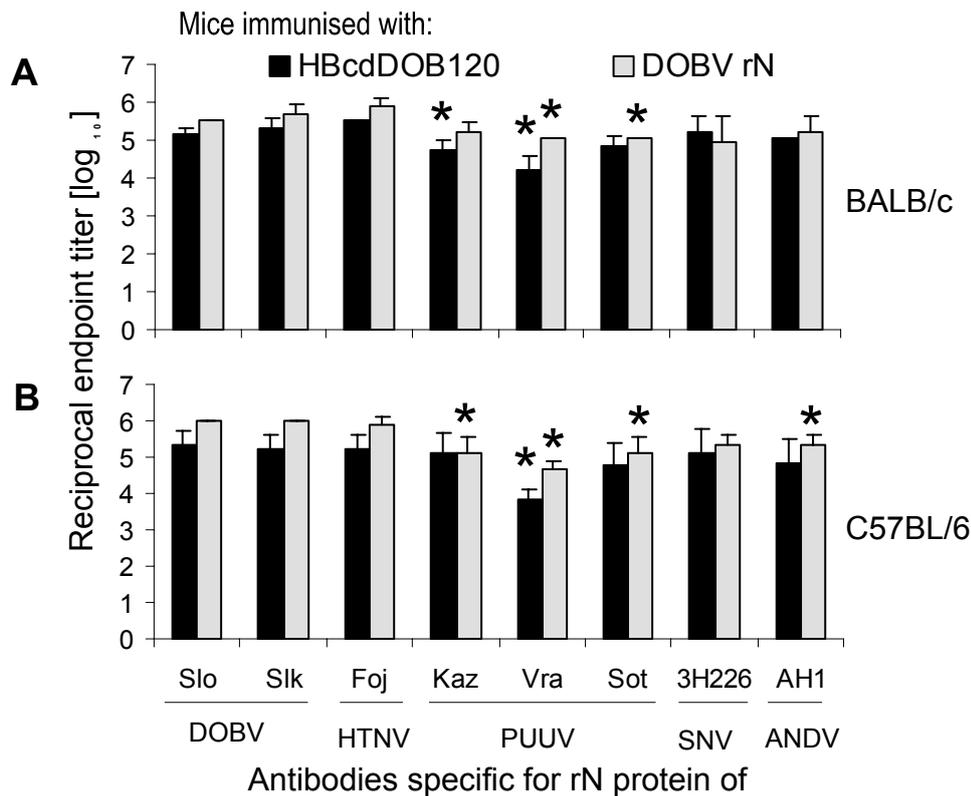
immunised with PBS (data not shown).

### **3.5 HBcdDOB120 and DOBV rN protein induced antibodies are highly cross-reactive to the rN proteins of other hantaviruses**

The reactivity of sera from mice immunised three times with HBcdDOB120 or DOBV-Slo rN protein to the rN proteins of other hantaviruses was analysed. Therefore, individual sera taken two weeks after the final immunisation with HBcdDOB120 or DOBV rN protein (both based on the N protein of DOBV-Slo) were tested for antibodies reacting with the rN proteins of DOBV-Slk, HTNV-Foj, PUUV-Vra, PUUV-Kaz and PUUV-Sot, SNV-3H226 and ANDV-AH1.

Sera of mice immunised with HBcdDOB120 were all reactive to the rN proteins of DOBV-Slk, HTNV-Foj and PUUV-Vra, PUUV-Kaz, and PUUV-Sot, ANDV-AH1 and SNV-3H226 when tested in ELISA (Fig. 8A,B). The pattern of cross-reactivity did not differ between BALB/c and C57BL/6 mice. The cross-reactivity was highest to the heterologous rN proteins of the more closely related HTNV. However, reactivities were significantly lower to the more distantly related PUUV-Kaz (only in BALB/c mice,  $p=0.032$ ) and PUUV-Vra ( $p=0.008$  BALB/c;  $p=0.016$  C57BL/6). Reactivities to PUUV-Sot, SNV and ANDV were not found to be significantly different to the reactivity to DOBV (Fig. 8A,B).

In immunofluorescence assay, a highly diluted (1:10,000) serum pool from HBcdDOB120 vaccinated BALB/c mice showed highest fluorescence intensity and more cells immunofluorescence positive on HTNV infected cells. Sera showed similar intensity on DOBV infected VeroE6 cells but much lower intensity of fluorescence and fewer immunofluorescence positive PUUV infected cells (data not shown). Interestingly, the level of cross-reactivity was lower when a serum pool was tested in Western Blot. Serum of mice immunised with HBcdDOB120 reacted to a low extent to the rN proteins of PUUV-Kaz and PUUV-Sot and did not react at all to rN protein of PUUV-Vra (data not shown). However, both tests are not quantitative as the intensity of immunofluorescence and the intensity of the bands in Western Blot could only be estimated.



**FIGURE 8: Analysis of the cross-reactivity of antibodies of BALB/c (A) and C57BL/6 (B) mice two weeks after the third immunisation with 50  $\mu$ g HBcdDOB120 or DOBV rN protein (scheme 3, Fig. 2B).** Antibodies induced by either of those two constructs based on the rN protein of hantavirus strain Dobrava Slovenia (DOBV-Slo) were tested on the reactivity with the rN proteins of the hantaviruses DOBV strain Slovakia (Slk), Hantaan virus (HTNV) strain Fojnica (Foj), Puumala viruses (PUUV) strains Kazan (Kaz), Vranica/Hällnäs (Vra), Sotkamo (Sot), Sin Nombre virus (SNV) strain 3H226 and Andes virus (ANDV) strain AH1. Endpoint titres (three times the background) of each animal was determined by ELISA. Shown are the arithmetic mean values and standard deviations of the reciprocal endpoint titres of five animals. Stars indicate significant differences in  $\log_{10}$  titres between DOBV-Slo-specific titres compared to antibody titres reacting to the N protein of other hantaviruses as determined by Mann Whitney U Test ( $p < 0.05$ ).

In general, the sera of mice immunised with DOBV-Slo rN protein reacted in ELISA strongly with all rN proteins (Fig. 8A,B). The pattern of cross-reactivity did not differ between BALB/c (Fig. 8A) and C57BL/6 (Fig. 8B) mice. The reactivity of the sera from both BALB/c and C57BL/6 mice immunised with DOBV-Slo rN protein to the rN protein of the more closely related DOBV-Slk and HTNV-Foj was slightly stronger when compared with the reactivity to the rN proteins of the PUUV strains (Fig. 8A,B). In both mouse strains, the reactivity was significantly lower to PUUV-Vra ( $p=0.008$ ), PUUV-Sot ( $p=0.008$  BALB/c;  $p=0.032$  C57BL/6). Only sera from C57BL/6, but not BALB/c mice immunised with DOBV rN reacted to significantly lower level to ANDV-AH1 rN protein ( $p=0.032$ ).

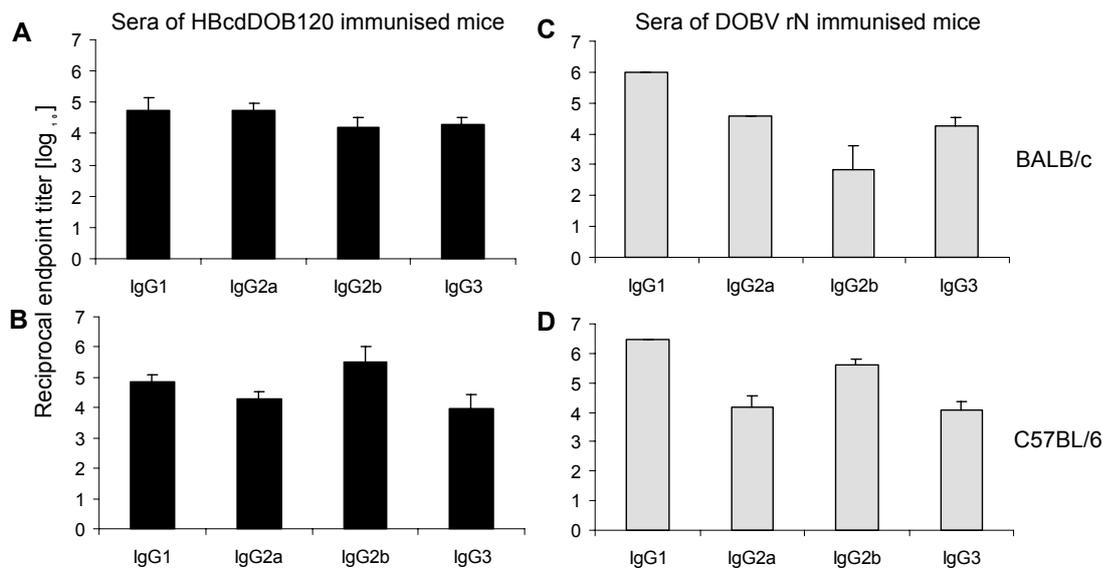
In immunofluorescence assay highly diluted (1:10,000) pool of sera from DOBV rN protein immunised BALB/c mice reacted strongly to HTNV and DOBV infected VeroE6 cells but weaker to PUUV infected cells (data not shown). In Western Blot, the serum pool of mice immunised with DOBV-Slo rN protein reacted with the rN proteins of both DOBV, HTN and all three PUUV strains (data not shown).

