Immunologische und epidemiologische Aspekte antigenspezifischer T- und B-Zell-Antworten gegen humanpathogene Parvoviren

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
der Naturwissenschaftlichen Fakultät III
- Biologie und Vorklinische Medizin -
der Universität Regensburg

vorgelegt von
Juha Lindner
aus Helsinki, Finnland

Regensburg, August 2008

Das Promotionsgesuch wurde am 30.07.2008 eingereicht.
Die Arbeit wurde von Prof. Dr. Susanne Modrow angeleitet.

Prüfungsausschuss:
Vorsitzende: Prof. Dr. Charlotte Förster
1. Gutachterin: Prof. Dr. Susanne Modrow
2. Gutachterin: Prof. Dr. Rosemarie Baumann
3. Prüfer: Prof. Dr. Stephan Schneuwly

Inhaltsverzeichnis

Publikationsübersicht

Im Rahmen der Arbeit gewonnene Ergebnisse wurden in den nachfolgend aufgeführten wissenschaftlichen Publikationen veröffentlicht oder zur Publikation eingereicht. Die relevanten Originalarbeiten (I-IV) und Übersichtsartikel (VII, VIII) sind der Arbeit im Anhang beigelegt und werden im Text an den entsprechenden Stellen zitiert.

Originalarbeiten:

I. **Lindner, J.**, Zehentmeier, S., Franssila, R., Schroeder, J., Barabas, S., Deml, L., und Modrow, S.; *CD4+ T Helper Cell Responses Against Human Bocavirus VP2 Virus-like Particles in Healthy Adults.* J. Infect. Dis. 2008. Im Druck.

II. **Lindner, J.**, Karalar, L., Zehentmeier, S., Plentz, A., Struff, W., Kertai, M., Segerer, H., und Modrow, S.; *Humoral Immune Responses Against Human Bocavirus VP2 Virus-like Particles.* Viral Immunol. 2008. Im Druck.

III. Karalar, L., **Lindner, J.**, Schimanski, S., Kertai, M., Segerer, H., und Modrow, S.; *Prevalence and Clinical Aspects of Human Bocavirus Infection in Children.* Eingereicht.

IV. **Lindner, J.**, Noutsias, M., Lassner, D., Schultheiss, H-P., Kuehl, U., und Modrow, S.; *Adaptive Immune Reponses against Parvovirus B19 in Patients with Myocardial Disease.* Eingereicht.

V. **Lindner, J.**, Barabas, S., Saar, K., Altmann, D., Pfister, A., Fleck, M., Deml, L., und Modrow, S.; *CD4+ T Cell-responses Against the VP1-unique Region in Individuals with Recent and Persistent Parvovirus B19 Infection.* J. Vet. Med. B. Infect. Dis. Vet. Public Health. 2005 Sep;52(7-8):356-61.

VI. Barabas, S., Gary, R., Bauer, T., **Lindner, J.**, Lindner, P., Weinberger, B., Jilg, W., Wolf, H., und Deml, L.; *Urea-mediated Cross-presentation of Soluble Epstein-Barr Virus BZLF1 Protein.* Eingereicht.

Übersichtsartikel:

VII. **Lindner, J.**, und Modrow, S.; *Human Bocavirus – A Novel Parvovirus to Infect Humans.* Intervirology. 2008 Jun 9;51(2):116-122.

VIII. **Lindner, J.**, Karlar, L., Schimanski, S., Pfister, H., Struff, W., und Modrow, S.; *Clinical and Epidemiological Aspects of Human Bocavirus Infection.* Eingereicht.

Für die aufgeführten Publikationen bestand der eigene Arbeitsanteil in:

(i) der Durchführung aller Versuche zur Produktion, Charakterisierung, und Aufreinigung von virusähnlichen Partikeln für HBoV und B19V (Publikationen I-IV).

(ii) der Zusammensetzung der Studienkollektive (Publikationen I, V) und der Bearbeitung der Probanden- und Patientenproben (Publikationen I-V).

(iii) der Etablierung, Planung und Durchführung aller ELISA und ELISPOT Versuche zum Nachweis zellulärer (gegen HBoV und B19V) und humoraler (gegen HBoV) Immunantworten (Publikationen I-V).

(iv) der Datenanalyse und dem Schreiben der Manuskripte (Publikationen I, II, IV, V, VII und VII).

(v) der Datenanalyse und dem Schreiben des Manuskripts in Zusammenarbeit mit Dr. med. cand. Lüdya Karalar und Prof. Dr. Susanne Modrow (Publikation III) .

(vi) der Durchführung von ELISPOT-Versuchen zum Nachweis virusspezifischer T-Zell-Antworten und der Beteiligung am Schreiben des Manuskriptes (Publikation VI).

Wissenschaftliche Präsentationen

Vorträge auf Kongressen:

- **Lindner, J.**, *et al.*; Human Bocavirus in Children with Infectious Diseases. "*XII International Parvovirus Workshop*", 1.-5.6.2008, Cordoba, Spanien.

- **Lindner, J.**, *et al.*; Human Bocavirus – a new pathogen frequent in children with respiratory disease. "*ESCV 2008 Clinical Virology Annual Meeting*", 12.-15.3.2008, Saariselkä, Finnland.

- **Lindner, J.**, *et al.*; Analysis of parvovirus B19 specific T-cell immune responses in healthy adult individuals and dilated cardiomyopathy patients. "*International Parvovirus Meeting 2007*", 27.-28.9.2007, Bari, Italien.

- **Lindner, J.**, *et al.*; CD4+ T helper cell responses against human bocavirus VP2 virus-like particles in healthy adults. "*International Parvovirus Meeting 2007*", 27.-28.9.2007, Bari, Italien.

- **Lindner, J.**, *et al.*; Analysis of Parvovirus B19 Specific $CD4^+$ T-Cell Responses Using B-cells Expressing Viral Genes as Antigen-specific Stimulators. "*5th Workshop of the Study group Immunobiology of Viral Infections*", 4.-6.10.2006, Zeilitzheim.

- **Lindner, J.**, *et al.*; Analysis of Parvovirus B19 Specific $CD4^+$ T-Cell Responses Using B-cells Expressing Viral Genes as Antigen-specific Stimulators. "*XIth Parvovirus Workshop 2006*", 27.-31.8.2006, Les Diablerets, Schweiz.

- **Lindner, J.**, *et al.*; Detection of $CD4^+$ T-cell responses against the VP1-unique region in individuals with different courses of parvovirus B19 infection using transiently transfected B-cells for in vitro restimulation of peripheral blood cells. "*Jahrestagung der Gesellschaft für Virologie*", 15.-18.3.2006 München.

- **Lindner, J.**, *et al.*; $CD4^+$ T cell-responses against the VP1-unique region in individuals with persistent or acute Parvovirus B19-infection. "*International Parvovirus Meeting 2005*", 15.-16.7.2005 Leipzig.

Ausgewählte Posterpräsentationen:

- **Lindner J.**, *et al.*; Analysis of parvovirus B19 specific t-cell responses using b-cells expressing viral genes as antigen-specific stimulators. "*13th International Congress of Immunology*", 21.-25.8.2007, Rio de Janeiro, Brasilien.

- **Lindner, J.**, *et al.*; Analysis of Parvovirus B19 specific T-cell responses using B-Cells expressing viral genes as antigen-specific stimulators. "*International Parvovirus Meeting 2007*", 27.-28.9.2007, Bari, Italien.

Abkürzungsverzeichnis

AAV	Adeno-assoziiertes Virus
b	Basen
B19V	Parvovirus B19
BPV	Bovines Parvovirus
CaMV	*Canine Minute Virus*
CD	*Cluster of Differentiation*
CPV	Canines Parvovirus
CsCl	Cäsiumchlorid
DNA	Desoxyribonukleinsäure
E. coli	*Escherichia coli*
ELISA	*Enzyme-linked immunosorbent assay*
ELISPOT	*Enzyme-linked Immuno Spot Technique*
EU	*Endotoxin units*
FPV	Felines Parvovirus
HBoV	Humanes Bocavirus
HIV	Humanes Immundefizienz-Virus
HLA	*Human leucocyte antigen*
IFN	Interferon
Ig	Immunglobulin
IL	Interleukin
kDa	Kilodalton
LPS	Lipopolysaccharid
mg	Milligramm
ml	Milliliter
nm	Nanometer
nt	Nukleotide
PARV4/5	Parvovirus 4 bzw. 5
PBMC	*Peripheral blood mononuclear cells*
PCR	*Polymerase chain reaction*
PLA2	Phospholipase A2
RNA	Ribonukleinsäure
RSV	*Respiratory syncytial virus*
SDS	Natrium Dodecylsulfat
SFC	*Spot forming cells*
Th-Zelle	T-Helfer-Zelle
TNF	Tumornekrosefaktor
VLP	virusähnliche Partikel
VP1u	*VP1 unique region*

Zusammenfassung

Die adaptive Immunantwort spielt bei der Kontrolle und Eliminierung von Infektionserregern eine zentrale Rolle und verleiht dem Organismus einen meist lebenslangen Schutz gegen Reinfektionen. Der Nachweis von Immunreaktionen gegen Pathogene ist unverzichtbar für die Forschung und klinische Diagnostik, da hierdurch erregerspezifische Therapien ermöglicht werden. Dies gilt auch für Infektionen mit Parvoviren, welchen zunehmend klinische Relevanz zugesprochen wird. In der Familie der *Parvoviridae* findet man zwei humanpathogene Vertreter: Das Parvovirus B19 (B19V) und das Humane Bocavirus (HBoV). Ersteres wurde vor drei Jahrzehnten als Verursacher der Ringelröteln in Kindern beschrieben. HBoV hingegen wurde erst kürzlich entdeckt und konnte bis heute nicht eindeutig mit Erkrankungen in Verbindung gebracht werden.

In dieser Arbeit sollten durch die Analyse der HBoV- und B19V-spezifischen Immunantwort (i) Informationen zur Epidemiologie und Krankheitsassoziation von HBoV-Infektionen in gesunden Probanden und Patienten mit Infektionserkrankungen gewonnen werden und (ii) untersucht werden, ob nachweisbare Mengen an B19V-Genomen in Herzbiopsien von Patienten mit akuter Myokarditis oder dilatativer Kardiomyopathie kausal mit der Entstehung von Herzerkrankungen in Verbindung gebracht werden können.

Hierfür wurden zunächst grundlegend ELISA- und ELISPOT-Methoden zum Nachweis der B19V- und HBoV-spezifischen humoralen und zellulären Immunantwort etabliert: Durch Infektion von High5-Insektenzelllinien mit den rekombinanten Baculoviren bacVP2/HBoV bzw. bacVP2/B19V wurden für HBoV und B19V virusähnliche Partikel produziert und gereinigt, welche aus viralen VP2-Kapsidproteinen bestanden. Für B19V wurden mittels des rekombinanten Baculovirus bacVP12/B19V zusätzlich Partikel hergestellt, die aus einer Mischung der beiden Kapsidproteine VP2 und VP1 zusammengesetzt sind. Alle virusähnlichen Partikel wiesen eine für Parvoviren charakteristische Morphologie auf und dienten als Antigene für die etablierten Testsysteme.

Studien zur Verbreitung HBoV-spezifischer IgG- und IgM-Antikörper in gesunden Probanden und in Kindern mit Infektionserkrankungen zeigten, dass HBoV-Infektionen in der deutschen Bevölkerung weit verbreitet sind: Kinder sind während der ersten Lebensmonate durch mütterliche HBoV-spezifische Immunglobuline geschützt, ab einem Alter von etwa zwei Jahren findet man eine Zunahme der Prävalenz virusspezifischer IgG-Antikörper, die sich in zweijährigen Kindern und in Erwachsenen auf Werte von 38% beziehungsweise 94% beläuft. Die hohe Prävalenz von HBoV-spezifischen

Immunantworten in der erwachsenen Bevölkerung wurde zusätzlich zu den serologischen Untersuchungen durch den häufigen Nachweis von virusspezifischen IFN-γ produzierenden $CD4^+$ T-Helfer-Zellen bestätigt. HBoV-spezifische IgM wurden ausschließlich in Kindern gefunden, in deren Blut mittels PCR auch HBoV-Genome als Anzeichen akuter HBoV-Infekte nachweisbar waren. In virämischen Kindern konnten am häufigsten Symptome der unteren Atemwege (14,6%) oder des Magen-Darm-Traktes (7,8%) beobachtet werden. Darüber hinaus wurden akute HBoV-Infektionen auch in 5,0% der untersuchten Kinder nachgewiesen, welche keine Anzeichen für Infektionserkrankungen zeigten.