### **3.6 HBcdDOB120 and DOBV rN induced N-specific antibodies of all IgG subclasses**

To get a first idea which kind of immune response is induced by HBcdDOB120 particles and DOBV rN protein in mice, we determined titres of N-specific antibodies of the IgG subclasses IgG1, IgG2a, IgG2b and IgG3. In addition, titre ratios of IgG1/IgG2a as well as titre ratios of all four IgG subclasses to total IgG were calculated to characterise the (mostly T cell secreted) cytokine environment when B cells were activated.

HBcdDOB120 induced in BALB/c and C57BL/6 mice N-specific antibodies of all IgG subclasses, IgG1, IgG2a, IgG2b and IgG3 to a similar extend (Figs 9A,B). The ratio of IgG1/IgG2a was between 1.0 and 1.2 in BALB/c and between 1.1 and 1.5 in C57BL/6 mice at four time points after the first immunisation (Tab. 4).

Besides IgG1 and IgG2a antibodies, IgG2b and IgG3 were measured in the sera of mice immunised with HBcdDOB120. In the sera taken the mean IgG2b/IgG ratios lay between 0.70 and 1.02 while the IgG3/IgG ratios were between 0.67 and 0.82 (Tab. 4).



**FIGURE 9: IgG subclass distribution of DOBV N-specific antibodies in sera of mice two weeks after the last of three immunisations with HBcdDOB120 or DOBV rN protein.** Shown are the arithmetic means and standard deviations of the reciprocal endpoint titres (three times the background) of five animals. Each titre was determined separately by ELISA for each animal. Comparability of the anti-IgG1, anti-IgG2a, anti-IgG2b and anti-IgG3 antibodies used in the ELISA was confirmed in a special ELISA (see chapter 2.4.1).

In mice sera after immunisation with DOBV rN protein N-specific IgG of all subclasses were found to be induced (Figs 9C,D). However, we observed a slight dominance of IgG1 over IgG2a, IgG2b and IgG3 in the sera of BALB/c mice (Fig. 9C). This dominance of IgG1 over the other IgG subclasses was detected in sera after each immunisation step (Tab. 4). In the sera of C57BL/6 mice IgG1 dominated over IgG2b, followed by IgG2a and IgG3 (Fig. 9D). As observed in BALB/c mice, this dominance could be seen in sera of C57BL/6 mice after each immunisation (Tab. 4).

In sera taken at four time points the mean IgG2b/IgG ratios laid between 0.48 and 0.61 for BALB/c and 0.84 and 0.93 for C57BL/6 without showing a time-dependent pattern (Tab. 4). The IgG3/IgG ratios were between 0.65 and 0.73, decreasing with time after immunisation (Tab. 4).

**TABLE 4: Ratios of N-specific antibody titre of IgG subclasses to total IgG and IgG1 to IgG2a ratios at various time points after immunisation of BALB/c and C57BL/6 mice with HBcdDOB120 particles or DOBV rN protein.** Animals were immunised three times (weeks 0, 3 and 6) with 50 µg in adjuvants according to scheme 3 (Fig. 2B) and bled at various time points. Shown are the means (in bold) and the standard deviation (s) of five animals of the ratios of antibody titres that as determined by ELISA.

time point <sup>a</sup>	mouse strain	immunisation antigen	IgG1/IgG <sup>b</sup>		IgG2a/IgG <sup>b</sup>		IgG2b/IgG <sup>b</sup>		IgG3/IgG <sup>b</sup>		IgG1/IgG2a <sup>c</sup>		
			mean <sup>d</sup>	s	mean <sup>d</sup>	s	mean <sup>d</sup>	s	mean <sup>d</sup>	s	mean <sup>d</sup>	s	
3	BALB/c	HBcdDOB120	<b>0.97</b>	0.11	<b><u>0.81</u></b>	0.09	<b><u>0.86</u></b>	0.11	<b>0.81</b>	0.10	<b><u>1.20</u></b>	0.12	
6			<b>0.98</b>	0.08	<b><u>0.90</u></b>	0.07	<b><u>0.83</u></b>	0.08	<b><u>0.81</u></b>	0.03	<b>1.10</b>	0.15	
8			<b><u>0.82</u></b>	0.06	<b>0.82</b>	0.06	<b><u>0.72</u></b>	0.08	<b>0.74</b>	0.05	<b><u>1.01</u></b>	0.13	
35			<b><u>0.90</u></b>	0.07	<b>0.84</b>	0.07	<b>0.70</b>	0.07	<b>0.72</b>	0.04	<b>1.08</b>	0.15	
3		DOBV rN	<b>1.08</b>	0.07	<b><u>0.68</u></b>	0.05	<b><u>0.60</u></b>	0.05	<b>0.73</b>	0.05	<b><u>1.58</u></b>	0.09	
6			<b>0.95</b>	0.04	<b><u>0.73</u></b>	0.05	<b><u>0.50</u></b>	0.10	<b><u>0.70</u></b>	0.04	<b>1.30</b>	0.08	
8			<b><u>1.02</u></b>	0.03	<b>0.77</b>	0.03	<b><u>0.48</u></b>	0.13	<b>0.72</b>	0.04	<b><u>1.31</u></b>	0.00	
35			<b><u>1.04</u></b>	0.08	<b>0.81</b>	0.05	<b>0.60</b>	0.11	<b>0.70</b>	0.02	<b>1.28</b>	0.06	
3		C57BL/6	HBcdDOB120	<b>0.93</b>	0.04	<b>0.64</b>	0.08	<b>0.93</b>	0.07	<b><u>0.82</u></b>	0.01	<b>1.47</b>	0.17
6				<b>0.92</b>	0.10	<b>0.68</b>	0.03	<b>0.90</b>	0.06	<b>0.79</b>	0.04	<b>1.35</b>	0.16
8				<b>0.89</b>	0.03	<b><u>0.79</u></b>	0.04	<b>1.02</b>	0.07	<b>0.73</b>	0.07	<b>1.14</b>	0.05
35				<b>0.77</b>	0.08	<b>0.57</b>	0.02	<b>0.98</b>	0.04	<b>0.67</b>	0.05	<b>1.36</b>	0.13
3			DOBV rN	<b>1.00</b>	0.07	<b>0.64</b>	0.04	<b>0.92</b>	0.04	<b><u>0.72</u></b>	0.03	<b>1.58</b>	0.13
6				<b>0.98</b>	0.03	<b>0.67</b>	0.03	<b>0.93</b>	0.03	<b>0.72</b>	0.03	<b>1.47</b>	0.08
8				<b>0.99</b>	0.03	<b><u>0.69</u></b>	0.05	<b>0.92</b>	0.00	<b>0.67</b>	0.04	<b>1.44</b>	0.13
35				<b>0.87</b>	0.04	<b>0.59</b>	0.04	<b>0.84</b>	0.09	<b>0.65</b>	0.04	<b>1.48</b>	0.04

<sup>a</sup> weeks after first immunisation

<sup>b</sup> ratios of the N-specific log<sub>10</sub> reciprocal antibody titre of IgG subclass to IgG total

<sup>c</sup> ratios of the N-specific log<sub>10</sub> reciprocal antibody titre of IgG1 to IgG2a

<sup>d</sup> The HBcdDOB120 induced ratios that significantly differ from DOBV rN induced ratios are in italic and underlined (p<0.05). Analysis of significant differences by Mann-Whitney U Test, done separately for BALB/c and C57BL/6 mice.

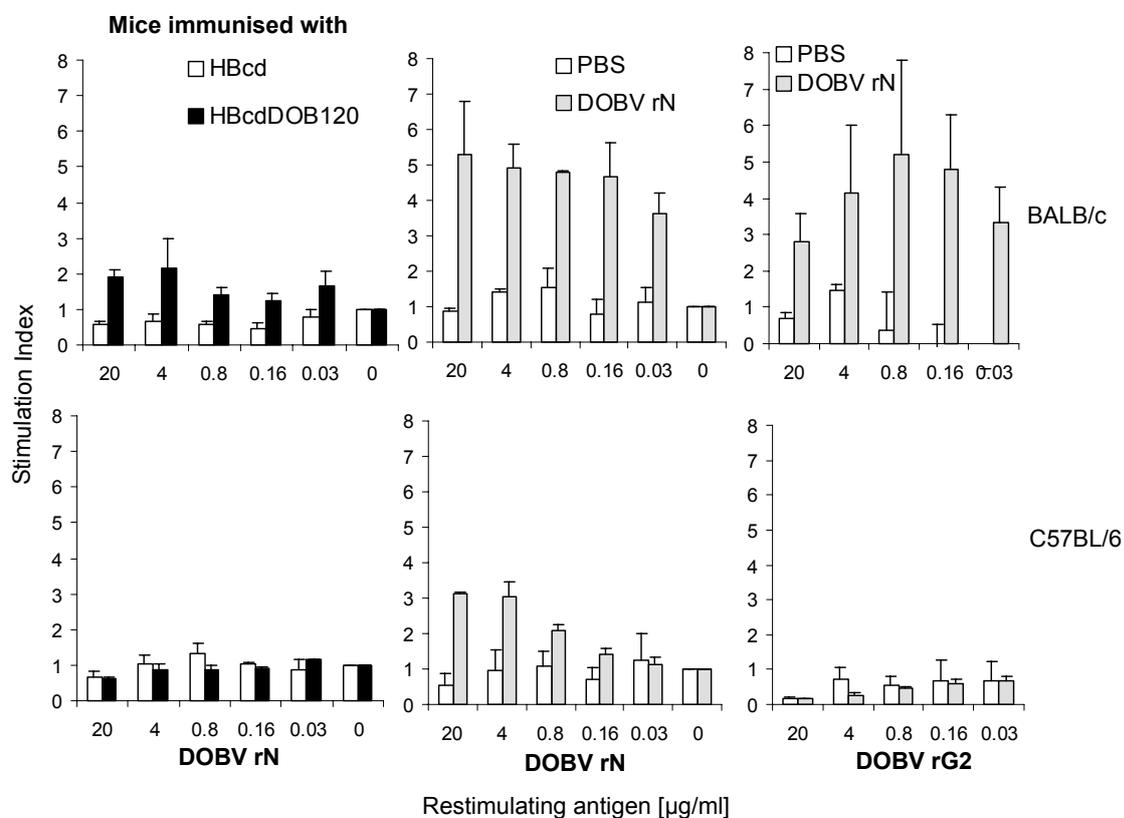
### 3.7 Proliferation of N-specific lymphocytes was low after immunisation with HBcdDOB120 or DOBV rN protein

To investigate the cellular immune response, mice immunised three times with HBcdDOB120 or DOBV rN protein were subsequently immunised with a low dose of DOBV rN protein in order to bring the N-specific lymphocytes back to the draining lymph nodes.