Im zweiten Teil der Arbeit wurde untersucht, ob man in Patienten mit Myokarditis oder dilatativer Kardiomyopathie, die sich durch das Vorhandensein von B19V-DNA im Herzgewebe auszeichnen, virusspezifische Immunreaktionen findet, welche auf eine Beteiligung von B19V an der Entstehung der Herzerkrankungen deuten. In insgesamt 20% der Patienten fanden sich mittels PCR-Analysen Hinweise auf akute, persistierende oder kürzliche B19V-Infektionen. Dies war bei 25% der Patienten mit Myokarditis und 14% der an dilatativer Kardiomyopathie erkrankten Personen der Fall. In den restlichen Patienten (80%) wurden keine Hinweise auf eine aktive Virusproduktion im Blut beobachtet. In keinen der Patienten fanden sich im Vergleich zu 30 gesunden, B19V-seropositiven Kontrollprobanden signifikante Unterschiede in der B19V-spezifischen T-Zell-Antwort. In zwei virämischen Patienten (66%) wurden neben dem Nachweis von B19V-DNA im Blut zusätzlich serologische Hinweise für akute, persistierende oder kürzliche B19V-Infektionen gefunden. In Patienten ohne nachweisbare B19V-Virämie war dies nur in einem Patienten (8%) der Fall, hier wurden Antikörper gegen lineare Epitope des VP2-Proteins als Anzeichen einer kürzlichen B19V-Infektion gefunden. Diese Antikörper konnten jedoch auch in 25% der gesunden Kontrollprobanden nachgewiesen werden. Aus den Ergebnissen lässt sich folgern, dass in seltenen Fällen eine Beteiligung akuter B19V-Infektionen an der Entstehung und/oder Aufrechterhaltung von Herzerkrankungen, insbesondere Myokarditis, in Betracht gezogen werden muss. Jedoch kann der alleinige Nachweis von B19V-Genomen in Myokardbiopsien ohne weitere Anzeichen für akute B19V-Infektionen nicht als ausreichend für die kausale Beziehung des Virus in der Krankheitspathogenese angesehen werden.

1. Einleitung

1.1. Die Familie der Parvoviridae

Die Familie der *Parvoviridae* umfasst zwei Unterfamilien, die der *Densovirinae* und der *Parvovirinae*, deren Vertreter zu den kleinsten bekannten viralen Erregern zählen (*parvus* = klein). Zu den *Densovirinae* gehören ausschließlich Parvoviren der Insekten, zu den *Parvovirinae* hingegen zählen zahlreiche tier- und humanpathogene Viren, welche sich in die Genera *Dependovirus, Erythrovirus, Parvovirus*, *Amdovirus* und *Bocavirus* unterteilen (Tabelle 1).

Wie bereits durch die Namensgebung impliziert, umfasste das Genus *Dependovirus* ursprünglich nur Vertreter der apathogenen Adeno-assoziierten Viren, welche sich ausschließlich bei gleichzeitiger Anwesenheit von Helferviren, z.B. Adeno- oder Herpesviren, replizieren können [1, 2]. Heute werden dem Genus aufgrund phylogenetischer Analysen zusätzliche, sich autonom replizierende Viren, so z.B. das Parvovirus der Gänse, zugerechnet [3, 4].

Zum Genus *Erythrovirus* gehören autonome Parvoviren mit einem engen Tropismus für erythroide Vorläuferzellen. Am besten untersucht ist das Parvovirus B19 (B19V), welches als Verursacher der Ringelröteln identifiziert wurde [5]. Es galt für lange Zeit als das einzige humanpathogene Parvovirus [6, 7].

Der Großteil aller veterinärmedizinisch relevanten Parvoviren wird dem Genus *Parvovirus* zugeordnet, so z.B. das porzine Parvovirus, das canine Parvovirus und das feline Panleukopenievirus. Als einziger Vertreter des Genus *Amdovirus* ist das Virus der Aleutenkrankheit der Nerze bekannt. Dem Genus *Bocavirus* gehören das *canine minute virus*, das Rinderparvovirus (BPV) und das im Jahr 2005 erstmals beschriebene Humane Bocavirus (HBoV) zu [8].

Neben B19V und HBoV wurden kürzlich zwei weitere miteinander sequenzverwandte Parvoviren in menschlichen Plasmaproben entdeckt, welche als PARV4 und PARV5 bezeichnet wurden [9, 10]. Inzwischen gilt PARV5 als ein weiterer Genotyp des PARV4. Eine genauere Verwandtschaftsanalyse und taxonomische Einordnung dieser Viren ist bisher jedoch noch nicht erfolgt.

Familie	Unterfamilie	Genus	Vertreter
Parvoviridae	Parvovirinae	Parvovirus	Hühnerparvovirus Felines Panleukopenievirus Canines Parvovirus Kilham Rat Virus Lapines Parvovirus *Minute Virus of Mice* Maus-Parvovirus 1 Porcines Parvovirus
		Erythrovirus	**Parvovirus B19** Parvovirus der Javaneraffen Parvovirus der Rhesusaffen Parvovirus der Schweinsaffen
		Amdovirus	Virus der Aleutenkrankheit der Nerze
		Dependovirus	Adeno-assoziierte Viren (AAV) 1, 4 **AAV 2**, **3**, **5** Bovines AAV Avines AAV Canines AAV Gänseparvovirus Entenparvovirus
		Bocavirus	*Canine minute virus* Bovines Parvovirus **Humanes Bocavirus**
	Densovirinae	Densovirus	
		Iteravirus	
		Brevidensovirus	

Tabelle 1: Die Virusfamilie der *Parvoviridae*. Humanpathogene Parvoviren sind in roter Schrift dargestellt. Die Viren PARV4 und PARV5 wurden aufgrund ihrer noch fehlenden phylogenetischen Einordnung nicht berücksichtigt.

1.2. Parvovirus B19

Das Parvovirus B19 wurde erstmals im Jahr 1975 bei Untersuchungen von Blutkonserven auf das Vorhandensein von Hepatitis-B durch die australische Virologin Yvonne Cossart zufällig entdeckt [6, 7].

1.2.1. Genomaufbau und Sequenzvariabilität

Das B19V besitzt ein für die *Parvoviridae* charakteristisches einzelsträngiges, lineares DNA-Genom mit einer Größe von 5594 Nukleotiden [11]. Die interne Kodierungsregion des Genoms wird sowohl am 3´- als auch am 5´-Ende von 383 Nukleotide umfassenden palindromischen Sequenzen, den *inverted terminal repeats*, flankiert. Diese können sich aufgrund ihrer komplementären Sequenzen haarnadel- oder pfannenstielartig aneinander lagern und dienen der Initiierung der Synthese des zweiten DNA-Stranges des viralen Genoms [12]. Weiterhin tragen sie zu einer erhöhten Genomstabilität bei [13].

Das Genom des B19V enthält zwei große offene Leserahmen, welche für das Nichtstrukturprotein NS1 und die viralen Kapsidproteine VP1 und VP2 kodieren (Abbildung 1) [14]. Insgesamt werden während der Replikation von B19V mindestens neun überlappende, alternativ gespleißte mRNA-Transkripte gebildet, deren Synthese von dem einzigen viralen p6-Promotor am 3´-Ende des Genoms kontrolliert wird [13, 15, 16]. Zusätzlich zu den NS1-, VP1- und VP2-kodierenden Transkripten werden im Rahmen einer B19V-Infektion unter anderen zwei RNA-Spezies gebildet, durch die zwei potentielle Proteine mit der Größe von 11 kDa und 7,5 kDa produziert werden könnten. Bisher konnten diese Proteine im Laufe einer B19V-Infektion nicht nachgewiesen werden, jedoch liefern kürzlich durchgeführte Deletionsstudien erste Hinweise auf einen möglichen positiven Einfluss von 11 kDa auf die Infektiosität des B19V [17].

Weltweit wurden drei Genotypen des Virus beschrieben, deren Sequenzen sich in den offenen Leserahmen um etwa 10% und im Promotorbereich um mehr als 20% unterscheiden [14, 18-21]. Die Sequenzvariabilität zwischen verschiedenen Isolaten eines Genotyps hingegen ist in der Regel mit etwa 1% Unterschied gering [22].

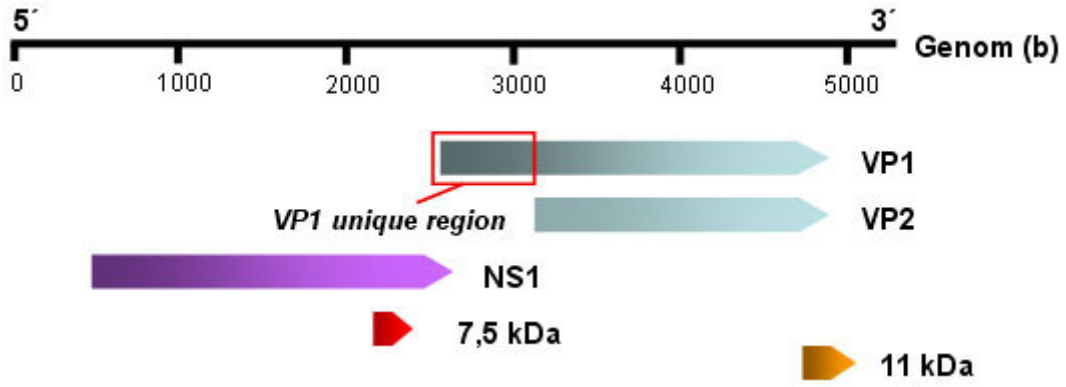

Abbildung 1: Schematische Darstellung der Genomorganisation des Parvovirus B19. Die Position und Länge der jeweiligen offenen Leserahmen der viralen Proteine sind als Pfeile dargestellt. b: Basen; kDa: Kilodalton.

1.2.2. Virusmorphologie und Struktur

Die ikosaedrischen, unbehüllten B19V-Partikel haben einen Durchmesser von etwa 18-25 nm und bestehen ausschließlich aus den beiden viralen Strukturproteinen VP1 (83 kDa) und VP2 (58 kDa). Das aus 60 Kapsomeren bestehende Kapsid wird hierbei zu etwa 95% von VP2 und zu 5% von VP1 gebildet [16]. Durch Röntgenkristallographie wurde gezeigt, dass B19V-Partikel im Gegensatz zu anderen Vertretern der *Parvoviridae*, so z.B. zum felinen und caninen Parvovirus, keine „spike-ähnlichen" Strukturen aufweisen und somit vor allem den Kapsiden des AAV-2 ähneln [23, 24]. Die für die Kapsidbildung von Parvoviren notwendige Domäne wurde mittels rekombinanter Produktion virusähnlicher Partikel in der Sequenz des VP2-Proteins lokalisiert [25, 26].

Die Sequenz des VP2 Strukturproteins ist mit der des VP1 identisch. Letzteres besitzt jedoch an seinem aminoterminalen Ende eine 227 Aminosäuren umfassende zusätzliche Domäne, die als VP1 *unique region* (VP1u) bezeichnet wird [14]. Diese Domäne weist eine Phospholipase A2-ähnliche Aktivität auf, welche für den Replikationszyklus und die Infektiosität des Virus essentiell ist [17, 27, 28].

Das virale Nichtstrukturprotein NS1 spielt eine wichtige Rolle bei der Regulation der Transkription und Replikation von B19V. Es besitzt eine Helikase- und Endonuklease-Aktivität und kann im Zellkern die Funktion des viralen p6-Promotors und zahlreicher zellulärer Promotoren, z.B. diejenigen der Interleukin (IL) 6 und Tumornekrosefaktor-alpha Gene, regulieren [29-32]. Weiterhin ist NS1 eng mit der Induktion von Kaspase-abhängigen apoptotischen Vorgängen in Wirtszellen verbunden [33].

1.2.3. Epidemiologie und Infektionszyklus

Die Infektion mit dem weltweit verbreiteten B19V erfolgt in den meisten Fällen im Kindesalter. Bei Kindern und Jugendlichen liegt die Durchseuchung in der Regel zwischen 20% (4-5 Jahre) und 60% (10-15 Jahre), bei Erwachsenen ab 18 Jahren können virusspezifische Antikörper bei etwa 70% bis zu 80% der untersuchten Individuen nachgewiesen werden [34-36]. Das B19V wird in der Regel durch Tröpfcheninfektion übertragen [37]. Da das sich in Erythrozytenvorläufern replizierende Virus eine hohe Stabilität aufweist und nur ungenügend durch Hitze oder Detergenzien inaktiviert werden kann, stellen viruskontaminierte Blutprodukte eine weitere Infektionsquelle dar [38-40]. Die Kontagiosität des B19V wird dabei durch die für die akute Infektionsphase charakteristische enorm hohe Viruslast von bis zu 10^{12} viralen Partikeln per Milliliter Blut unterstützt.

B19V kann sich ausschließlich in erythroiden Vorläuferzellen der Stadien BFU-E (*erythrocyte burst forming unit*) bis CFU-E (*erythrocyte colony forming unit*) [41], in Erythroblasten im Knochenmark [42] sowie in der fetalen Leber vermehren [43]. Hierbei dienen dem Virus das Blutgruppenantigen P [44] sowie eine Vielzahl weiterer Kofaktoren, z.B. das Autoantigen Ku80 [45] und das α5β1-Intergin [46], als Rezeptoren. Diese werden neben erythroiden Vorläuferzellen zusätzlich auf vielen anderen Zellen, beispielsweise auf reifen Erythrozyten, Endothelzellen sowie Megakaryozyten, gefunden [47]. Da diese Zellen von B19V jedoch nicht produktiv infiziert werden können, scheint der Replikationszyklus des Virus von weiteren, bisher unbekannten Faktoren mitbestimmt zu werden.

Die Aufnahme des B19V in die Zielzellen erfolgt vermutlich über rezeptorvermittelte Endozytose, wie es für CPV gezeigt werden konnte [48]. Das Blutgruppenantigen P, auch Globosid oder Globotetraosylceramid genannt, dient hierbei hauptsächlich der Bindung des B19V-Virions an die Zelle, der Zelleintritt wird hingegen durch den Korezeptor α5β1-Intergin vermittelt [46]. Durch die Phospholipase A2-ähnliche Aktivität des VP1-Proteins werden die Viren anschließend vermutlich aus Endosomen bzw. Endolysosomen freigesetzt und mittels eines Kernlokalisationssignals im VP1-Protein in den Nukleus transportiert [49]. Dort wird das virale Genom mit der Hilfe zellulärer Enzyme repliziert und transkribiert [50]. Anschließend werden im Laufe des Infektionszyklus die viralen Proteine NS1, VP1 und VP2 synthetisiert.