Mice previously immunised with HBcd and PBS, respectively, were also immunised with the same dose of DOBV rN to guarantee the "sub-immunogenic" nature of this low dose. Proliferation of cells was determined using  $6 \times 10^5$

(BALB/c) or  $4 \times 10^5$  (C57BL/6) cells per well.

In mice immunised with HBcdDOB120 only small (BALB/c) or no (C57BL/6) proliferative response after restimulation with DOBV rN could be detected (Fig. 10). The highest proliferative response, a stimulation index (SI) of 2.2 developed after a restimulation with 4  $\mu\text{g/ml}$  DOBV rN protein. These results are representative for three other experiments conducted on cells from BALB/c mice treated the same way as in the experiment shown here (data not shown). Restimulation of cells with 4  $\mu\text{g/ml}$  ConA resulted in strong proliferation with SIs of 4 to 14 (BALB/c) or 40 (C57/BL6) (data not shown).



**FIGURE 10: Analysis of N-specific proliferation of lymph node cells.** One Mouse was immunised three times sc with HBcdDOB120 or DOBV rN protein (scheme 3, Fig 2B). Seven months after the last immunisation these mice were injected with a sub-immunogenic dose of 2  $\mu\text{g}$  DOBV rN protein to induce the N-specific lymphocytes to home to the draining lymph nodes. Control mice previously immunised with HBcd or PBS were immunised the same way. Four days after the injection of the sub-immunogenic dose doublets of  $6 \times 10^5$  (BALB/c) or  $4 \times 10^5$  (C57BL/6) cells from the pooled inguinal, axial and brachial lymph nodes were restimulated for 72 h *in vitro* with different concentrations of DOBV rN protein or left untreated in complete RPMI medium. In addition, cells from animals immunised with DOBV-Slo rN were restimulated with rG2 protein to assess if the His tag or potential yeast contaminations have an impact on the proliferation. Level of proliferation was determined by means of brom-desoxyuridine incorporation (Roche). The stimulation index was calculated as the proliferation induced by the respective antigen concentration divided by the proliferation induced by medium alone. Shown are means of doublets with the respective standard deviations.

Immunisation of mice with DOBV rN protein resulted in proliferative responses in BALB/c and C57BL/6 mice after restimulation with DOBV rN protein (Fig. 10). The strongest N-specific responses, with SIs of over 5 (BALB/c) and over 3 (C57BL/6), were found after restimulation with 4 µg/ml and 20 µg/ml DOBV rN protein.

In BALB/c mice restimulation with His-tagged rG2 protein purified in a similar way as DOBV rN protein also induced cells that were immunised with DOBV rN protein to proliferate (Fig. 10). The proliferation of cells from BALB/c mice immunised with DOBV rN protein caused by rG2 protein was variable and in some antigen concentrations higher than the proliferation caused by DOBV rN protein (Fig. 10). The same was seen in the other three experiments (data not shown).

In contrast, lymph node cells of C57BL/6 mice immunised with DOBV rN protein did not proliferate at all when restimulated with rG2 protein. The same was the case for the lymph node cells from C57BL/6 and BALB/c mice immunised with PBS after restimulation with DOBV rN protein or rG2 protein (Fig. 10 and data not shown). Cells from mice immunised with HBcdDOB120 did also not proliferate after restimulation with 20 µg/ml rG2 protein (data not shown). Restimulation of cells with 4 µg/ml Con A resulted in strong proliferation (see 3.4.1.1).

### **3.8 Higher cytokine levels were secreted after immunisation with DOBV rN protein than after immunisation with HBcdDOB120**

Cytokines IL-2, IL-4 and IFN- $\gamma$  were measured in the supernatant of mouse cells used in the proliferation assay (see above). As in the proliferation assay, cytokine concentrations in the supernatant of BALB/c cells were not comparable to the cytokine concentrations in the supernatant of C57BL/6 cells because less cells of the latter per well were cultured (see above).

The cells of BALB/c mice (Figs 11, 13) and a C57BL/6 (Fig. 12) mouse immunised with HBcdDOB120 secreted only very little amounts of IL-2 after restimulation with DOBV rN protein. The highest levels of IL-2, 80 pg/ml in BALB/c (Fig. 11) and 7 pg/ml in C57BL/6 (Fig. 12) cell supernatants, were detected after restimulation with 20 µg/ml DOBV rN protein.

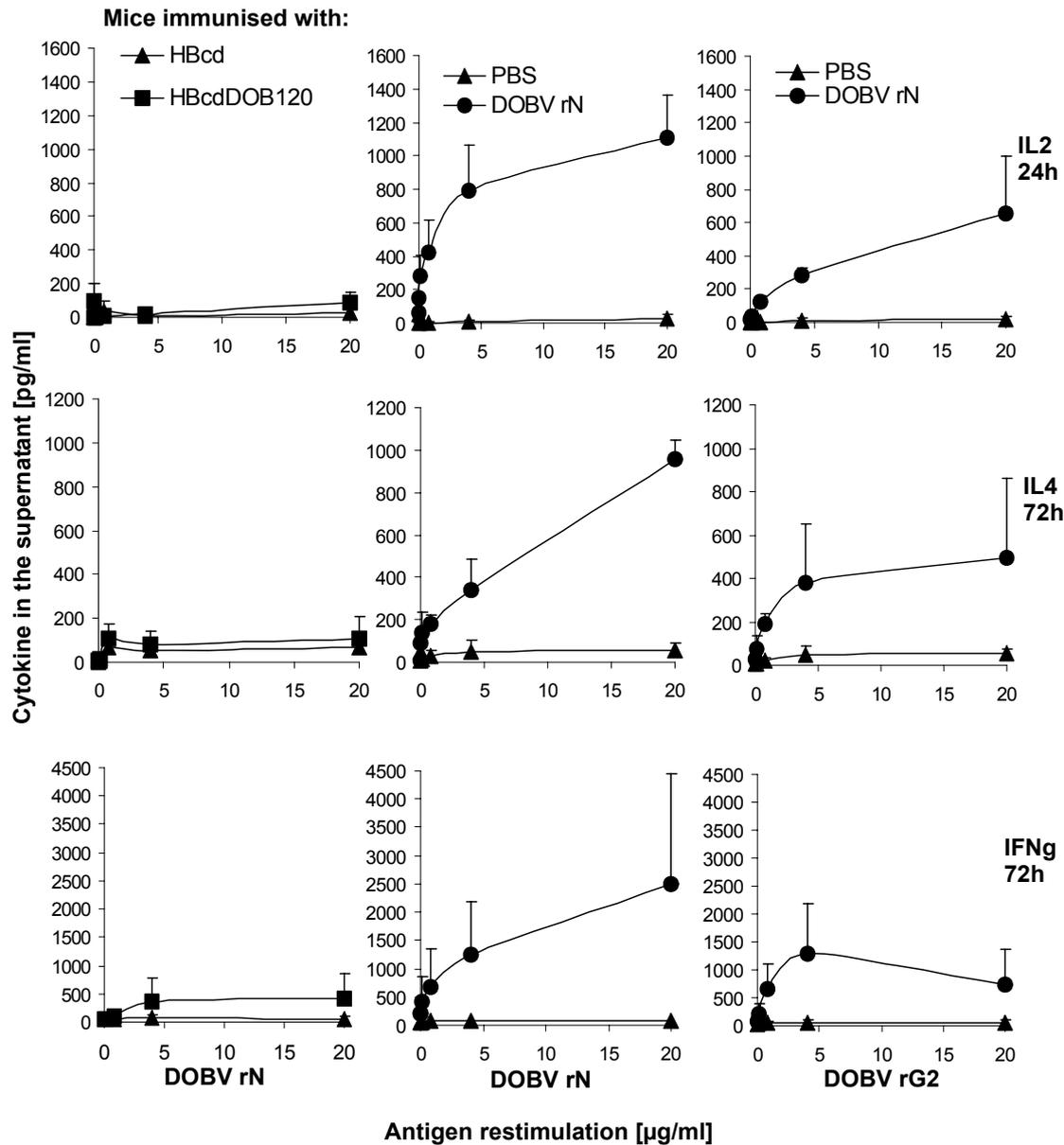
Cells of mice immunised with HBcdDOB120 secreted very little (BALB/c) or no

(C57BL/6) IL-4 and IFN- $\gamma$  after restimulation with DOBV rN protein. Like IL-2, BALB/c cells secreted the highest amount of IFN- $\gamma$  (230 pg/ml) after restimulation with 20  $\mu$ g/ml DOBV rN (Fig. 11) At this concentration lymphocytes secreted the most IL-4 and IFN- $\gamma$  after 48 h (Fig. 13) and 72 h (Fig. 11) of restimulation. However, in one experiment highest IL-4 concentrations (80 pg/ml) were found after restimulation of lymphocytes with 5  $\mu$ g/ml DOBV rN (data not shown).

Lymphocytes from control mice immunised with HBcd secreted also low amounts of cytokines after restimulation with DOBV rN protein (Figs 11-13). Highest concentrations of IL-4 (< 70 pg/ml) and IFN- $\gamma$  (< 90 pg/ml) were emitted by BALB/c lymphocytes after restimulation with DOBV rN protein for 72 h (Figs 11).

The cells from BALB/c mice (Fig. 11, 13) and a C57BL/6 mouse (Fig. 12) immunised with DOBV rN protein both secreted IL-2 after restimulation with DOBV rN protein. However, level of IL-2 was much higher in the supernatant of BALB/c lymphocytes compared to the supernatant of C57BL/6 lymphocytes. The highest level of secretion was observed after restimulation with 20  $\mu$ g/ml, 1,500 pg/ml in BALB/c cells and 75 pg/ml in C57BL/6 cells. Restimulation of the lymph node cells from BALB/c, but not the C57BL/6 mouse with rG2 control protein resulted in the detection of IL-2 secretion of more than half of the level after restimulation with rN protein (Figs 11, 12).

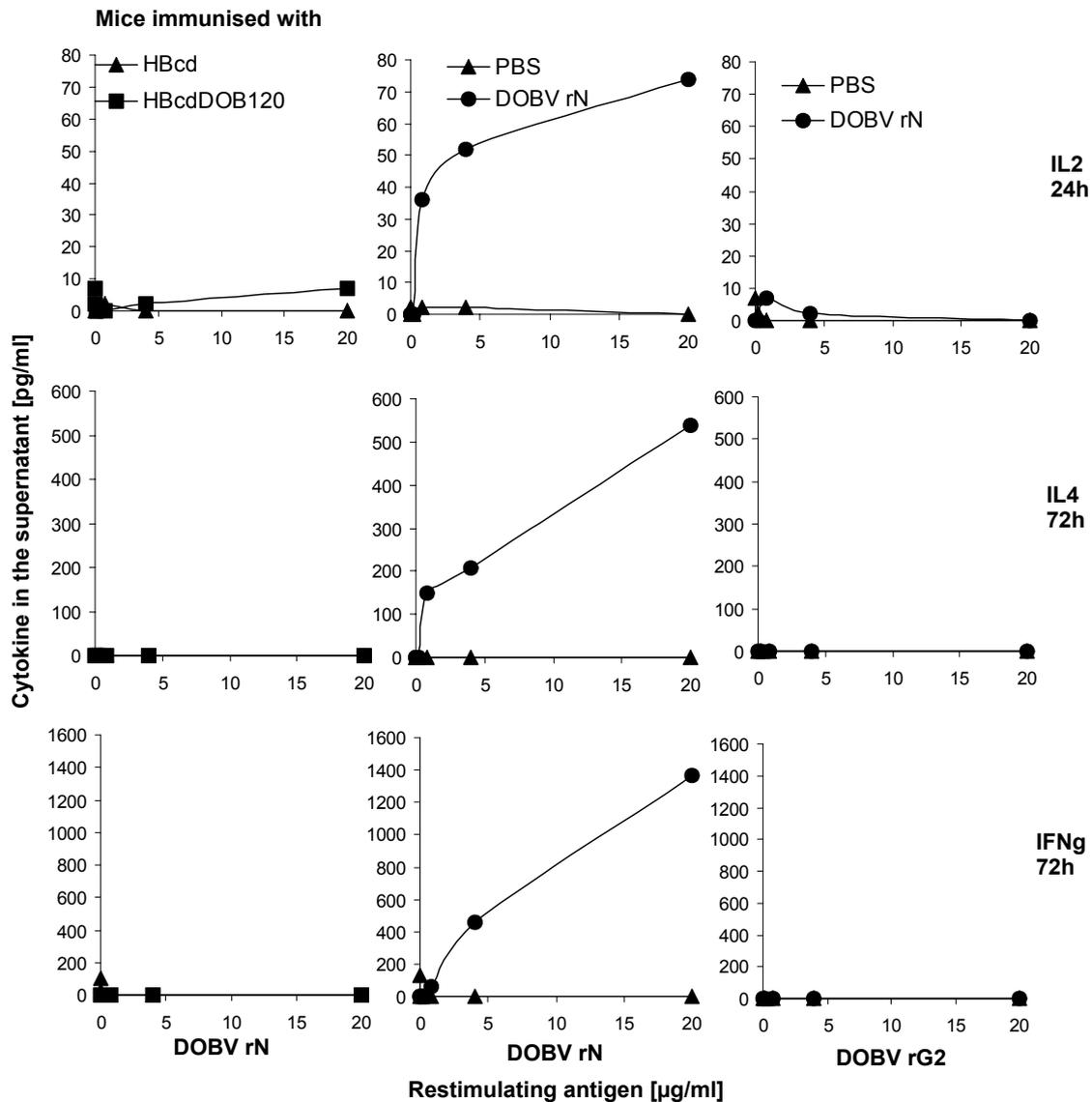
Immunisation of BALB/c and C57BL/6 mice with DOBV rN protein induced lymph node cells to secrete high amounts of IL-4 and IFN- $\gamma$  after restimulation with DOBV rN protein (Figs 11-13). Highest secretion of IL-4 was 930 pg/ml (BALB/c, Fig. 11) and 540 pg/ml (C57BL/6, Fig. 12) after restimulation of cells with 20  $\mu$ g/ml DOBV rN protein. Also IFN- $\gamma$  was secreted most after restimulation of cells with 20  $\mu$ g/ml, 4,800 pg/ml in BALB/c (Fig. 11) and 1,400 pg/ml in C57BL/6 cells (Fig. 12). In the same line, BALB/c lymphocytes secreted the highest amounts of IL-4 and IFN- $\gamma$  after 48 h of restimulation with 20  $\mu$ g/ml rN protein (Figs 13).



**FIGURE 11: Analysis of IL-2, IL-4 and IFN- $\gamma$  secreted by lymphocytes from four BALB/c mice.** Mice were immunised with HBcdDOB120 or DOBV rN protein and supernatant from cultured lymphocytes was collected after 24h (IL-2) or 72 h (IL-4, IFN- $\gamma$ ) of restimulation with different concentrations of DOBV rN protein. Negative control mice were immunised with HBcd and PBS, respectively. Cells from mice immunised with DOBV rN protein were additionally restimulated with rG2 protein to estimate cytokine secretion due to cells reacting to His-tag or potential yeast contaminations. Supernatants were taken from cells used in the proliferation assay (Fig. 10) and cytokine concentrations were determined by sandwich ELISA (chapter 2.4.4).

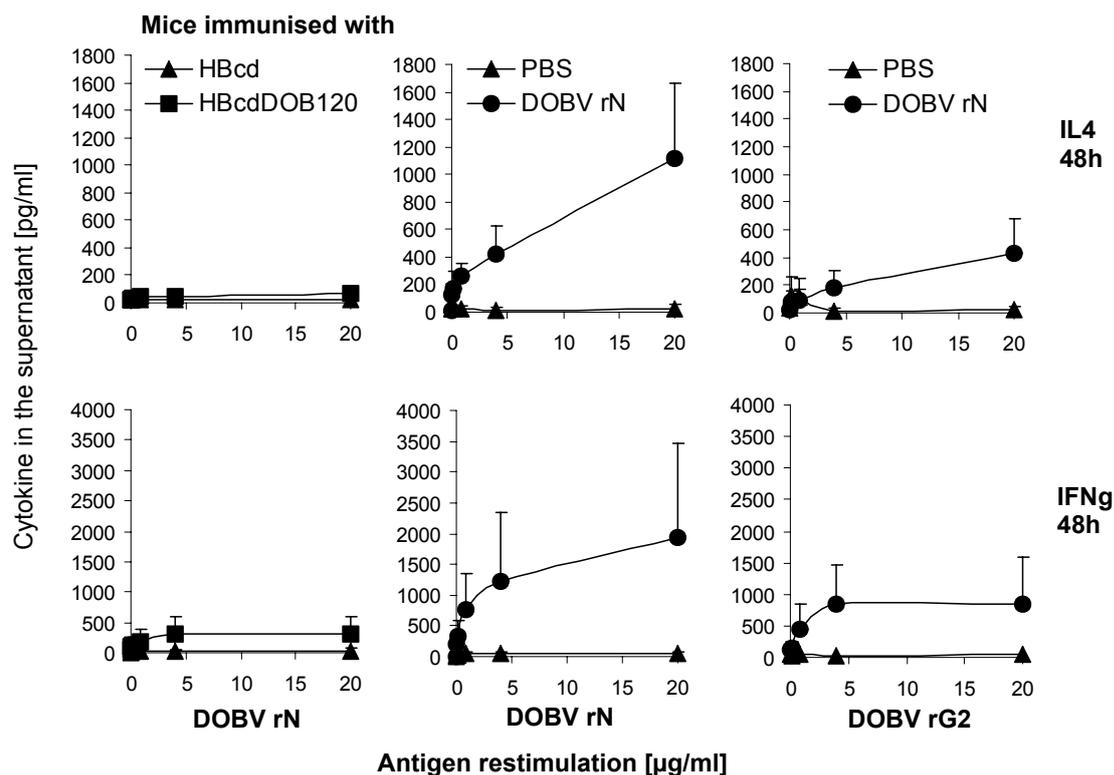
Restimulation with rG2 protein also caused the cells from BALB/c, but not cells from the C57BL/6 mouse immunised with DOBV rN protein to secrete high amounts of IL-4 and IFN- $\gamma$  (Figs 11, 12). However, the level of IL-4 and IFN- $\gamma$  induction was lower at most restimulating antigen concentration when compared to

rN protein stimulation (Figs 11, 12).