Die der Morphogenese und Freisetzung von reifen Viruspartikeln zugrunde liegenden molekularen Mechanismen sind weitgehend ungeklärt. Es wird jedoch vermutet, dass

Viruspartikel durch NS1-induzierte Zellzerstörung aus den Wirtszellen freigesetzt werden [33].

1.2.4. Virale Persistenz

B19V wird im Zuge von akuten Infektionen in der Regel innerhalb des ersten Monats nach Erregerkontakt von der einsetzenden Immunantwort im Organismus eliminiert. In seltenen Fällen kann sich das Virus jedoch über mehrere Monate und/oder Jahre vermehren und eine persistierende Infektion etablieren. Des Weiteren vermehren sich Anzeichen für eine häufige, potentiell lebenslange Persistenz des vollständigen Virusgenoms in verschiedenen Geweben nach dem Ablauf einer akuten B19V-Infektion. So wurde B19V-DNA bereits im Knochenmark [51, 52], im Synovialgewebe [53], in der Leber [54], im Myokard [55-57] und in der Haut nachgewiesen [58, 59]. Der grundlegende Mechanismus dieser DNA-Latenz und ihr möglicher Einfluss auf pathophysiologische Prozesse ist bisher unklar. Es ist weiterhin nicht bekannt, ob das virale Genom spontan reaktiviert werden kann und so als potentielle endogene Quelle des B19V betrachtet werden muss.

1.2.5. Klinische Relevanz

Infektionen mit B19V verlaufen in bis zu 68% aller Fälle asymptomatisch [60]. Klinisch relevante Infekte sind mit einer Vielzahl unterschiedlich schwerer Erkrankungen in Verbindung gebracht worden (Tabelle 2). Im Folgenden werden die häufigsten und für die Arbeit relevanten Symptomatiken aufgeführt.

Erkrankungen bei immunkompetenten Personen

Häufig	Allgemeines Krankheitsgefühl
	Erythema infectiosum – Ringelröteln – *Fifth disease* – *Slapped cheek disease*
	Transiente Anämie
	Transiente Mono-, Oligo- und Polyarthritis – Arthropathien
Selten	Mono-, Oligo- und Polyarthritis
	Thrombo-, Granulo- und Panzytopenie
	Akutes Leberversagen – Hepatitis
	Vaskulitis – *Giant cell arteritis* – *Polyarteritis nodosa* – Myositis
	Myokarditis
	Glomerulonephritis
	Chronic fatigue syndrome – Meningitis – Enzephalitis
	Gullian-Barré Syndrom

Patienten mit speziellen Problemen und Grunderkrankungen

Schwangere	Spontanabort – Totgeburt
	Hydrops fetalis
	Intrauteriner Kindstod
Patienten mit gestörter Bildung/Reifung von Erythrozyten	Schwere Anämie
	Aplastische Krise
Immundefiziente Patienten	Chronische Anämie
	Erythroblastopenie (*pure red cell aplasia*)
	Chronische Thrombo-, Granulo- und Panzytopenie
	Chronische Arthritis
	Myo- und Perikarditis – akutes Herzversagen
	Akutes Leberversagen – Hepatitis
	Meningitis – Enzephalitis

Tabelle 2: Übersicht von mit B19V-Infektionen assoziierten Krankheiten.

1.2.5.1. Erythema infectiosum

Als häufigste Manifestation einer B19V-Infektion gelten die mild verlaufenden Ringelröteln, auch *Erythema infectiosum* oder „*fifth disease*“ genannt. Es handelt sich hierbei um eine Kinderkrankheit, die in der Regel mit einem allgemeinen Krankheitsgefühl, grippeähnlichen Symptomen wie Fieber und Kopfschmerzen sowie einem charakteristischen ringel- und girlandenförmigen makulopapulösem Exanthem assoziiert ist. Das Exanthem tritt üblicherweise etwa eine Woche nach Kontakt mit dem Virus im Gesichtsbereich auf und breitet sich im weiteren Krankheitsverlauf über den Rumpf und die Extremitäten der Patienten aus. Die Symptome des *Erythema infectiosum* klingen in der Regel innerhalb 1-2 Wochen nach Krankheitsausbruch ab.

Durch experimentelle Infektionsstudien an freiwilligen B19V-seronegativen Probanden konnte das Krankheitsbild des *Erythema infectiosum* erfolgreich induziert werden [61]. Hierbei wurde gezeigt, dass das Auftreten des Exanthems mit dem Eingreifen der virusspezifischen humoralen Immunantworten korreliert. Daraus schloss man, dass der Entstehung des Exanthems die Ablagerung von Virus-Antikörper-Immunkomplexen in Kapillaren der Haut zugrunde liegt [37, 62].

1.2.5.2. Arthritiden und Arthropathien

Besonders bei Erwachsenen führen Infektionen mit B19V häufig zu schweren Arthritiden und Arthropathien der Hand-, Knie- und Fußgelenke. Etwa 60% aller infizierten Frauen und 30% der Männer sind davon betroffen [63, 64]. Bei Kindern ist eine B19V-Infektion in etwa 10% der Fälle mit meist transienten Gelenkentzündungen verbunden [65]. Normalerweise klingt das Erkrankungsbild innerhalb 3 Wochen nach Infektion ab, bei bis zu 20% der Patienten können die Gelenkentzündungen jedoch über Jahre hinweg persistieren und wiederkehrende Beschwerden verursachen [63]. Diese Infektionsverläufe ähneln dann häufig dem Krankheitsbild der rheumatoiden Arthritis [66-70]. Die direkte Assoziation von B19V mit rheumatoider Arthritis ist jedoch weitgehend unerforscht und umstritten, da bisher keine eindeutigen Hinweise von viralen Einflüssen in der Krankheitspathogenese beschrieben worden sind [37, 71].

1.2.5.3. Aplastische Krisen und Anämie

Bei Personen mit gestörter Bildung und Reifung der roten Blutkörperchen, z.B. bei Patienten mit Sichelzellenanämie, führen B19V-Infektionen zu sehr schweren und lebensbedrohlichen aplastischen Krisen [72-74]. Diese können in der Regel effizient durch Bluttransfusionen behandelt werden [72, 73]. Bei immunsupprimierten Personen verursacht B19V oft eine chronisch persistierende Infektion, welche durch die frühzeitige Verabreichung virusspezifischer Immunglobuline therapiert werden kann [75-77].

1.2.5.4. Parvovirus B19-Infektionen in der Schwangerschaft

In etwa 50% aller Infektionen im zweiten oder dritten Trimenon einer Schwangerschaft wird eine transplazentäre Übertragung des B19V auf den Fötus beobachtet [78]. Eine fötale Infektion kann asymptomatisch ohne Komplikationen verlaufen, kann jedoch auch bis hin zum Tod des Fötus führen. Hierbei entwickelt sich in dem Fötus eine schwere Anämie, die gelegentlich mit einer Myokarditis verbunden ist [79, 80]. Aufgrund der Virusreplikation in den Erythrozytenvorläufern der fötalen Leber kommt es zur Störung des fötalen Blutkreislaufes, welche zu Flüssigkeitsansammlungen und zur Ausbildung des *Hydrops fetalis* führen kann [80-84].

1.2.5.5.Parvovirus B19-assoziierte Herzerkrankungen

In seltenen Fällen sind bei Kindern [85-87] und Erwachsenen [88-90] Myokarditiden als Folge einer akuten B19V-Infektion beschrieben worden. Deren Pathogenese ist bis heute

weitgehend ungeklärt, da das Virus eine hohe Wirtsspezifität zu sich rapide teilenden Erythrozytenvorläufern aufweist und bisher keine virale Replikation in Myozyten beobachtet worden ist. Es wurde jedoch gezeigt, dass der zelluläre Rezeptor des B19V, das Blutgruppenantigen P, auch auf Zellen des Myokards zu finden ist [91].

Neben den auf akuten B19V-Infektionen basierenden Myokarditiden wird seit kurzem die Rolle der im Herzmuskel häufig auffindbaren viralen DNA bei der Entstehung von Herzerkrankungen untersucht [56, 57]. Diesbezüglich wird diskutiert, ob das latent im Herzgewebe vorhandene Virusgenom in Personen mit einer abgelaufenen B19V-Infektion reaktiviert werden kann und so als eine endogene Quelle von infektiösem B19V in Betracht gezogen werden muss. Bisher wurde jedoch keine aktive Synthese von B19V beziehungsweise viralen Proteinen als Anzeichen einer lokalen Reaktivierung in Herzbiopsien nachgewiesen.

In ersten PCR-Studien wurden im Vergleich zu Kontrollgruppen signifikant erhöhte Prävalenzen von B19V-Genomen im Herzgewebe von Patienten mit einer dilatativen Kardiomyopathie gefunden [92, 93]. Hierbei war die Schwere des Krankheitsverlaufes direkt mit der Abnahme der Viruslast im Gewebe assoziiert [92]. Dieser kausale Zusammenhang konnte jedoch in einer weiteren Studie nicht bestätigt werden [55]. Da es im Rahmen einer B19V-Infektion häufig zur Etablierung einer Latenz der viralen Genome in zahlreichen Geweben kommt [51-54], ist die Aussagekraft des Nachweises viraler DNA im Herz bisher fragwürdig.

1.2.6. Diagnostik und Therapie von B19V-Infektionen

Akute B19V-Infektionen weist man neben der symptombezogenen Diagnostik grundsätzlich über virusspezifische Antikörper und/oder virale DNA im Blut von Patienten nach [94]. Der Nachweis von spezifischen IgG- und IgM-Antikörpern gegen Virusproteine erfolgt hierbei durch ELISA und/oder durch Western blot-Analysen [95, 96].

Die klinische Behandlung einer akuten Parvovirus B19-Infektion ist nur symptombezogen bei dem Auftreten schwerwiegender Komplikationen notwendig [37]. So müssen beispielsweise im Falle von schweren Anämien oder aplastischen Krisen Patienten mit Bluttransfusionen behandelt werden. Immunsupprimierte Patienten können darüber hinaus bei Verdacht einer akuten B19V-Infektion mit virusspezifischen Immunglobulinpräparaten therapiert werden, wodurch schwere und eventuell lebensbedrohliche Krankheitsverläufe vermieden werden können. Falls während einer Schwangerschaft der Verdacht auf einen akuten B19V-Infekt besteht, müssen Frauen in kurzen Abständen mittels Ultraschall auf

die Ausbildung hydropischer Ödeme untersucht werden. Bei Bedarf können beim Fötus intrauterine Bluttransfusionen durchgeführt werden [97, 98]. Eine Impfung gegen B19V steht bisher nicht zur Verfügung.

1.3. Humanes Bocavirus

Fast drei Jahrzehnte lang repräsentierte das B19V den einzigen humanpathogenen Vertreter der *Parvoviridae*. Im Herbst 2005 beschrieb eine schwedische Arbeitsgruppe ein neues Virus, welches aus respiratorischen Proben von Kindern mit Lungenerkrankungen isoliert und aufgrund seiner Genomsequenz der Familie der Parvoviren zugeordnet wurde [8]. Da das Virus eine nahe Verwandtschaft mit dem *bovinen* Parvovirus und dem *canine minute virus* aufwies, erhielt es die Bezeichnung „Humanes Bocavirus".

1.3.1. Genomaufbau und Sequenzvariabilität

Das Genom des HBoV hat eine Größe von 5217 Nukleotiden (*Stockholm 1* Isolat) und umfasst die für die *Parvoviridae* charakteristischen offenen Leserahmen für die VP1, VP2 und NS1 Proteine (Abbildung 2). Im Unterschied zum B19V besitzt HBoV einen zusätzlichen offenen Leserahmen, welcher für das bei tierpathogenen Parvoviren häufig vorkommende Protein NP1 kodiert.

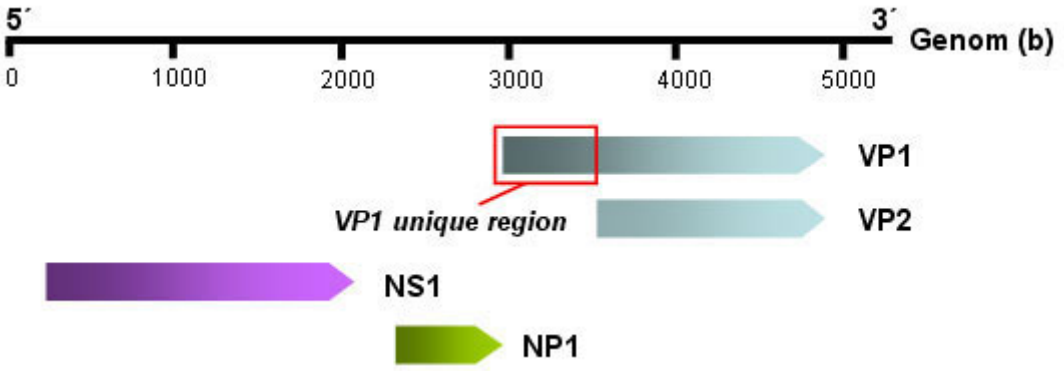

Abbildung 2. Schematische Darstellung der Genomorganisation des Humanen Bocavirus. Die Position und Länge der jeweiligen offenen Leserahmen der viralen Proteine sind als Pfeile dargestellt. b: Basen.