**FIGURE 12: Analysis of IL-2, IL-4 and IFN- $\gamma$  secreted by lymphocytes from one C57BL/6 mouse.** Mice were immunised with HBcdDOB120 or DOBV rN protein and supernatant from cultured lymphocytes was collected after 24h (IL-2) or 72 h (IL-4, IFN- $\gamma$ ) of restimulation with different concentrations of DOBV rN protein. Negative control mice were immunised with HBcd and PBS, respectively. Cells from mice immunised with DOBV rN protein were additionally restimulated with rG2 protein to estimate cytokine secretion due to cells reacting to His-tag or potential yeast contaminations. Supernatants were taken from cells used in the proliferation assay (Fig. 10) and cytokine concentrations were determined by sandwich ELISA (chapter 2.4.4).

Cells of mice immunised with PBS did not secrete any IL-2, IL-4 or IFN- $\gamma$  (Figs 11-13).



**FIGURE 13: Analysis of IFN- $\gamma$  and IL-4 secreted by lymphocytes from four BALB/c mice.** Mice were immunised with HBcdDOB120 or DOBV rN protein and supernatant from cultured lymphocytes was collected after 48 h of restimulation with different concentrations of DOBV rN protein. Negative control mice were immunised with HBcd and PBS, respectively. Cells from mice immunised with DOBV rN protein were additionally restimulated with rG2 protein to estimate cytokine secretion due to cells reacting to His-tag or potential yeast contaminations. Supernatants were taken from cells used in the proliferation assay (Fig. 10) and cytokine concentrations were determined by sandwich ELISA (chapter 2.4.4).

## 4 Discussion

### 4.1 A preexisting immunity to the carrier protein rather boosts the immunity to the antigenic insert

Hepatitis B is a widespread infection resulting in a high prevalence of anti-HBc antibodies in the human population worldwide. These pre-existing anti-HBc antibodies might influence the success of a vaccine based on HBc as a carrier protein. In this study data are presented that a preexisting immunity to HBc induced by immunisation with particles formed by entire core protein did not abrogate the DOBV N-specific antibody response after immunisation with HBcdDOB120. Similarly, in mice preexisting antibodies to HBV small surface

antigen (HBs) only slightly suppressed the antibody response against part of the hepatitis C virus envelope presented on HBs particles [Netter, 03]. In line, in mice immunised with a *Haemophilus influenzae* type B (HiB) polysaccharide/BSA vaccine, an enhancing effect of BSA carrier protein-specific preexisting antibodies on the anti-HiB immune response was observed [Schneerson, 80]. In children that were primed with a diphtheria toxin also an enhanced immunogenicity of a HiB polysaccharide coupled to diphtheria toxin was found [Granoff, 93].

The antigen dose used for priming the anti-carrier immune response has been reported to influence the outcome whether the immune response to the respective antigen presented on the carrier is enhanced or suppressed [Peeters, 91]. Therefore, additional studies are needed to investigate if the titre of preexisting anti-core antibodies might influence the results of the immunisation with chimeric particles. Additionally it should be investigated, if the dose of HBcdDOB120 influences DOBV N-specific titre in mice with anti-core immunity.

These data are in contrast to the negative influence of a preexisting immunity to the carrier when trying to deliver a vaccinia virus construct encoding HTNV N and glycoproteins to vaccinia virus-immune volunteers [McClain, 00]. In the same line, in mice with preexisting immunity to an adenovirus (AdV) carrier, the expression of luciferase provided by an AdV-luciferase gene vector was significantly decreased compared to mice lacking an AdV-specific immunity [Vlachaki, 02]. Preexisting antibodies against human papillomavirus (HPV) capsid proteins L1/L2 also abolished the protection from a tumor challenge in mice immunised with chimeric HPV-L1/L2 VLPs harbouring a tumor protein segment expressed by the tumor [Da Silva, 01c; Da Silva, 03b].

Likely, the presence of carrier-specific antibodies might result in the opsonisation of most particles, of both viral vectors and VLPs, and their uptake by antigen presenting cells (APCs) via Fc receptors. Therefore, a possible explanation for the different influence of a preexisting immunity on DNA-based vectors versus nucleic acid-free VLPs might be the following. When vectors or particles are opsonised with antibodies, foreign protein-encoding DNA in the vector gets degraded. On the other hand the foreign protein insert of chimeric VLPs will get presented better on MHC class I and II. This enhanced MHC class II presentation could induce a stronger insert-specific antibody response. As such an enhancement of peptide

presentation on MHC class I and II has been shown after covering protein with antibodies [Zheng, 01a; Zheng, 01b; Rafiq, 02a] this could be an explanation for the higher antibody response in mice with preexisting carrier-specific antibody titre. However, this does not explain the lower protectivity against tumors in mice with a preexisting L1/L2-specific immune response after immunisation of mice with chimeric L1/L2/tumor antigen particles [Da Silva, 01b; Da Silva, 03a]. It seems that particles that consist on carrier proteins that confer receptor dependent uptake, like papillomavirus L1/L2 [Da Silva, 01a] induction of insert specific immune response is hampered by preexisting carrier specific immune response. This is in contrast to particles based on carrier proteins HBc (this study) and small HBs [Netter, 03].

Here, only the influence of a preexisting antibody response to the carrier protein on the insert-specific antibody response was measured. But, as preimmunisation induced HBc-specific IgG of all subclasses, it is likely that the preimmunisation also induced HBc-specific T-cells, as would happen in a natural HBV infection. This suggests that an immunisation with HBc-derived VLPs would be successful in people that once were infected with HBV. However, it remains to be proven if HBc-specific T cells have an abolishing effect on the induction of insert-specific immunity or protection. Additionally it should be proven if the immune response obtained in mice with a preexisting anti-core immunity can be confirmed in the situation of a preexisting immunity due to HBV infection, e.g. in a primate model.

#### **4.2 DOBV N proteins induce a similar immune response as other hantavirus N proteins**

In south-east Europe the *A. flavicollis*-adapted DOBV is the major cause of severe clinical courses of HFRS. In addition, experimental inoculation of this virus was found to kill suckling mice [Klingström, 03]. While the immunogenicity and protectivity of PUUV rN protein in rodents has been investigated in several studies [de Carvalho Nicacio, 01b; Dargeviciute, 02; de Carvalho Nicacio, 02], less is known about the immunogenicity of DOBV rN protein. Therefore, in this study the immunogenicity of two different protein derivatives based on the N protein of an *A. flavicollis*-adapted DOBV, HBcdDOB120 and DOBV rN protein was investigated.

In line with initial immunisation studies in rabbits [Razanskiene, 04], high antibody titres were induced in BALB/c and C57BL/6 mice strains by immunisation with HBcdDOB120 and DOBV rN protein. This strong immunogenicity is also reflected by the fact, that already after the primary immunisation a high-titred antibody response was observed. These data are similar to those found for *E. coli*-expressed PUUV-Kaz and yeast-expressed PUUV-Vra rN proteins in the same mice strains [de Carvalho Nicacio, 01a; A. Zvirbliene et al., unpublished data]. The homologous antibody titres observed by us after immunisation with DOBV rN protein were comparable to homologous antibody titres in bank voles after immunisation with PUUV-Kaz rN protein, but much higher than those found in bank voles after immunisation with DOBV-Slo rN protein [de Carvalho Nicacio, 02]. If this difference is caused by the different expression systems used to produce the rN proteins or by a suboptimal detection of bank vole antibodies by anti-mouse IgG remains to be elucidated.

As previously observed for entire *E. coli*-expressed rN proteins of different hantaviruses (de Carvalho Nicacio et al., 2002), immunisations of mice with chimeric HBc particles and entire DOBV rN protein resulted in a highly cross-reactive antibody response. As discussed below (chapter 4.4), this confirms again previous data about a highly cross-reactive region located in the amino-terminus of the N protein.