Zahlreiche Isolate des HBoV sind nach der Entdeckung des Virus weltweit beschrieben worden [8, 99, 100]. Diese weisen eine hohe Sequenzhomologie mit bis zu 99% Identität auf [101]. Hierbei wurden die Gene für NS1 und NP1 im Unterschied zu den variablen

VP1 bzw. VP2-Regionen als hochkonservierte Domänen des viralen Genoms beschrieben [100].

1.3.2. Virusmorphologie und Struktur

Die genaue Struktur des HBoV ist bisher wenig erforscht. In einer ersten Studie wurden virale Partikel in respiratorischen Proben einer akut mit HBoV infizierten Person elektronenmikroskopisch nachgewiesen [102]. In der vorliegenden Arbeit konnte die Generierung HBoV-ähnlicher Partikel mittels Überexprimierung des viralen VP2-Gens in Insektenzellen gezeigt werden (Publikation I) [103].

Ähnlich wie bei B19V (siehe 1.2.2.), ist das VP1-Protein des HBoV bis auf die VP1u-Domäne sequenzidentisch mit dem VP2-Strukturprotein [8]. Auch bei HBoV wird VP1u, welche im Vergleich zum B19V mit 14,6 kDa ein geringeres Molekulargewicht aufweist, eine Ca^{2+}-abhängige Phospholipase A2-ähnliche Aktivität zugeschrieben [104].

Über die Funktion der Nichtstrukturproteine des HBoV ist bisher wenig bekannt. Da jedoch in der Sequenz des NS1-Proteins des HBoV im Vergleich zu B19V zahlreiche konservierte Domänen beobachtet werden können, ist zu vermuten, dass das Protein ähnliche transaktivierende und zytotoxische Funktionen im Laufe der Virusreplikation ausüben kann (siehe 1.2.2.). Auch das NP1-Protein wird vermutlich der Regulation von Genaktivitäten während des viralen Replikationszyklus dienen, da ihm im verwandten BPV eine DNA-bindende Eigenschaft zugewiesen wurde [105].

1.3.3. Epidemiologie und Infektionszyklus

HBoV-Infektionen kommen weltweit vor und wurden mittels PCR-Nachweis in bis zu 19% der untersuchten Kinder, die meist aufgrund respiratorischer Infekte hospitalisiert wurden, gefunden [8, 99, 100, 106-155]. Die Mehrzahl dieser Kinder war zum Zeitpunkt der Infektion jünger als 2 Jahre [110, 113, 119, 120, 135, 156]. Bisher sind nur einzelne Fälle von HBoV-Infektionen bei meist immunsupprimierten Erwachsenen bekannt [134, 137, 157]. Infektionen mit HBoV wurden hauptsächlich in den Wintermonaten diagnostiziert [8, 111, 116, 121, 130, 158], wenige Studien beschreiben jedoch auch eine erhöhte Prävalenz des Virus während des Frühlings und/oder des Sommers [114, 122, 131].

Über den Übertragungsmechanismus und den Infektionszyklus des HBoV ist bisher wenig bekannt. Für verwandten Parvoviren erhobene Daten und die mit der HBoV-Infektion assoziierten Symptomatiken lassen jedoch vermuten, dass das Virus über

Tröpfcheninfektion und/oder Schmierinfektion übertragen werden kann (siehe 1.2.3.). Über die Zielzellen und den zellulären Rezeptor des Virus sind bisher keine Informationen vorhanden.

1.3.4. Virale Persistenz

Im Vergleich zu B19V-Infektionen wurden bei HBoV-Infekten bisher geringere Viruslasten von <500 bis 10^8 Genomkopien/ml Probe gefunden [126]. Es wurde postuliert, dass vor allem Patienten mit geringen Mengen an nachweisbaren HBoV-Genomen an persistierenden Infektionen leiden könnten [159]. In ersten Studien konnte weiterhin eine Korrelation zwischen der Schwere der klinischen Symptome und der Höhe der Viruslast nachgewiesen werden [126]. Da diese jedoch in anderen Studien nicht bestätigt wurde [120], ist die Theorie einer möglichen persistierenden HBoV-Infektion umstritten.

Im Unterschied zur B19V-Infektion gibt es bisher keine Anzeichen für die Etablierung einer umfangreichen DNA-Latenz von HBoV (siehe 1.2.4.). So konnte virale DNA bisher nicht oder nur in einzelnen Fällen in verschiedenen Geweben mittels PCR nachgewiesen werden [158] (Kuethe F., Lindner J. *et al.*, in Vorbereitung).

1.3.5. Klinische Relevanz des HBoV

Infektionen mit HBoV werden mit Erkrankungen des Respirationstraktes in Verbindung gebracht. Häufig beobachtete Symptome in HBoV-positiven Patienten sind Pneumonien, Bronchitiden, grippeähnliche Syndrome und allgemeine Unwohlheit.

Die Assoziation von HBoV mit Krankheiten ist jedoch bisher nicht eindeutig bewiesen, da bis zu 69% aller HBoV-infizierten Patienten gleichzeitig mit anderen viralen oder bakteriellen respiratorischen Pathogenen koinfiziert sind [145]. So wird das Virus gehäuft z.B. mit Adenoviren, dem Metapneumovirus, dem *respiratory syncytial virus*, aber auch mit Streptokokken nachgewiesen [160]. Weiterhin handelt es sich bei den meisten klinischen Befunden ausschließlich um retrospektiv gewonnene Daten, wodurch in vielen Fällen nur mangelhafte Informationen über den langfristigen Krankheitsverlauf vorhanden sind.

Zusätzlich zu Atemwegserkrankungen wird eine Rolle des HBoV bei der Entstehung von Gastroenteritiden untersucht, da das Virus häufig in Stuhlproben von an Durchfall leidenden Kindern nachgewiesen werden kann [116, 118, 161, 162]. Jedoch erschweren auch hierbei hohe Koinfektionsraten mit klassischen Durchfallerregern, so z.B. mit Humanen Rotaviren, die eindeutige Krankheitsassoziation.

1.3.6. Diagnostik von HBoV-Infektionen

Die Diagnose von HBoV-Infektionen beruht bislang hauptsächlich auf dem Nachweis von viralen Genomen in verschiedenen klinischen Proben, so z.B. in Serum oder Plasma, in respiratorischen Aspiraten, und Stuhl- oder Urinproben. Hierzu beschriebene PCR-Methoden weisen eine hohe Heterogenität auf und basieren auf einer Vielzahl unterschiedlicher Oligonukleotide spezifisch für die viralen Gene VP1 bzw. VP2 [110, 162], NP1 [8, 133] und NS1 [108, 134].

1.4. Immunologische Abwehr bei parvoviralen Infekten

1.4.1. Allgemeine immunologische Grundlagen

Das zelluläre Immunsystem wird aufgrund der Geschwindigkeit und Spezifität der gegen infektiöse Erreger aufgebrachten Schutzreaktion in zwei Gruppen unterteilt (Abbildung 3).

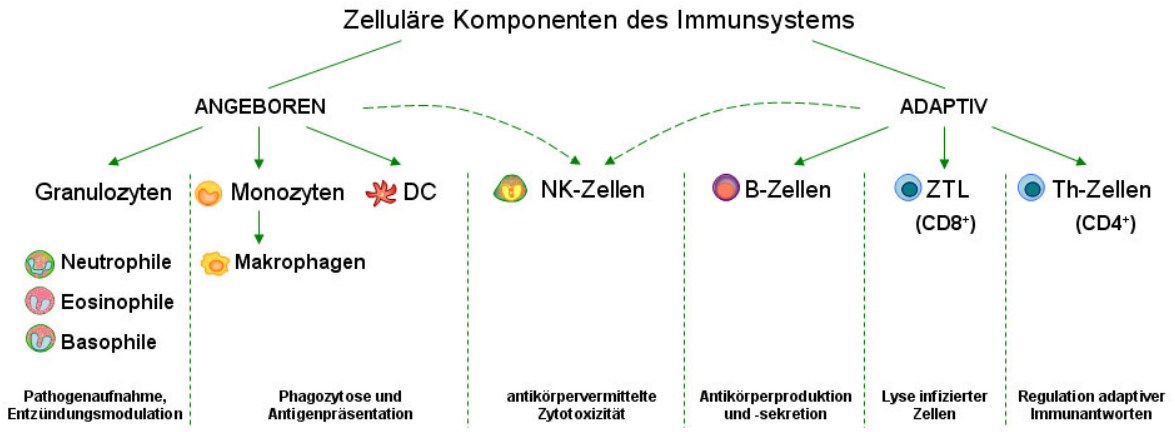

Abbildung 3. Übersicht der zellulären Komponenten des Immunsystems. DC: Dendritische Zellen; NK-Zellen: natürliche Killerzellen; Th-Zellen: T-Helfer-Zellen; ZTL: zytotoxische T-Lymphozyten.

Hierbei stellt die angeborene Immunität mit einer Fülle kurzlebiger, jedoch rasch abrufbarer Immunantworten die erste Abwehrlinie des Organismus gegen Pathogene dar. Als zelluläre Komponenten dieses komplexen Systems können unter anderen Phagozyten (z.B. Granulozyten, Makrophagen und Monozyten inklusive deren Vorläufer) und natürliche Killerzellen (NK-Zellen) genannt werden. Als nicht-zelluläre Faktoren des angeborenen Immunsystems zählen beispielsweise Akutphase-Proteine, das aus

zahlreichen Bestandteilen bestehende Komplementsystem und eine Vielzahl von Zytokinen und/oder Chemokinen.

Einen dauerhaften Schutz gegen Pathogene mit der Eigenschaft eines immunologischen Gedächtnisses sichert die nach Tagen beziehungsweise Wochen nach der Infektion einsetzende adaptive Immunabwehr, auf dessen einzelne humorale und zelluläre Bestandteile und deren Funktion bei parvoviralen Infekten im Folgenden näher eingegangen werden soll.

1.4.1.1. Die spezifische humorale Immunantwort

Unter dem Begriff der adaptiven humoralen Immunantwort wird die Produktion von Antikörpern durch B-Lymphozyten zusammengefasst. Diese auch als Immunglobuline (Ig) bezeichneten Moleküle kommen in unterschiedlichen Klassen vor (IgM, IgD, IgG_{1-4}, IgA_{1-2}, IgE) und dienen dank ihrer hochvariablen Fab-Domänen (*fragment antigen binding*) der Erkennung und Opsonisierung von Erregern im Organismus [163]. Hierbei weisen die Antikörperklassen verschiedene biochemische und funktionelle Charakteristika auf und dienen der Erkennung von unterschiedlichen Fremdstrukturen. Vermittelt über ihre konservierten Fc-Domänen (*fragment constant* oder *crystalline*) können freie oder bereits mit ihrem Antigen komplexierte Antikörper von einer Vielzahl von Immunzellen mittels Fc-Rezeptoren erkannt werden.

Im einleitenden Schritt der adaptiven humoralen Immunantwort wird das Zielantigen von naiven B-Lymphozyten aufgenommen. Hierfür verfügen die B-Zellen über einen in der Zytoplasmamembran verankerten B-Zell-Rezeptor, welcher spezifisch die Erkennung und Internalisierung von Fremdstrukturen vermitteln kann. Nachfolgend reifen B-Zellen, unterstützt von zytokinproduzierenden T-Helfer-Zellen, zu aktivierten Plasmazellen aus, welche für die Sekretion antigenspezifischer Immunglobuline zuständig sind.

Im Zuge eines erstmaligen Erregerkontaktes kommt es zunächst zur gehäuften Produktion von Antikörpern der Klasse IgM. Diese weisen eine pentamere Struktur aus fünf durch kurze *Joining*-Peptide miteinander verbundenen Antikörpereinheiten auf [163]. IgM-Antikörper wirken neutralisierend, induzieren die klassische Aktivierung der Komplementkaskade und können aufgrund ihrer geringen Affinität mit mehreren, einander ähnlichen antigenen Strukturen reagieren [164]. Sie repräsentieren somit eine erste breite Front der adaptiven humoralen Immunantwort, welche jedoch nach Abklingen der akuten Infektion zeitnah durch verschiedene hochaffine Antikörper ersetzt wird.

Antikörper der Klasse IgG repräsentieren mit etwa 75% der Gesamtimmunglobuline die größte Antikörperpopulation im Blut [163]. Sie besitzen eine hohe Affinität zu ihren jeweiligen Antigenen und verleihen dem Organismus bei wiederholtem Kontakt mit den gleichen Erregern eine schützende Immunität. IgG-Antikörper werden in vier unterschiedliche Subklassen unterteilt (IgG_{1-4}), die je nach Art und Beschaffenheit der Zielstruktur und des Erregertyps gebildet werden [165]. Bei den meisten viralen Infekten werden vor allem IgG der Subklassen 1 und 3 produziert. Der Nachweis von IgG_4-Antikörpern deutet häufig auf einen wiederholten oder lang andauernden Erregerkontakt, so z.B. bei chronischen Infektionen, hin [166-168]. Die Bildung von IgG_2-Antikörpern wird hauptsächlich durch Kontakt mit bakteriellen Polysacchariden und Carbohydraten induziert.

1.4.1.2. Die spezifische zelluläre Immunantwort

Die zelluläre Immunantwort umfasst alle direkten und über Botenstoffe vermittelten immunologischen Reaktionen, welche von T-Zellen koordiniert und durchgeführt werden. Ihren Namen verdanken diese immunologisch wichtigen Zellen dem Ort ihrer Reifung, dem Thymus. Von dort aus gelangen die T-Zellen über die Blutbahn in die Peripherie des Körpers, wo sie solange zwischen Blut und lymphatischen Gewebe zirkulieren, bis sie auf ihr spezifisches Antigen treffen und zur Proliferation angeregt werden [169]. Zur spezifischen Erkennung ihrer Antigene besitzen T-Zellen den T-Zell-Rezeptor, ein heterodimeres Transmembranprotein, das aus zwei Proteinketten besteht [170]. Damit T-Zellen jedes beliebige körperfremde Antigen erkennen können, ist eine große Zahl von T-Zell-Rezeptoren mit unterschiedlichen Spezifitäten notwendig. Diese enorme Variabilität wird, ähnlich wie bei der Generierung der Antikörpervielfalt, auf genetischer Ebene durch somatische Rekombination ermöglicht [171, 172].

Im Unterschied zu B-Zellen sind T-Zellen nicht in der Lage, ihr spezifisches Antigen in löslicher Form in ihrer Umgebung zu erkennen, sondern benötigen deren Präsentation im Komplex mit HLA (*human leukocyte antigen*) –Molekülen. Für die erfolgreiche Bindung und Erkennung zwischen dem T-Zell-Rezeptor und den HLA-Molekülen ist ein weiterer Korezeptor notwendig, anhand dessen T-Zellen in die zentralen Effektorpopulationen der CD4- und CD8-positiven T-Helfer-Zellen (Th) bzw. zytotoxischen T-Lymphozyten unterteilt werden.

CD8-positive T-Zellen können Antigenfragmente auf einer Vielzahl von Zellen im Komplex mit HLA-I Molekülen erkennen, welche auf fast allen kernhaltigen Körperzellen

exprimiert werden. Hierbei dient die Präsentation intrazellulär abstammender Peptide auf HLA-I Proteinen der körperinternen immunologischen Kontrolle von potentiell infizierten oder entarteten Zellen. Diese werden nach einer erfolgreichen Antigenerkennung durch CD8-positive T-Zellen durch Ausschüttung von zytotoxischen Stoffen, so z.B. Perforin und Granulysin, abgetötet [173, 174].

CD4-positive Th-Zellen erkennen im Unterschied zu zytotoxischen T-Lymphozyten exogen aufgenommene Proteine, deren Peptidabschnitte im Komplex mit HLA-II Molekülen auf der Oberfläche von speziellen antigenpräsentierenden Zellen, z.B. Dendritischen Zellen, Monozyten, Makrophagen und B-Zellen, präsentiert werden (Abbildung 4).

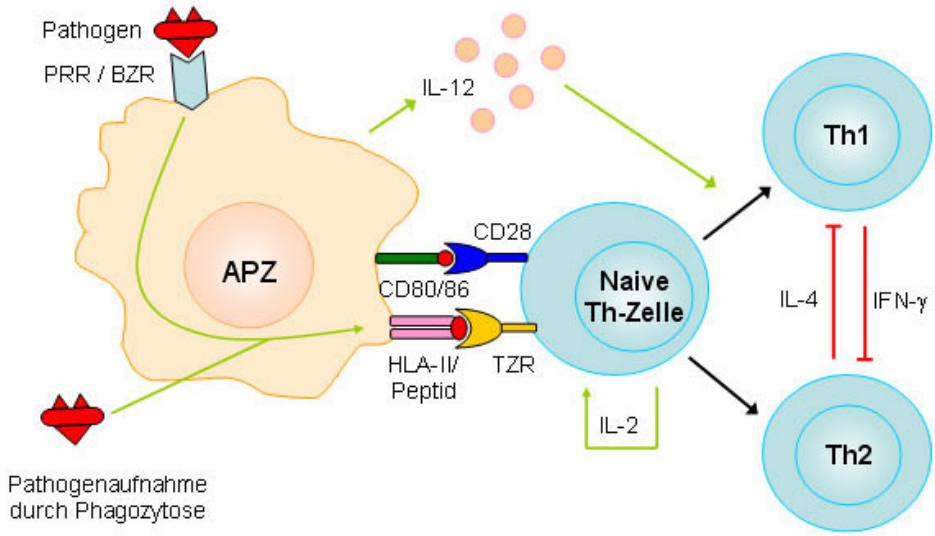

Abbildung 4. Initiierung CD4$^+$ zellulärer Immunantworten. Die Abbildung zeigt die von antigenpräsentierenden Zellen abhängige Aktivierung CD4$^+$ Th-Zellen. Hierbei zur Geltung kommende fördernde und hemmende regulatorische Faktoren sind als grüne bzw. rote Pfeile dargestellt. APZ: antigenpräsentierende Zelle; BZR: B-Zell-Rezeptor; CD80/86/28: Für die Aktivierung von T-Zellen notwendige kostimulatorische Moleküle; HLA: *human leukocyte antigen*; IFN: Interferon; IL: Interleukin; PRR: *pathogen recognition receptor*; Th(1/2)-Zellen: T-Helfer-Zellen der Subpopulationen Th1 bzw. Th2; TZR: T-Zell-Rezeptor.

Als Reaktion auf die spezifische Fremderkennung reagieren Th-Zellen mit der Produktion von charakteristischen Zytokinen, anhand derer sie in zwei größere Subpopulationen eingeteilt werden können (Abbildung 5): Den Th1- und Th2-Zellen.

Die Th1-Zellen sind vor allem durch die Produktion des Zytokins Interferon-gamma (IFN-γ) charakterisiert, welches eine Vielzahl von Immunzellen zur Sekretion des

Tumornekrosefaktor-alpha und verschiedener Chemokine stimulieren kann [175]. Zusätzlich induziert IFN-γ in Immunzellen eine erhöhte Expression der HLA-I und –II Moleküle und fördert somit die weitere Aktivierung von $CD4^+$ und $CD8^+$ T-Zellen [176].

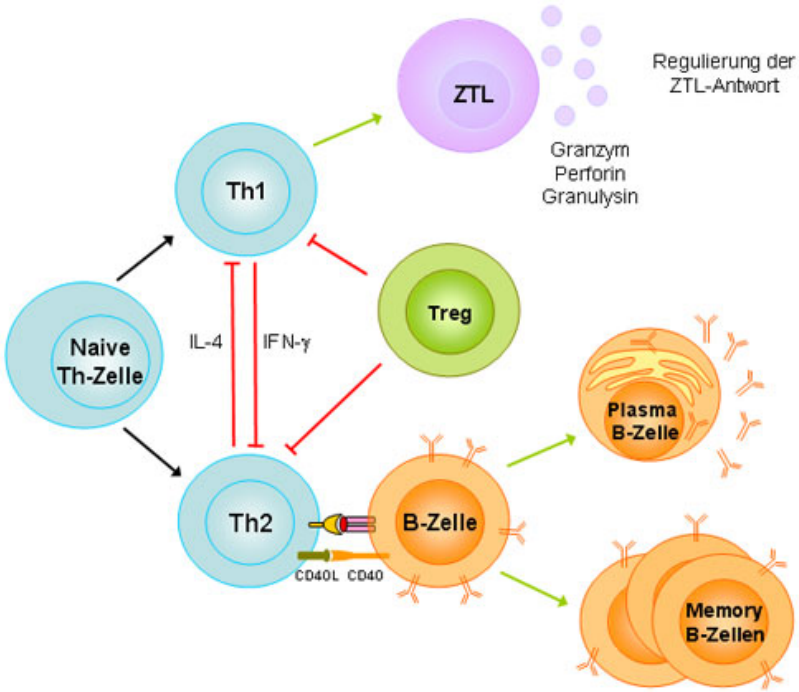

Abbildung 5. Regulation adaptiver Immunantworten durch $CD4^+$ T-Zellen. Die Abbildung zeigt die unterschiedlichen Subpopulationen $CD4^+$ T-Zellen und deren Funktionen in der Regulation von adaptiven Immunantworten. Hierbei zur Geltung kommende fördernde und hemmende regulatorische Faktoren sind als grüne bzw. rote Pfeile dargestellt. APZ: antigenpräsentierende Zelle; CD40L/40: Kostimulatorische Moleküle; Th(1/2)-Zelle: T-Helfer-Zellen der Subpopulationen Th1 bzw. Th2; Treg: regulatorische T-Zelle; ZTL: zytotoxische T-Zelle.

Im Unterschied hierzu fördern Th2-Zellen, die durch die Produktion der Zytokine IL-4, IL-5, IL-6, IL-10 und IL-13 charakterisiert sind, die humorale Immunantwort. Sie regulieren unter anderem den „*class switch*“ von z.B. IgM- zu IgG-Antikörpern [177, 178]. Neben aktivierenden Funktionen sind Th2-Zellen in der Lage, die Funktion und Ausreifung von Th1-Zellen zu unterdrücken. Hierbei spielen vor allem IL-4 und das vielfältige inhibitorische Funktionen vorweisende IL-10 eine zentrale Rolle, da sie unter anderem die Sekretion der Th1-typischen Zytokine IL-12 und IFN-γ verhindern [179, 180].

1.4.2. Immunantworten gegen Parvovirus B19

1.4.2.1. Humorale Immunantwort gegen B19V

Die Hauptaufgabe bei der Eliminierung des Parvovirus B19 während einer akuten Infektion kommt der humoralen Immunabwehr zu. So werden virusspezifische Immunglobuline zur erfolgreichen Therapie und Minderung der Viruslast während chronischer B19V-Infekte bei immunsupprimierten Patienten eingesetzt [77, 181].

Etwa eine Woche nach Kontakt mit dem Virus lassen sich IgM-Antikörper gegen die Strukturproteine VP1 und VP2 des B19V nachweisen (Abbildung 6) [61, 73, 182]. Häufig können in infizierten Personen auch IgM-Antikörper gegen das NS1-Protein beobachtet werden [183]. Nach ungefähr zwei Wochen sind anschließend im Serum der Patienten erste IgG-Antikörper gegen VP1 und VP2 nachweisbar. Diese bleiben meist lebenslang erhalten und bieten einen dauerhaften Schutz gegen Neuinfektionen. Der Hauptteil der virusspezifischen IgG-Antikörper weist einen Subtyp der Klasse 1 (IgG_1) auf [184]. Zusätzlich treten in etwa der Hälfte aller Patienten nach Beginn der klinischen Symptome B19V-spezifische IgA-Antikörper auf [182], seltener werden auch IgE-Moleküle gegen das Virus detektiert [185].

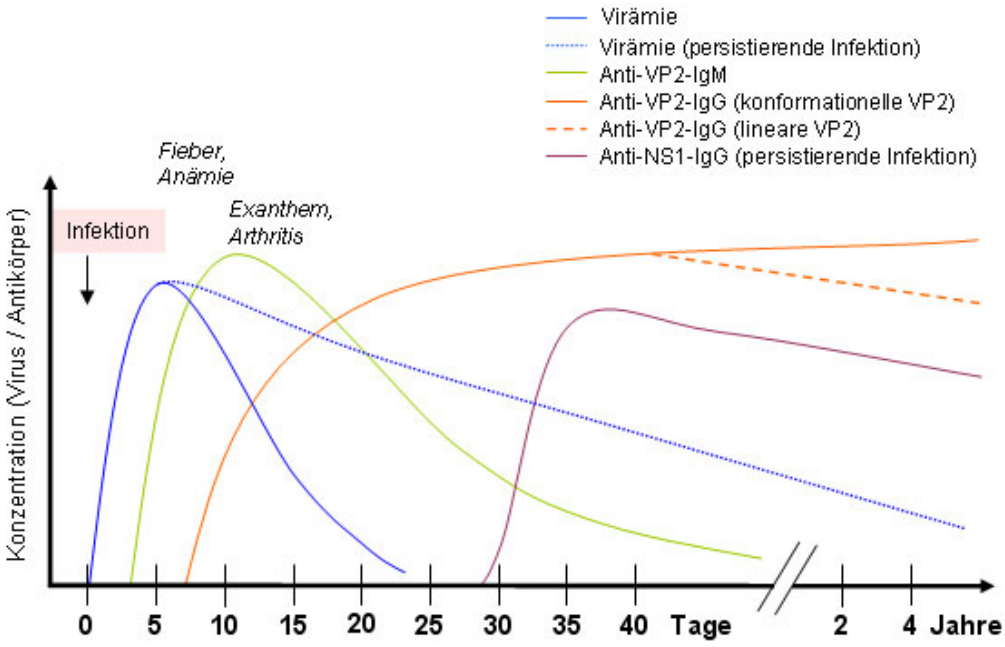

Abbildung 6. Humorale Immunantworten während akuter und persistierender Infektionen mit Parvovirus B19. Die Darstellung zeigt die zeitlichen Phasen einer B19V-Infektion, in denen Virusproteine beziehungsweise virusspezifische Antikörper im Blut von Patienten nachweisbar sind. NS1-spezifische Antikörper (Anti-NS1-IgG) treten vor allem im Rahmen persistierender B19V-Infektionen auf. Ig: Immunglobulin.