In the same line, rabbit sera raised against yeast-expressed rN proteins of DOBV and HTNV [Razanskiene, 04] and sera from mice immunised with DOBV-Slo rN and HBcdDOB120 protein (this study) showed the same pattern of cross-reactivity. The sera reacted to high extent to the rN proteins of HTNV and DOBV and to slightly lower extent to PUUV, SNV and ANDV. The same lower reactivity to PUUV and ANDV rN protein was found in sera of DOBV-Slo rN protein immunised bank voles [de Carvalho Nicacio, 02]. For the sera of DOBV rN and HBcdDOB120 immunised mice from this study this pattern of cross-reactivity was not only observed in ELISA, but also in immunofluorescence analysis using HTNV- and PUUV-infected Vero E6 cells. When looking at PUUV immunised rabbits [Razanskiene, 04] and PUUV or ANDV immunised bank voles [de Carvalho Nicacio, 02] the reverse cross-reactivity can be seen, with lower reactions to rN proteins of DOBV and HTNV.

Interestingly, this very high level of cross-reactivity could not be found by Western Blot analysis of sera from HBcdDOB120 immunised mice; the sera reacted only weakly with rN proteins of PUUV-Sot and PUUV-Kaz and not at all with PUUV-Vra. This might mean that the cross-reactive antibody bind to conformational epitopes that were destroyed by the reducing SDS buffer used in the gels. On the other side this could reflect the lower sensitivity of Western Blots compared to ELISA. Thus, further investigation are needed to investigate if the conformational nature of the epitopes of cross-reactive antibodies induced by the immunisation with HBcdDOB120 is responsible for the lack of reactivity to denatured PUUV rN.

In this study N-specific IgG of all subclasses were detected in mice after immunisation with DOBV rN protein. In the same line, N-specific antibodies of the subclasses IgG1, IgG2a and IgG2b were found in BALB/c and C57BL/6 mice after two immunisations with PUUV rN protein, supplemented with the same adjuvants as used in this study [de Carvalho Nicacio, 01g]. However, in contrast to this study, no IgG3 was found. If this difference to the data outlined in this study is due to intrinsic properties of the rN proteins from different hantaviruses, due to the differences in expression systems, *E. coli* [de Carvalho Nicacio, 01h] and yeast (this study) or due to differences in the affinity of the detection antibody used remains to be investigated.

HBcdDOB120, as DOBV rN protein also induced N-specific IgG of all four subclasses. In line with that, immunisation of mice with chimeric HBcdHTN120 or HBcdPUUV120 VLPs or chimeric HaPyV VP1-derived VLPs harbouring 120 aa of PUUV-Vra N protein [Gedvilaite, 04] also resulted in the induction of N-specific antibodies of all four IgG subclasses. The presence of all all IgG subclasses, especially IgG1 and IgG2a, suggests the induction of a mixed Th1/Th2 response (see chapter 4.5).

In respect to cellular immune response, HBcdDOB120 (this study) induced the same low levels of IL-2 and IL-4 secretion as the chimeric HaPyV VP1 VLPs harbouring 120 aa of PUU N protein [Gedvilaite, 04]. However, immunisation of bank voles with *E. coli*-derived rN proteins of DOBV, PUUV and ANDV [de Carvalho Nicacio, 02] resulted in the generation of PUUV N-specific lymphocytes. In contrast, this study showed that immunisation of mice with yeast-derived DOBV rN protein resulted in low levels of DOBV N-specific lymphocytes (further

discussed in chapter 4.5). In the future it should be investigated what determines this difference in N-specific lymphocyte induction. Thus, proliferative response of DOBV N-specific lymphocytes of mice or bank voles immunised with *E. coli*- and yeast-derived DOBV rN protein, respectively, should be investigated in the same experiment.

Taken together, besides the induction of only low levels of N-specific lymphocytes after DOBV rN protein found in this study, HBcdDOB120 and DOBV rN proteins induce a similar immune response as other recombinant hantavirus constructs.

#### **4.3 Freund's adjuvants enhances the immune response, but does not seem to modify the N-specific Th1/Th2 cell ratio**

The antibody titres found after immunisation with HBcdDOB120 with adjuvant [data presented here and Geldmacher, 04e] were half to one order of magnitude higher compared to antibody titres in mice immunised with HBcdDOB120 without adjuvants (Geldmacher et al., unpubl. data). The immunisation scheme without adjuvant was in dosis and time schedule the same as the immunisation scheme described in Geldmacher et al., 2004b. Similarly, the N-specific antibody titre after immunisation of mice with HaPyV VP1-derived VLPs harbouring 120 aa of the PUU-Vra N protein with adjuvant was about one order of magnitude higher than that observed by immunisation with the same particles but without adjuvant [Gedvilaite, 04].

The use of CFA and IFA as adjuvants during immunisations not only amplifies the immune response, but can lead to a more Th1 and more Th2 dominated immune response, respectively [Billiau, 01a]. Therefore the induction of a mixed Th1/Th2 response by HBcdDOB120 and DOBV rN protein as shown in this study might be due to the use of adjuvants. However, immunisation of mice without adjuvants with chimeric HBcdDOB120, HBcdHTN120 or HBcdPUUV120 particles (Geldmacher et al., unpubl. data, see paragraph above) or DOBV rN protein without adjuvant (Niedrig and Geldmacher, unpubl. data) also resulted in the induction of all IgG subclassed and thereby in a mixed Th1/Th2 response. The same was found after immunisation of mice with chimeric HaPyV VP1-derived VLPs harbouring 120 aa of PUUV-Vra N protein [Gedvilaite, 04]. This suggests that the chimeric particles

and entire rN protein are able to mediate the induction of a mixed Th1/Th2 response and that the induction of subclasses are not biased by the presence of the added adjuvant.

Vaccination of mice with DOBV rN and HBcdDOB120 protein both resulted in the induction of a long-lasting N-specific immunity. Even eight months after the last immunisation high N-specific antibody titres in both mice strain were found, which suggests a possible induction of memory B cells. Alternatively, long term storage of rN protein in the organism, as is believed to be caused by the adjuvants CFA and IFA [see Billiau, 01b], and thereby repeated activation of B cells could be responsible. The half life of IgG in the serum of mice is only several days [Vieira, 86; Vieira, 88] and thereby too short to explain the presence of such high antibody titre in the serum months after immunisation without the presence of N-specific B cells.

To study if memory B cells are induced, mice should be vaccinated with the two rN protein derivatives and the immune response should be characterised after several months, e.g. by means of a B cell ELISPOT. In the B cell ELISPOT, B cells get activated to secrete antigen-specific antibodies, including effector memory B cells as well as central memory B cells [Crotty, 04]. The same should be done for immunisation of mice without adjuvants.

The data indicates that adding CFA and IFA as adjuvants in this study had increased the N-specific immunity but did not seem to bias the immune response towards Th1 or Th2. However, immunisations of DOBV rN protein without adjuvants was investigated in a very small number of mice (Niedrig and Geldmacher, unpubl. data). Thus, mice should be immunised with DOBV rN protein without adjuvants to test further if the same kind of immune response is induced as with adjuvants. Additionally, lymphocytes of these mice should be tested on their cytokine secretion pattern (e.g. IFN- $\gamma$  versus IL5) by ELISPOT assay, intracellular cytokine staining or cytokine secretion assay to characterise the N-specific Th1/Th2 response more directly.

#### **4.4 The antibody response induced by chimeric HBcd protein resembles the one induced by entire rN protein**

In the bank vole challenge model [Lundkvist, 96], animals have been challenged two weeks after the third immunisation. The antibody titres detected in the present study two weeks after the third immunisation with complete rN protein (429 aa) are slightly higher than titres found after immunisation with HBcdDOB120 particles carrying 120 amino acids of the rN protein. The same slightly higher antibody titre is seen in BALB/c mice immunised with complete rN of PUUV-Vra compared to titres of mice immunised with VLPs carrying 120 aa of the PUUV rN protein [Gedvilaite, 04, Zvirbliene unpubl. data].

An immunisation with lower doses (10 µg, 20 µg) of HBcdDOB120 resulted in C57BL/6 mice in an about one fold lower reciprocal log antibody titre than BALB/c mice [Geldmacher, 04d, Geldmacher et al., unpubl. data and 4.3]. In contrast, the immunisation with a high dose (50 µg) of HBcdDOB120 induced the same N-specific antibody titre in BALB/c and C57BL/6 mice. The lower antibody response in C57BL/6 mice when immunised with a low dose of particles might be explained by the fact that C57BL/6 mice, compared to BALB/c mice, represent weaker responders to the HBc antigen [Milich, 87]. However this lower response in C57BL/6 mice could be overcome by immunising mice with a higher dose of chimeric particles as shown in this study.

The titres of N-specific antibodies in mice immunised with HBcdDOB120 particles were only slightly lower than those observed in mice immunised with the entire yeast-expressed rN protein of DOBV-Slo. On the one hand this outcome reflects the fact that the amino-terminus of the N protein represents the immunodominant region of this protein [Jenison, 94b; Lundkvist, 96; Elgh, 96b; Gött, 97]. On the other hand these slightly lower titres might be explained by the presence of additional, more carboxy-terminally located epitopes in N protein [for references see Lundkvist, 02]. The strong cross-reactivity of sera from mice immunised with HBcdDOB120 or DOBV rN protein found in this study is also confirmed by binding of mAbs, directed against the amino-terminal region of N, to heterologous N proteins [Dzagurova, 95; Yoshimatsu, 96c; Razanskiene, 04].

Taken together, besides the slightly lower antibody titre and the lack of cross-

reactivity to denatured PUU-Vra rN protein in the sera of HBcdDOB120 immunised sera, HBcdDOB120 and DOBV rN protein induce very similar antibody response.