In der frühen Phase der Infektion richten sich neutralisierende Antikörper mit geringer Avidität gegen lineare und konformationelle Epitope der viralen Strukturproteine. Im weiteren Verlauf der Infektion und während der Konvaleszenz findet anschließend eine Aviditätsreifung der IgG-Moleküle statt [186]: Es kommt zu einer starken Abnahme und/oder zum Verschwinden von IgG-Antikörpern, welche gegen lineare Epitope des VP2-Proteins gerichtet sind. Der genaue Mechanismus und Grund dieses als „*epitope-type specificity*“ bekannten Phänomens ist weitgehend ungeklärt, jedoch konnten ähnliche Befunde bei Immunantworten gegen nichtverwandte Viren, so z.B. gegen HIV oder das *equine infectious anaemia virus*, beobachtet werden [187, 188].

Im Laufe einer B19V-Infektion und vor allem während der Konvaleszenz kommt es darüber hinaus häufig zur Bildung von IgG-Antikörpern gegen das virale Nichtstrukturprotein NS1. Diese werden häufig bei Patienten mit verzögerter Viruseliminierung aus dem Blut, z.B. bei chronisch persistierenden Infektionen, mit einer Seroprävalenz von bis zu 80% gefunden [189-191]. Bei gesunden Individuen mit einer abgelaufenen B19V-Infektion hingegen sind NS1-spezifische IgG-Antikörper in der Regel nur in etwa 20-35% der Fälle nachweisbar [183, 189, 192].

1.4.1.2. Zelluläre Immunantworten gegen B19V

Über die T-Zell-vermittelte Immunantwort gegen B19V und deren Einfluss auf die Pathogenese virusassoziierter Erkrankungen ist im Vergleich zur humoralen Immunität nur wenig bekannt. Durch Reaktivierungsexperimente von Blutzellen bei Patienten mit abgelaufener oder akuter B19V-Infektion mit rekombinant produzierten Virusproteinen konnten in ersten Studien B19V-spezifische $CD4^+$ Th-Zellen zur Proliferation [193] und zur Produktion der Zytokine IFN-γ und IL-10 stimuliert werden [194, 195]. Nachfolgend konnten gegen VP1u gerichtete T-Zellen in Patienten mit akuten oder kürzlich zurückliegenden B19V-Infektionen nachgewiesen werden, jedoch nahm deren Frequenz im Vergleich zu VP2-spezifischen Th-Zellen nach Abklingen der Infektion rasch ab (Publikation V) [196]. Der Mechanismus und die Funktion dieser Abnahme ist bisher unverstanden, da VP1u als immundominantes Ziel der humoralen Immunantwort gilt und deshalb eine antigenspezifische Th-abhängige Immunregulation der Antikörperantwort notwendig erscheint.

Im Unterschied zu den Kapsidproteinen dient das virale Nichtstrukturprotein NS1 als Ziel für $CD8^+$ zytotoxische T-Zellen, welche für die Lyse infizierter und virusproduzierender

Zellen zuständig sind. In mittels Peptidstimulationen durchgeführten Studien konnten in Individuen, welche über HLA-B35 Moleküle verfügen, NS1-spezifische zytotoxische T-Zellen nachgewiesen werden [197]. In weiteren Studien wurden anschließend zahlreiche weitere, in der Sequenz von NS1 beinhaltete Epitope mit einer breit gefächerten HLA-Restriktion beschrieben [198, 199].

1.4.3. Immunantworten gegen HBoV

1.4.3.1. Humorale Immunantwort gegen HBoV

Über HBoV-spezifische Immunantworten ist bisher nur wenig bekannt. Zeitgleich mit den Ergebnissen dieser Arbeit konnten in ersten Studien Antikörper gegen die Strukturproteine VP1 und VP2 mittels Immunfluoreszenz [200], Western blot-Analysen [201] oder ELISA (Publikation II, III) [103] nachgewiesen werden. Hierbei wurden IgG-vermittelte humorale Immunantworten altersabhängig mit einer hohen Prävalenz von mehr als >80% bereits bei kleinen 2 bis 3 Jahre alten Kindern und Erwachsenen beobachtet (Publikationen II, III) [200]. In weiteren Arbeiten wurde eine HBoV-Seroprävalenz von 35% bis 79% bei gesunden Probanden und bei Patienten mit Lungenerkrankungen beschrieben [103, 201]. Interessanterweise scheint die VP1 *unique region* des HBoV im Unterschied zur VP1u-Domäne des B19V in der virusspezifischen humoralen Immunantwort im Vergleich zum VP2-Protein eine untergeordnete Rolle zu spielen. So wurden VP1u-gerichtete IgG- und IgM-Antikörper bei nur 7% bzw. 2% der untersuchten Kindern mit Infektionen des Respirationstraktes gefunden [201].

1.5. Zielsetzung der Arbeit

In der Arbeit sollten grundlegend Testsysteme zur Analyse der B19V- und HBoV-spezifischen zellulären (ELISPOT) und humoralen (ELISA) Immunantworten etabliert und validiert werden. Als Antigene hierfür sollten für HBoV und B19V virusähnliche Partikel in Insektenzellen produziert, aufgereinigt und charakterisiert werden.
Unter Verwendung der etablierten Testsysteme war das Ziel der Arbeit:

(i) die Häufigkeit und Verbreitung von akuten HBoV-Infektionen und virusspezifischen humoralen und zellulären Immunantworten in der deutschen Bevölkerung zu bestimmen. Weiterhin sollten Informationen zur Krankheitsassoziation des HBoV in Kindern mit akuter Infektion gewonnen werden.

(ii) zu untersuchen, ob man in Patienten mit Myokarditis oder dilatativer Kardiomyopathie, die sich durch das Vorhandensein von B19V-DNA im Herzgewebe auszeichnen, virusspezifische adaptive Immunreaktionen findet, welche auf eine Beteiligung von B19V-Infektionen an der Entstehung der Herzerkrankungen deuten.

2. Ergebnisse

Die Ergebnisse werden entsprechend der im Anhang aufgeführten wissenschaftlichen Publikationen I-IV nachstehend in Übersicht zusammengefasst.

2.1. Produktion virusähnlicher Partikel des B19V und HBoV

Für den Nachweis virusspezifischer adaptiver Immunantworten in den entsprechenden Testsystemen wurden virusähnliche Partikel (VLP) des B19V und HBoV hergestellt. Hierzu wurde zunächst ein rekombinantes Baculovirus (bacVP2/HBoV) zur Expression des HBoV VP2-Gens in Insektenzellen generiert (Publikation I). Für die Herstellung von B19V-VLP wurden die bereits in der Arbeitsgruppe vorhandenen rekombinanten Baculoviren bacVP2/B19V beziehungsweise bacVP12/B19V verwendet. Diese ermöglichten die Produktion von VLP, die entweder aus dem VP2-Protein (B19V/VP2) oder aus einer Mischung der VP1- und VP2-Proteine (B19V/VP12) des B19V bestehen.
Zur Produktion der virusähnlichen Partikel wurden zunächst High5-Insektenzellen bei einer *multiplicity of infection* von 3 mit den Baculovirusstämmen bacVP2/HBoV, bacVP2/B19V oder bacVP12/B19V infiziert und für 72 Stunden kultiviert. Danach wurde mittels SDS-Gelelektrophorese und Western blot-Analysen die Produktion der jeweiligen VP2-Proteine nachgewiesen. Die Aufreinigung und Charakterisierung der VLP erfolgte anschließend durch Ultrazentrifugation über CsCl-Gradienten und Elektronenmikroskopie (Abbildung 7).

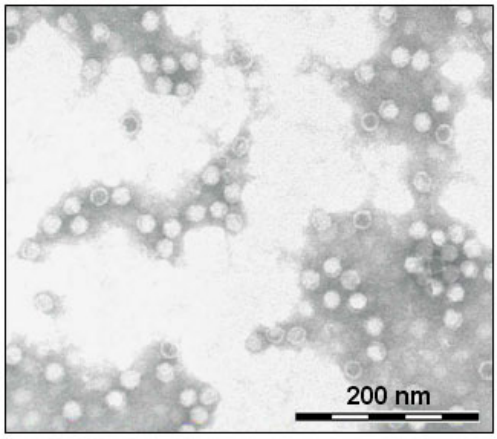

Abbildung 7. Elektronenmikroskopische Analyse virusähnlicher Partikel des Parvovirus B19. Abgebildet ist eine repräsentative elektronenmikroskopische „*negative stain*“-Aufnahme von mittels CsCl-Ultrazentrifugation gereinigten B19V/VP2-VLP.

Hierbei wiesen die HBoV/VP2-VLP eine isopyknische Dichte von 1,33 g/cm3 und einen für Parvoviren charakteristischen Durchmesser von 21-25 nm auf (Publikation I).
Um die produzierten VLP für die Analyse virusspezifischer T-Zell-Antworten einzusetzen, mussten mögliche LPS-Kontaminationen der Antigene ausgeschlossen werden. Ab Konzentrationen von mehr als 10 EU/mg Protein würden diese zu unspezifischen, positiven Reaktionen in immunologischen Versuchen führen. Die LPS-Konzentrationen der Proben lagen jedoch mit 8,2 EU/mg (HBoV/VP2), 4,2 EU/mg (B19V/VP12) und 1,57 EU/mg (B19V/VP2) unter diesem Grenzwert. Sie konnten somit für die in der Arbeit durchgeführten Versuche vernachlässigt werden.

2.2. Analyse HBoV-spezifischer humoraler Immunantworten

2.2.1. Entwicklung und Validierung eines quantitativen ELISA zum Nachweis HBoV-spezifischer Antikörper

Für den quantitativen Nachweis HBoV-spezifischer IgG- und IgM-Antikörper wurde ein auf der Verwendung von HBoV/VP2-VLP basierender ELISA entwickelt (Publikation II). Der Test wies in Experimenten eine hohe Reproduzierbarkeit und Validität mit *intra*- und *inter assay*-Variationen zwischen 0,2% und 10% auf. Weiterhin wurde untersucht, ob die im Serum vorhandenen HBoV-spezifischen IgG mögliche Kreuzreaktivitäten mit dem nahverwandten B19V aufweisen könnten. Deswegen wurden in vergleichenden Studien die Seren von 22 B19V-seronegativen und 34 B19V–seropositiven Probanden untersucht. Hierbei ließen sich HBoV-spezifische IgG mit medianen Werten von 394 bzw. 478 relativen Einheiten in beiden Probandengruppen nachweisen (p=0,4503). Daraus konnte gefolgert werden, dass B19V- und HBoV-spezifische Antikörper keine Kreuzreaktivitäten miteinander aufweisen.

2.2.2. Charakterisierung der Probanden- und Patientenkollektive für die Bestimmung der Seroprävalenz und Krankheitsassoziation des HBoV

Zur Analyse der HBoV-Seroprävalenz in Erwachsenen wurden in Zusammenarbeit mit Dr. Wilhelm Struff (DRK Blutspendedienst West, Münster) Serumproben von 299 gesunden Blutspendern (195 Männer, 104 Frauen, Altersspanne: 19-78 Jahre, Durchschnittsalter: 42 Jahre) gesammelt und getestet (Publikation II).
Um zusätzlich die Prävalenz HBoV-spezifischer Antikörper in Kindern zu bestimmen und weiterhin erste Krankheitsassoziationen für HBoV durchführen zu können, wurden in Zusammenarbeit mit Herrn Prof. Hugo Segerer (Klinik St. Hedwig, Regensburg)

insgesamt 297 Kinder (160 Jungen, 137 Mädchen, Altersspanne: 1-190 Monate, Durchschnittsalter: 57,4 Monate) mit Infektionserkrankungen auf HBoV-spezifische humorale Immunantworten und auf Anzeichen akuter HBoV-Infekte mittels ELISA und PCR untersucht (Publikation III). Hierbei wurden 156 bzw. 64 der Kinder mit Infektionen des Respirations- bzw. Gastrointestinaltraktes am Klinikum St. Hedwig (Regensburg) aufgenommen und behandelt. Weitere 77 Probanden wiesen ein breites Spektrum unterschiedlicher Symptome auf. Insgesamt 60 Kinder (38 Jungen, 22 Mädchen, Altersspanne: 1 – 194 Monate, Durchschnittsalter: 77,9 Monate) ohne Anzeichen von akuten Infektionen wurden als Kontrolle in die Studie mit aufgenommen (Publikation II, III).

2.2.3. Prävalenz und klinische Assoziation von akuten HBoV-Infektionen

Akute HBoV-Infektionen wurden bei 6,7% (20/297) aller Kinder mit und bei 5,0% (3/60) derjenigen ohne Infektionserkrankungen beobachtet (Publikation III). Virusgenome als Anzeichen einer akuten HBoV-Infektion wurden in keinen der Seren von erwachsenen Blutspendern gefunden (Publikation II). Die höchste Prävalenz viraler DNA wurde in Proben von Patienten gefunden, welche aufgrund von Erkrankungen des Respirationstraktes hospitalisiert wurden. HBoV-DNA war in 14,6% (24/48) und 10,0% (5/50) der Proben von Kindern mit Infekten der unteren Atemwege bzw. Pneumonie nachweisbar. In zwei dieser Kinder konnte eine Koinfektion mit RSV bzw. A-Streptokokken diagnostiziert werden. Im Vergleich zu Kindern ohne Infektionserkrankungen (5,0%) wiesen zusätzlich Seren von Probanden mit Erkrankungen des Magen-Darm-Traktes eine leicht erhöhte Prävalenz akuter HBoV-Infektionen auf (7,8%, 5/64). Die Koinfektionsrate mit Rotaviren lag in diesen Kindern bei 60% (3/5).