#### **4.5 Chimeric HBc particles as well as entire rN protein induce some N-specific lymphocytes**

A switch in IgG subclasses indicates the presence of T helper cells (Th cells). The occurrence of the subclass IgG2a in mouse sera is strongly correlated to IFN- $\gamma$ -secreting Th1 cells while the presence of IgG1 is strongly correlated to IL-4-secreting Th2 cells [Isakson, 82; Snapper, 87]. So far, a similarly strong correlation between the subclasses IgG2b and IgG3 to any cytokine has not been found [Snapper, 97]. Nevertheless, IgG subclass switching to IgG2b has been thought of being forced by TGF- $\beta$  produced by several cell types [reviewed by Benoist, 98]. However, IgG2b generation is not driven by Th1 nor Th2 cells [Stevens, 88]. The production of IgG3 has been induced by IFN- $\gamma$  in the presence of anti-IgD antibodies coupled to dextran and IL-5 (Snapper 1992), but inhibited by IFN- $\gamma$  in the presence of LPS [Snapper, 87]. In addition Th1 as well as Th2 cells can induce an IgG subclass switch towards IgG3 [Stevens, 88]. The latter experiments indicated that an IgG subclass switch in B cells does not entirely depend on cytokine regimes.

The majority of IFN- $\gamma$  and IL-4 that induce IgG subclass switches is secreted by Th1 and Th2 cells, respectively. NK, NKT and mast cells also secrete IFN- $\gamma$  and IL-4, respectively, but are present in only small amounts in the lymph nodes and spleen, where B cell isotype switching is thought to mainly take place.

The N-specific IgG subclass distribution after HBcdDOB120 vaccination might have been induced solely by the presentation of N protein on HBcd particles and not by the intrinsic antigenicity of the N insert. Truncated HBcd particles have been proven before to induce, compared to full length HBc, a IgG1 dominated IgG subclass response [Milich, 97a], due to the lack of the RNA binding domain in truncated HBcd [Riedl, 02]. However, as complete DOBV rN is also inducing a similar IgG subclass distribution, the IgG subclass distribution might be caused by an intrinsic property of the N protein and not by HBcd carrier characteristics.

HBcdDOB120 VLPs and DOBV rN protein both induced N-specific IgG antibodies of all subclasses. The IgG1 to IgG2a ratio is slightly smaller in the serum of animals immunised with HBcdDOB120 compared to the ratio in sera of animals immunised with DOBV rN protein. As outlined above, this indicates a stronger influence of IFN- $\gamma$  in the mice immunised with HBcdDOB120 compared to the influence of IFN- $\gamma$  in mice immunised with DOBV rN protein. Nevertheless the ratio of IgG1 to IgG2a at all time points is above 1 also in HBcdDOB120 immunised animals suggesting dominance of IL-4 and thus Th2 cells. This bias towards Th2 is expected when immunising with protein, as external proteins are known to induce a stronger antibody and Th2 cell than CTL and Th1 cell response. However, lymph node cells of animals immunised with HBcdDOB120 showed very little proliferation and cytokines after restimulation with DOBV rN.

T cells, secreting IFN- $\gamma$  and IL-4, leading to N-specific IgG2a and IgG1 switch, respectively (see above) have most probably been present during B cell activation after HBcdDOB120 and DOBV rN protein immunisation. It is possible that HBcd-specific T cells helped N-specific T cells in terms of proliferation by secreting IL-2. However, HBcd-specific T cell proliferation is low after immunisation with HBe [Milich, 97b], a protein very similar to the C-terminally truncated HBcd used in this study. However some HBc-specific T cell proliferation has been observed after immunising BALB/c mice with HBcd [Borisova, 93a]. Therefore, in this study the cytokines present at the IgG subclass switch of N-specific B cells probably could have come partly from N-specific and partly from HBcd-specific T cells. However, levels of N-specific T cells were low, as seen by the low proliferation and low secretion of IL-2, IL-4 and IFN- $\gamma$ .

It has been found that part of the HBV surface antigen (HBs) inserted into a C-terminally truncated HBcd induced more insert-specific T cell proliferation [Borisova, 93b] compared to this study. In the same line, it has been shown that a papillomavirus E17 protein epitope inserted into particles composed of full length HBc protein can induce E17-specific T cell proliferation as well as IL-2 and IL-4 secretion [Tindle, 94]. In both cases, the epitope was inserted at the same region of HBc as the 120 aa of the DOBV N protein into the C-terminally truncated HBcd used in this study. As has been shown previously, HBc/epitope fusion proteins elicited low or no epitope-specific CTL responses in mice [Street, 99; Storni, 02]

indicating limited induction of a Th1/CTL response. The two protein derivatives used in this study also did not generate any cytolytic T cells in five experiments (Geldmacher unpubl. data). However, those experiments should be repeated with a positive control group of mice immunised with an N protein construct that induces N-specific CTLs. This would make it possible to investigate whether the target cells that expressed N protein could be lysed by N-specific CTLs.

In contrast to this study, a stronger induction of N-specific T cells was found in bank voles after immunisation with *E. coli*-derived DOBV rN protein [de Carvalho Nicacio, 02, see chapter 4.2]. One explanation for this difference in T cell induction could be the difference in time from immunisation to the sacrificing of the animals. When a moderately high proliferation of N-specific spleenocytes were found after immunisation with *E. coli*-derived rN proteins days after immunisation [de Carvalho Nicacio, 02], while a longer time schedule was used in this study (see Fig. 2B). However, two other experiments in which BALB/c mice were sacrificed a few days after HBcdDOB120 and DOBV rN protein immunisation (Geldmacher, unpubl. data) the same proliferation as shown in Fig. 10 was found: no or hardly any N-specific proliferation after HBcdDOB120 and an SI of up to 5 in lymph node cells from DOBV rN protein immunised mice. This indicates that the difference in N-specific proliferative response when comparing the data described by De Carvalho Nicacio [de Carvalho Nicacio, 02] and the data described in this study is not due to differences in immunisation schedule.

Lymphocytes from mice immunised with DOBV rN proliferated after restimulation with DOBV rN, but proliferated in some antigen concentrations as strongly after restimulation with a yeast-expressed negative control protein, rG2. However, it has recently been found out that there is a problem with measuring the concentration of rG2, as it is not well coloured by Bradford reagent nor by Coomassie and thereby concentration is often underjudged (Razanskiene, personal communication). Therefore, if amounts of restimulating G2 was underestimated by two fold, then some N-specific T cells were induced after DOBV rN protein immunisation. Another proof for the induction of N-specific T cells after immunisation with DOBV rN protein is the high N-specific IL-2 secretion by lymphocytes. However, further investigations studying the induction of N-specific T cells are needed, e.g. by measuring proliferation using a different protein as

negative control.

#### **4.6 Compared to DOBV rN protein HBcdDOB120 seems to need less T cell help to induce an N-specific immune response.**

After the first immunisation the antibody titre in BALB/c and C57BL/6 mice immunised with HBcdDOB120 is slightly higher than in animals immunised with complete rN protein. The slightly higher titre of animals immunised with HBcdDOB120 after the first immunisation could be due to the T cell independent B cell activation by the repetitive nature of the HBc antigen as described for nude B10.BR mice [Milich, 86a]. In another study, chimeric HBc particles have been shown to induce T cell independent insert-specific antibodies in mice [Fehr, 98]. When antigens, so called T cell independent antigens, do not require T cells for activating B cells, they are thought to induce a more rapid antibody response compared to T cell dependent antigens [DeFranco, 98].

Besides the particulate nature, LPS contamination of HBcdDOB120 [Geldmacher, 04c] is another possible cause for the more rapid antibody response induced by HBcdDOB120 compared to the antibody response induced by DOBV rN protein. LPS can also act as a T cell independent antigen and polyclonally activate B cells [reviewed by DeFranco, 98]. When immunising mice with HBcdDOB120 particles, the contaminating LPS might still be in the proximity of the particles after immunisation. The LPS could then activate DOBV N-specific B cells that would otherwise be activated much later, after N-specific T cell help became available.

In nude mice, which lack most T cells, HBc particles are able to induce IgG antibodies, most of which were of the IgG2b subclass [Milich, 87]. Other studies showed a predominance of IgG3 in the sera of fully immunocompetent animals immunised with T cell independent antigens [Snapper, 87; Snapper, 92; Snapper, 97]. When comparing the ratio of the DOBV rN-induced IgG2b and IgG3 antibody titre to the total IgG antibody titre a slightly higher ratio in sera of animals immunised with HBcdDOB120 compared to the ratio in sera of animals immunised with DOBV rN protein were observed. In addition to the rapid antibody response after immunisation with HBcdDOB120 described above, these high IgG2b and IgG3 titres might be further evidence for the partially T cell independent nature of

HBcdDOB120 particles. Additionally, the high IgG3 titre indicated that not LPS contamination is causing the T cell independent nature of the HBcdDOB120 particles as the presence of IFN- $\gamma$  seems to inhibit IgG3 in LPS-activated B cells [Snapper, 87]. However, only the ratios of IgG2b, but not IgG3 to total IgG were significantly higher in HBcdDOB120 immunised BALB/c (but not C57BL/6) mice compared to animals immunised with DOBV rN, a protein that is very probably not a T cell independent B cell activator.