2.2.4. Seroprävalenz des HBoV in gesunden Blutspendern und Kindern mit Infektionserkrankungen

Die Prävalenz HBoV-spezifischer IgG-Antikörper in untersuchten Kindern mit bzw. ohne Anzeichen von Infektionserkrankungen betrug 71,4% (255/357). In gesunden Erwachsenen (Alter: 19-78 Jahre) wurden HBoV VP2-spezifische IgG in 94% (280/299) der Serumproben gefunden. In allen Probandengruppen ließen sich die HBoV-spezifischen Antikörper als Immunglobuline des Subtyps IgG_1 charakterisieren (Publikation II).

Die IgG-Prävalenz reichte von 23% in Säuglingen mit 7 bis 9 Monaten bis zu mehr als 90% in Kindern ab dem dritten Lebensjahr und Erwachsenen (Publikation II, III). Vor allem in Kindern zwischen 2 und 3 Jahren konnte eine starke Zunahme der Häufigkeit

virusspezifischer IgG-Antikörper von 37% auf 94% beobachtet werden, was auf eine erhöhte Anzahl von akuten Infektionen in dieser Altersgruppe hinweist. Dieser Befund konnte durch einen gehäuften Nachweis viraler DNA als Anzeichen akuter HBoV-Infektionen in den entsprechenden Altersgruppen bestätigt werden (Publikation III).
IgM-Antikörper wurden ausschließlich in Seren von Kindern gefunden, welche positiv auf virale DNA getestet wurden. Hierbei wiesen 39% (9/23) und 22% (5/23) der Proben positive bzw. grenzwertige Reaktivitäten im ELISA auf. In Erwachsenen wurden VP2-spezifische IgM-Antikörper nur vereinzelt gefunden (1%, 2/299).

2.3. Analyse HBoV-spezifischer $CD4^+$ Th-Zell-Antworten

Zusätzlich zu HBoV-spezifischen humoralen Immunantworten sollte in der Arbeit untersucht werden, ob in seropositiven Probanden neben Antikörpern auch HBoV-spezifische T-Zellen als Anzeichen einer abgelaufenen Infektion nachgewiesen werden können (Publikation I).

2.3.1. Charakterisierung des Probandenkollektivs zur Messung HBoV-spezifischer T-Zell-Antworten

69 gesunde Erwachsene (39 Männer, 30 Frauen, Altersspanne: 23-73 Jahre, Durchschnittsalter: 39 Jahre) wurden in die Analyse der HBoV- und B19V-spezifischen zellulären Immunantwort einbezogen (Publikation I). Im Vergleich zu negativen Referenzseren (medianer OD_{450} Wert: 0,056) waren bei allen Probanden IgG-Antikörper gegen das VP2-Protein des HBoV nachweisbar (medianer OD_{450} Wert: 0,863). Mittels diagnostischer ELISA und RecomLine® Tests (Mikrogen GmbH, Neuried) wurden B19V-spezifische IgG-Antikörper in 68% (47/69) der Probanden gefunden. B19V- bzw. HBoV-spezifische IgM-Antikörper oder virale DNA wurden in keinen der untersuchten Proben nachgewiesen.

2.3.2. Analyse virusspezifischer $CD4^+$ Th-Zell-Antworten

Zur Analyse virusspezifischer T-Zell-Antworten wurden aus Probandenblut isolierte PBMC (*peripheral blood mononuclear cells*) mit B19V/VP2-VLP bzw. HBoV/VP2-VLP stimuliert und anschließend mittels ELISPOT die Anzahl IFN-γ produzierender Zellen bestimmt (Publikation I). Bei HBoV-seropositiven Probanden wurden nach Stimulation mit HBoV/VP2-VLP im Vergleich zu durchgeführten Kontrollen (3 *spot forming cells* (SFC)/$2x10^5$ PBMC) signifikant erhöhte mediane Zahlen an IFN-γ sezernierenden Zellen

gezählt (20 SFC/2x10^5 PBMC; p<0,0001). Analog hierzu war in B19V-seropositiven Individuen (38 SFC/2x10^5 PBMC; p<0,0001) im Vergleich zu seronegativen Kontrollprobanden (6 SFC/2x10^5 PBMC) eine spezifische Aktivierung von IFN-γ produzierenden Zellen nach Stimulation mit B19V/VP2-VLP nachweisbar. Keine Korrelation wurde zwischen der Anzahl HBoV-spezfischer T-Zellen und der stärke der virusspezifischen IgG-Antwort gefunden (p=0,5809). Mittels magnetischer Zellseparation konnten $CD4^+$ Th-Zellen als die gegen HBoV/VP2-VLP reagierende Zellpopulation identifiziert werden. Bei keinem der durchgeführten Experimente konnten Kreuzreaktivitäten zwischen HBoV- und B19V-spezifischen T-Zellen beobachtet werden.

2.4. B19V-spezifische adaptive Immunantworten in Patienten mit akuter Myokarditis oder dilatativer Kardiomyopathie

Im Unterschied zu HBoV ist die adaptive Immunantwort gegen B19V weitgehend untersucht. Bisher ist jedoch unklar, ob und welche Rolle individuelle Unterschiede in der B19V-gerichteten Immunantwort bei der Entstehung von virusassoziierten Erkrankungen spielen. Des Weiteren ist nicht geklärt, welche biologischen und immunologischen Funktionen der für B19V beschriebenen DNA-Latenz im Herzgewebe zugrunde liegen und ob diese kausal mit der Krankheitspathogenese von Myokarditiden oder dilatativer Myokarditis in Verbindung gebracht werden können. Deshalb wurden in der Arbeit B19V-spezifische adaptive Immunantworten in seropositiven gesunden Erwachsenen und Patienten mit Herzerkrankungen und nachweisbaren B19V-Genomen im Myokardgewebe miteinander verglichen (Publikation IV). Hierbei sollte untersucht werden, ob nachweisbare Mengen an B19V-Genomen in Herzbiopsien von Patienten mit akuter Myokarditis oder dilatativer Kardiomyopathie kausal mit der Entstehung von Herzerkrankungen in Verbindung gebracht werden können.

2.4.1. Charakterisierung der Patienten mit Myokarditis und dilatativer Kardiomyopathie

8 Patienten mit einer akuten Myokarditis (7 Männer, 1 Frau, Altersspanne: 24-65 Jahre, Durchschnittsalter: 50 Jahre) und 7 Männer mit einer dilatativen Kardiomyopathie (Altersspanne: 42-69 Jahre, Durchschnittsalter: 57 Jahre) wurden auf Anzeichen von akuten, persistierenden oder zurückliegenden B19V-Infektionen untersucht. In Kooperation mit Dr. Michel Noutsias (Kardiovaskuläre Medizin, Charité Berlin) wurden in den Myokardbiopsien aller Patienten B19V-Genome in Mengen von <10^2 bis 1,2x10^5

Genomkopien/µg isolierter DNA gefunden. Parallel hierzu wurden 51 gesunde Probanden (27 Männer, 24 Frauen, Altersspanne: 23-73 Jahre, Durchschnittsalter: 35 Jahre) in die Studie einbezogen. Im Blut aller Patienten mit Myokarditis oder dilatativer Kardiomyopathie waren B19V-spezifische IgG-Antikörper nachweisbar (100%, 15/15), 59% aller Kontrollprobanden waren seropositiv für das Virus (30/51). Keine B19V-DNA konnte im Blut der gesunden Probanden nachgewiesen werden.

2.4.2. Analyse indikativer Parameter für B19V-Infektionen in Patienten mit Herzerkrankungen

Anzeichen für akute, persistierende oder kürzliche B19V-Infektionen wurden in insgesamt 25% (2/8) bzw. 14% (1/7) der untersuchten Patienten mit Myokarditis oder dilatativer Kardiomyopathie mittels PCR und serologischen Analysen gefunden. In zwei Patienten mit Myokarditis wurden zu zwei unabhängigen Krankheitszeitpunkten virale DNA mit Lasten zwischen 10^2-10^3 und $4{,}4 \times 10^3$ Genomkopien/ml Serum nachgewiesen. Im Blut einer dieser Patienten konnten zusätzlich B19V-spezifische IgM-Antikörper detektiert werden. Ein Proband mit dilatativer Kardiomyopathie wurde zum Zeitpunkt der Erstpräsentation im Krankenhaus positiv auf B19V-DNA (10^2-10^3 Genomkopien/ml Serum) und NS1-spezifische IgG im Blut getestet. Im Serum eines weiteren Patienten mit Myokarditis waren Antikörper gegen lineare Epitope des viralen VP2-Proteins nachweisbar. Diese wurden jedoch nicht als ausreichender Hinweis für den Zusammenhang einer B19V-Infektion mit der Herzerkrankung bewertet, da Antikörper mit ähnlicher Spezifität häufig auch in Kontrollseren gefunden wurden (25%, 7/28).

Trotz eindeutiger Anzeichen einer B19V-Infektion in drei Patienten waren in diesen keine Unterschiede bei den Herzfunktionsparametern LVEF (linksventrikuläre Ejektionsfraktion; p=0,2256) und LVEDD (linksventrikulärer enddiastolischer Durchmesser; p=0,5714) im Vergleich zu den restlichen Patienten zu beobachten.

2.4.3. Analyse von B19V-spezifischen $CD4^+$ Th-Zell-Antworten in Patienten mit Herzerkrankungen

In seropositiven Kontrollprobanden (32,5 SFC/2×10^5 PBMC) und Patienten mit Myokarditis (18 SFC/2×10^5 PBMC) oder dilatativer Kardiomyopathie (25,5 SFC/2×10^5 PBMC) wurde in Stimulationsexperimenten mit B19V/VP2-VLP eine signifikant erhöhte mediane Anzahl IFN-γ produzierender Zellen im Vergleich zu seronegativen Individuen beobachtet (3 SFC/2×10^5 PBMC). Stimulationen mit B19V/VP12-VLP ergaben

vergleichbare Ergebnisse, hier konnte die Anzahl antigenspezifischer Zellen sowohl in Patienten mit Myokarditis als auch dilatativer Kardiomyopathie auf 18 SFC/2x10^5 PBMC bestimmt werden. Mittels magnetischer Zellseparation ließen sich in Analogie zu HBoV $CD4^+$ Th-Zellen als antigenspezifisch reagierende Zellpopulation identifizieren (Abbildung 8).

Keine signifikanten Unterschiede in der Anzahl antigenspezifischer T-Zellen wurden bei Patienten mit Myokarditis oder dilatativer Kardiomyopathie mit oder ohne Anzeichen von akuten bzw. kürzlichen B19V-Infektionen beobachtet (p=0,3439 für B19V/VP2). Des Weiteren konnte in keinen der Patienten eine Korrelation zwischen der nachweisbaren Menge an B19V-DNA in Myokardbiopsien und der Anzahl reaktiver T-Zellen gefunden werden.

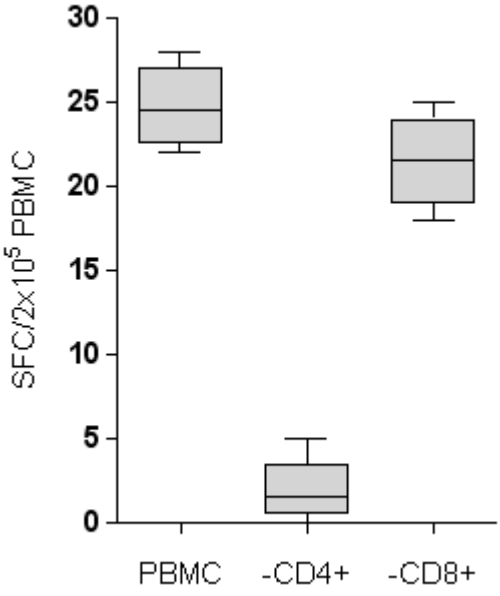

Abbildung 8. Identifikation $CD4^+$ Th-Lymphozyten als reaktive Population B19V-spezifischer zellulärer Immunität. Aus Patientenblut isolierte periphere mononukleäre Blutzellen (PBMC) wurden mit B19V/VP2-VLP oder B19V/VP12-VLP für 72 Stunden stimuliert und die Anzahl IFN-γ produzierender Zellen (*spot forming cells*, SFC) mittels ELISPOT ermittelt. Um die spezifisch reagierende Zellpopulation zu bestimmen, wurden aus den Zellkulturen vor der Antigenstimulation jeweils Th- (-CD4+) bzw. zytotoxische T-Lymphozyten (-CD8+) mittels magnetischer Zellseparation entfernt.