Additional studies are needed to tackle the question of the T cell independent nature of HBcdDOB120. Thus immunisations without adjuvants might elucidate if antibody titres in sera of HBcdDOB120 primed mice are higher than in DOBV rN protein primed mice. Furthermore immunisation of mostly T cell deficient nude mice with HBcdDOB120 particles are needed to investigate if this more rapid response is due to the T cell independent nature of the particles. Additionally, HBcdDOB120 without LPS contamination [as described in Geldmacher, 04b] and non-particulate HBcdDOB120 could be generated to see if the immune response is induced by the particulate nature of the protein or due to the T cell independent nature of the LPS contaminating the particles.

#### **4.7 Protection against hantaviruses can be conferred by N-specific T cells as well as N-specific antibodies**

In 1985 it was suggested that T cell mediated immunity plays a crucial role in fighting hantavirus infection in laboratory rodents that - in contrast to the natural rodent reservoir - cannot be persistently infected [Nakamura, 85a]. The possibility to readily infect T cell deficient nude mice but only transiently infect immunocompetent BALB/c mice with HTNV [Asada, 87a] also indicated that T cells are important in protection against hantavirus infection. However, T cells are needed for an efficient B cell response. Consequently the lack of a T cell help to B cells might be in part responsible for the persisting infection of nude mice and for the failure to persistently infect immunocompetent mice with hantaviruses.

In the same line it has been shown in adoptive transfer experiments that CD4<sup>+</sup> and CD8<sup>+</sup> cells are of importance in the protection against a HTNV challenge in mice [Asada, 87e]. The biggest drop in protection was observed when Lyt1<sup>+</sup> (CD5<sup>+</sup>)

cells were lysed before transferring spleen cells from HTNV immunised mice to naive mice. As CD5 is expressed on T cells and a subset of B cells this underlines again the necessity for these cell types in protection against hantavirus infection. However, the specificity the transferred cells had not been studied.

Splenocytes of bank voles immunised with rN proteins of different hantaviruses proliferated to a similar extent after restimulation with PUUV rN protein [de Carvalho Nicacio, 02]. However, N-specific stimulation indices were slightly higher after immunisation with rN protein of PUUV and Topographov (TOPV). As the rN protein of these two hantaviruses protected all 16 bank voles against PUUV infection, this suggests a role for N-specific T cells in protection [de Carvalho Nicacio, 02]. In contrast to N-specific splenocyte proliferation, N-specific antibody response was not directly associated to protection. PUUV N-specific antibody response was highest in PUUV rN protein, but second highest in the ANDV rN protein immunised group, in which only three of eight bank voles were protected against a PUUV challenge [de Carvalho Nicacio, 02]. This might suggest a stronger importance of N-specific T cells than of N-specific antibodies in protection against the virus. However, the level of N-specific lymphocyte proliferation as well as N-specific antibody titre both did not correlate significantly with protection. Additionally, splenocytes of DOBV rN protein immunised bank voles proliferated to a similar level as cells from ANDV rN protein immunised animals, even though DOBV rN protein protected twice as many animals (7 of 10) as ANDV rN protein (3 of 8) [de Carvalho Nicacio, 02]. As the titre of PUUV N-reactive antibodies was higher in ANDV rN protein immunised bank voles, antibody level could not explain this difference in protection. One explanation for the higher protection of DOBV rN protein immunised animals could be that DOBV rN protein harbours more conserved CTL epitopes that are identical in the PUUV N protein. However, as protein immunisation does normally not induce a lot of CTLs (discussed in chapter 4.5), this explanation seems unlikely.

For some enveloped viruses there is evidence that nucleocapsid specific antibodies can protect against a virus infection: p17-specific antibodies protected cells against HIV [Papsidero, 89], nucleocapsid specific antibodies against rabies virus [Lafon, 87] and against the Toscana virus, a representative of the genus *Phlebovirus*, family of Bunyaviridae [Cusi, 01]. For hantaviruses, it was shown *in*

*vitro* (VeroE6 cells) and *in vivo* (suckling mice model) that protection against infection can be mediated by N-specific mAbs from infection. Yoshimatsu did not measure the exact amount of antibodies used in passive transfer in the suckling mouse model [Yoshimatsu, 93] but did show some delay in death of mice challenged with HTNV that got passively transferred N-specific serum [Yoshimatsu, 96b]. In the same line, N-specific antibodies have been demonstrated to protect adult bank voles from a hantavirus infection (Lundkvist, personal communication). But, large amounts of mabs (0.5 mg) were needed for getting 50 % of animals protected and most of the N-specific mabs did not protect even when given in such a high amount (Lundkvist personal communication). The same level of protection was caused by passive transfer of spleen cells from mice immunised with HTN rN protein, which protected 43 % of suckling mice from an HTNV infection [Yoshimatsu, 93]. Therefore, protection against a hantavirus challenge induced by immunisation with N-constructs can partly be due to N-specific antibodies.

The mechanism by which antibodies specific for the internal N protein protect against the enveloped virus is not known. In the *in vitro* protection experiments mentioned above, the antibodies were scrape loaded onto the cells. Thereby, the N-specific antibodies can go into the cell and it was postulated that they inhibit uncoating or translation of the RNA [Yoshimatsu, 96a]. *In vivo* however, this mechanisms can not be imagined on big scale. ADCC was suggested as another potential protective mechanism for N-specific antibodies [de Carvalho Nicacio, 01i], i.e. by binding of antibodies to cell surface located N protein and thereby marking these cells for destruction by NK cells. However, in contrast to the glycoproteins [Ogino, 04], N protein could not be found on the surface of HTNV infected cells [Yoshimatsu, 93]. As Old World hantaviruses are thought to bud at the Golgi compartment (see chapter 1.1) it is unlikely that N protein is located on the surface of PUUV infected cells.

A possibly more likely explanation for the protection of N-specific antibodies against a hantavirus infection is the following. Antibodies bind to uncomplete virions, e.g. nucleocapsids, that are released by hantavirus infected cells and that these antibody/nucleocapsid complexes are more rapidly taken up by Fc receptor bearing APCs. These so called immune complexes might have been presented on

MHC class I and II better than protein alone [Rafiq, 02b]. Thereby nucleocapsid would be presented more rapidly and in bigger quantities on MHC class I and II to T cells. As the incubation time from hantavirus infection to outbreak of disease is relatively long (> 10 days), the rapid Fc dependent uptake of nucleocapsid protein leads to a more rapid and more vigorous T cell response that then can protect against the virus.

Taken together, the immune response induced by HBcdDOB120 is similar to the immune response induced by DOBV rN protein as shown in this study. Thus, the immunological mechanisms induced by HBcdDOB120 and DOBV rN protein leading to protection against a hantavirus infection may be similar. The same derivatives based on PUUV rN protein induced protection against a hantavirus challenge. Immunisations with HBcdPUU120 protected 88 % (Lundkvist unpub. data) and PUUV rN protein protected 100% of bank voles against a PUUV challenge [Dargeviciute, 02]. The proteins used in this study should be tested in an DOBV challenge model. Suckling mice can be infected with DOBV [Klingström, 03]. But, the immune system of suckling mice is not fully developed.. Recently it was shown that C57BL/6 mice might be used as a DOBV challenge model [Klingström, 04], even though the infection seems to be only transient. Thus both models are not ideal as a DOBV challenge model for the evaluation of vaccine candidates

In conclusion it is not clear what kind of immune response is needed to fight hantaviruses, but it can be deduced from the studies mentioned above and the results from this investigation, that N-specific antibodies as well as T cells can play a role in protecting against a hantavirus infection. Further investigations are needed to address the question of which arm of the immune response is most important in protection against hantaviruses.

## 5 Literature

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## 6 Appendix

### 6.1 Abbreviations

aa	amino acid
ADCC	antibody-dependent cytotoxicity
ANDV	Andes virus
APC	antigen presenting cell
BSA	bovine serum albumin
CFA	complete Freund's adjuvant
ConA	concanavalin A
CTL	cytotoxic T cells
DOBV	Dobrava virus
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
G1, G2	hantavirus glycoproteins 1 and 2
h	hour
HBc	entire core protein of hepatitis B virus (aa 1-183)
HBcd	carboxy-terminally truncated HBc (aa 1-144)
HBV	hepatitis B virus
HCPS	hantavirus cardiopulmonary syndrome
HLA	human leukocyte antigen
HFRS	haemorrhagic fever with renal syndrome
HTNV	Hantaan virus
HRP	horse radish peroxidase

IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
mAb	monoclonal antibody
MHC	major histocompatibility complex
N	nucleocapsid
NE	nephropathia epidemica
OD	optical density
ORF	open reading frame
PBS	phosphor-buffered saline
PUUV	Puumala virus
RdRp	RNA-dependent RNA polymerase
rG2	recombinant glycoprotein 2
rN	recombinant nucleocapsid
sc	subcutaneous
SEOV	Seoul virus
SI	stimulation index
SNV	Sin Nombre virus
Th cell	T helper cell
VLP	virus-like particle

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### 6.3 Publications

Geldmacher A., Skrastina D., Borisova G., Petrovskis I., Krüger D.H., Pumpens P., Ulrich R. **2005**: A hantavirus nucleocapsid protein segment exposed on hepatitis B virus core particles is highly immunogenic in mice when applied without adjuvants or in the presence of pre-existing anti-core antibodies. *Vaccine* 23: 3973-3983

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#### **6.4 Eidesstattliche Erklärung**

Ich erkläre, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

Astrid Geldmacher

Berlin, den 15. Oktober 2004