3. Diskussion

Die adaptive Immunantwort spielt bei der Kontrolle und Eliminierung von Krankheitserregern eine wichtige Rolle und verleiht dem Organismus nach Infektionen meist einen lebenslangen Schutz gegen Reinfektionen. Vermittelt werden diese Funktionen über die Zellpopulationen der B- und T-Lymphozyten, welche für die Produktion von spezifischen, den Erreger neutralisierenden Antikörpern und die Zerstörung von infizierten Zellen zuständig sind [163]. Die komplexe Aktivität dieser Zellen kann sich jedoch unter Umständen auch nachteilig für den Organismus auswirken, so beispielsweise im Rahmen von Autoimmunerkrankungen oder Abstoßungsreaktionen nach Transplantationen. In den letzten Jahrzehnten sind Methoden zum Nachweis und zur Charakterisierung unterschiedlicher Lymphozytenpopulationen und deren funktionellen Moleküle zu einer unverzichtbaren Voraussetzung für das Verständnis der Wechselwirkungen zwischen Pathogenen und dem Wirtsorganismus in der Forschung und klinischen Diagnostik geworden [202]. Diese liefern umfangreiche Informationen über den Gesundheitszustand und den pathogenspezifischen „Schutzstatus“ von Patienten und ermöglichen hierdurch idealerweise eine individuelle erregerspezifische Therapie von Infektionskrankheiten. Hierbei gewonnene Erkenntnisse fließen zusätzlich häufig in die Entwicklung von neuen oder verbesserten Impfstoffen mit ein, bei denen als zentrales Ziel die Induktion einer breit gefächerten und schützenden Immunantwort steht. Weiterhin ermöglichen immunologische Analysen in großen Kollektiven die Erhebung epidemiologischer Daten, welche zum besseren Verständnis der Erregerverbreitung und -evolution beitragen und wichtige Informationen für das öffentliche Gesundheitssystem liefern.

Wie bei den meisten viralen Infekten spielt die Analyse virusspezifischer Immunantworten auch bei Parvoviren eine äußerst wichtige Rolle. So können z.B. nicht diagnostizierte und unbehandelte akute B19V-Infektionen in Schwangeren zu schwerwiegenden Komplikationen bis hin zum Tod des Fötus führen [37]. Der Nachweis akuter Parvovirus B19-Infektionen beruht neben der symptombezogenen Diagnostik grundsätzlich auf der Detektion von virusspezifischen Antikörpern und viraler DNA im Serum oder Plasma von Patienten [94]. Die serologische Diagnostik von B19V-Infektionen beruht in der Regel auf ELISA und/oder Western blot-Analysen, welche eine hohe Spezifität aufweisen und mit einem geringen Kostenaufwand und wenig Probenmaterial routinemäßig durchgeführt werden können [95, 96]. Weiterhin ermöglicht die Analyse der Antikörperavidität in Kombination mit der Bestimmung der erkannten Zielepitope die Eingrenzung des ungefähren Zeitpunktes akuter B19V-Infektionen [96, 186, 203]. Der Nachweis von

Virusgenomen in Patienten liefert im Vergleich zur serologischen Analyse eine deutlich höhere Sensitivität und ermöglicht darüber hinaus die Diagnose von persistierenden B19V-Infektionen, bei denen die Antikörpertiter bereits stark abgesunken sein können.
Über die T-Zell-vermittelte Immunantwort gegen Parvovirus B19 und deren Einfluss auf die Pathogenese virusassoziierter Erkrankungen ist im Vergleich zur humoralen Immunität wenig bekannt. In eigenen Vorarbeiten wurde zum Nachweis dieser Reaktionen eine Methode etabliert, welche auf der transienten Expression von viralen Genen in antigenpräsentierenden Zellen basierte (Publikation V). Hierbei konnten zwar virusspezifische $CD4^+$ T-Zellen erfolgreich in Patienten mit kürzlicher B19V-Infektion nachgewiesen werden, jedoch wurde die Routineanwendung dieser Methode erschwert, da für die Analyse teuere Reagenzien und große Blutvolumen von Patienten benötigt wurden.

3.1. Produktion virusähnlicher Partikel als Antigen zum Nachweis Parvovirus-spezifischer Immunantworten

Um detaillierte Untersuchungen virusspezifischer adaptiver Immunantworten durchführen zu können, wurden in der vorliegenden Arbeit neue Methoden zum Nachweis HBoV- und B19V-spezifischer T- und B-Zell-Antworten etabliert. Als Methoden der Wahl wurden hierfür der ELISA für den Nachweis erregerspezifischer Antikörper und der ELISPOT zur Analyse virusgerichteter T-Zell-Antworten gewählt. Beide finden bereits in der Routinediagnostik von viralen und bakteriellen Infektionen Anwendung und bieten im Vergleich zur Durchflusszytometrie und zu Western blot-Untersuchungen eine hochspezifische und kostengünstige Alternative zur Analyse zahlreicher Patientenproben [204, 205].
Als virale Zielantigene für die etablierten Testsysteme wurden die VP2-Kapsidproteine des B19V und HBoV gewählt. Für B19V war VP2 bereits als immundominantes Ziel der humoralen und zellulären Immunantwort bekannt [196, 206]. Weiterhin war für mehrere Parvoviren gezeigt, dass die Expression des VP2-Gens in Insektenzellen aufgrund spezieller Aggregationsdomänen in den VP2-Proteinen zur Bildung nichtinfektiöser virusähnlicher Partikel führt [25, 26, 207]. Diese eignen sich besonders zum diagnostischen Nachweis von virusspezifischen Immunantworten, da durch ihre partikuläre Struktur (i) konformationelle Epitope erhalten bleiben und (ii) sie zur effizienten Restimulation von T-Lymphozyten verwendet werden können [186, 196].
B19V/VP2-, B19V/VP12- und HBoV/VP2-VLP wurden in Insektenzellen durch Infektion mit rekombinanten Baculoviren erfolgreich produziert und durch zonale

Ultrazentrifugation gereinigt. HBoV/VP2-VLP, welche in dieser Arbeit erstmals beschrieben wurden, wiesen hierbei in elektronenmikroskopischen Analysen eine für Parvoviren typische Morphologie und einen charakteristischen Durchmesser von etwa 21-25 nm auf (Publikation I) [102, 208].

3.2. Analyse der HBoV-spezifischen humoralen Immunantwort

Im Unterschied zu B19V waren zu Beginn der Arbeit keine Informationen über die Prävalenz von akuten HBoV-Infektionen und der virusspezifischen Immunantwort in der deutschen Bevölkerung bekannt. Weiterhin waren nur wenige Informationen über mögliche Krankheitsassoziationen des HBoV vorhanden. Deswegen wurden Methoden entwickelt, mit welchen epidemiologische Daten zur Verbreitung des HBoV in Deutschland erhoben werden konnten. Hierzu wurde basierend auf rekombinant produzierten HBoV/VP2-VLP ein ELISA zum quantitativen Nachweis HBoV-spezifischer Antikörper etabliert. Dieser wies in Experimenten eine hohe Reproduzierbarkeit auf und erfüllte somit die für diagnostische Tests vorgeschriebenen Richtlinien (Publikation II) [209]. Um die Spezifität der vom Test gelieferten Ergebnisse sicherzustellen, wurden mögliche Kreuzreaktivitäten zwischen HBoV- und B19V-spezifischen Antikörpern ausgeschlossen (Publikation II). Weiterhin stellte sich die Frage, ob gegen das Rinderparvovirus gerichtete Immunglobuline zu falschpositiven Ergebnissen beim Nachweis von HBoV-spezifischen Antikörpern beitragen könnten. BPV-spezifischen IgG wurden bei bis zu 27% der Menschen beschrieben und lassen sich vermutlich auf den Konsum von viruskontaminierter Milch und Milchprodukte zurückführen [210, 211]. Mögliche Kreuzreaktivitäten zwischen BPV- und HBoV-spezifischen Antikörpern wurden jedoch als unwahrscheinlich angesehen, da der Großteil der HBoV-seronegativen Individuen in Altersgruppen (Kinder zwischen 1 und 3 Jahren) mit einem starken Konsum von Milchprodukten gefunden wurde (Publikation III). Auch kann heute nicht mehr ausgeschlossen werden, dass es sich bei den ursprünglich als BPV-spezifisch beschriebenen Antikörpern in Wirklichkeit um gegen HBoV gerichtete Immunglobuline handelt, welche jedoch aufgrund ähnlicher Erkennungsstrukturen an BPV-Antigene binden.

HBoV-spezifische Antikörper wurden mit einer Prävalenz von mehr als 90% in gesunden Erwachsenen gefunden (Publikation II). Es handelte sich dabei ausschließlich um Antikörper der IgG_1-Subklasse. Diese stellen bei mehreren viralen Infektionen die prominenteste Antikörpersubklasse dar, so z.B. bei Infekten mit B19V oder HBV [184,

212, 213]. Bei Säuglingen und kleinen Kindern zwischen 6 Monaten und 3 Jahren betrug die Seroprävalenz des HBoV 23% bis über 90%. Bei bis zu 77% der Kinder unter 6 Monaten wurden gehäuft HBoV-spezifische Antikörper nachgewiesen (Publikation III). Bei diesen handelt es sich vor allem um mütterliche Immunglobuline, die während der Schwangerschaft von der Mutter auf den Fötus übertragen werden („Nestschutz“) und in der Regel in den ersten Monaten nach der Geburt im Kind nachweisbar sind [214]. Dieser häufige Nachweis maternaler Antikörper in Säuglingen ist als weiterer Hinweis auf die hohe Seroprävalenz des HBoV in Erwachsenen zu werten.

Die epidemiologischen Daten wurden zeitgleich durch Studien anderer Arbeitsgruppen bestätigt. Sie beschreiben eine Prävalenz HBoV-spezifischer IgG von bis zu 95% in Erwachsenen [103, 200, 215]. In finnischen Kindern mit etwa 2 Jahren wurden HBoV-spezifische Antikörper in 73% aller Probanden mittels Western blot beobachtet. Dabei wurde jedoch im Unterschied zu den Ergebnissen dieser Arbeit eine Abnahme der Prävalenz virusspezifischer IgG mit zunehmendem Probandenalter vermerkt [201]. Dieser Befund könnte durch eine Veränderung der Epitopspezifität HBoV-reaktiver IgG erklärt werden: Für das nahverwandte B19V wurde das Phänomen der „*epitope type specificity*“ beschrieben, bei dem es mit zunehmender Zeit nach einer B19V-Infektion zum Verschwinden der IgG gegen lineare VP2-Epitope kommt [186]. Parallel hierzu werden in Probanden durch Spezifitätsreifung entstehende IgG nachweisbar, welche ausschließlich konformationelle Epitope des VP2-Proteins erkennen. Ähnliche Prozesse bei der humoralen Immunantwort gegen HBoV hätten zur Folge, dass mittels denaturierter VP2-Proteine bei Western blot-Analysen ausschließlich kürzlich abgelaufene HBoV-Infektionen in meist kleinen Kindern detektiert werden könnten.

Der Großteil der akuten HBoV-Infektionen wurde in ein bis drei Jahre alten Kindern mittels eines PCR-Nachweises von viralen Genomen im Serum diagnostiziert. In zahlreichen Untersuchungen war HBoV bereits weltweit in Kindern dieser Altergruppe nachgewiesen worden, jedoch beschränkten sich die meisten Arbeiten hierbei auf den Nachweis viraler Genome in Proben des Respirationstraktes [8, 99, 100, 106-155]. Bei virämischen Probanden wurden IgM-Antikörper mit einer hohen Prävalenz von bis zu 61% als Anzeichen von akuten HBoV-Infektionen gefunden. Diese Ergebnisse belegen, dass HBoV in der Lage ist eine systemische Infektion im Menschen zu verursachen. Im Unterschied hierzu beschränken sich andere respiratorische Pathogene, z.B. das *respiratory syncytial virus*, auf eine meist lokale Virusverbreitung im Lungenepithel [216]. Deren Nachweis wird folglich bevorzugt in Lungenaspiraten anstatt von Patientenblut

durchgeführt. Für HBoV stellt Serum hingegen ein geeignetes Probenmaterial zum Infektionsnachweis und für weitere epidemiologische Studien dar.

Akute HBoV-Infektionen wurden mit einer ähnlichen Prävalenz in Kindern mit oder ohne Anzeichen von Infektionserkrankungen beobachtet (Publikation III). In Analogie zu B19V lässt das auf einen häufigen asymptomatischen Verlauf der Infektion schließen [60]. Am häufigsten wurde eine HBoV-Virämie bei Probanden mit Erkrankungen der unteren Atemwege bzw. Pneumonie mit einer Prävalenz von bis zu 14,6% nachgewiesen (Publikation III). Zusammen mit Ergebnissen aus zeitgleich durchgeführten prospektiven Studien anderer Arbeitsgruppen wurde hierdurch eine Assoziation des HBoV mit Lungenerkrankungen postuliert [8] [126]. Diese wurde durch die Tatsache verstärkt, dass die in der Arbeit untersuchten Patienten mit akuter HBoV-Infektion im Unterschied zu Studien anderer Gruppen nur vereinzelt mit zusätzlichen respiratorischen Pathogenen koinfiziert waren. Der Grund hierfür mag eine kontrollierte Entnahme von klinischen Proben zu Beginn des Krankenhausaufenthaltes sein, bevor Kinder mit weiteren Erregern koinfiziert werden können.

Zusätzlich zu Patienten mit Atemwegserkrankungen wurde im Vergleich zur asymptomatischen Kontrollgruppe eine leicht erhöhte Prävalenz akuter HBoV-Infekte bei Kindern mit Magen-Darm-Erkrankungen verzeichnet. Auch in anderen kürzlich durchgeführten Studien konnte HBoV in Stuhlproben von Kindern mit akuter Gastroenteritis nachgewiesen werden [116, 118, 161, 162]. Ob und in welcher Weise HBoV-Infektionen an der Pathogenese von Gastroenteritiden beteiligt sind, ist jedoch weitgehend unbekannt, da eine eindeutige Krankheitsassoziation aufgrund hoher Koinfektionsraten mit weiteren Durchfallerregern, z.B. dem Humanen Rotavirus, erschwert wird (Publikation III) [217]. So kann der Nachweis von HBoV-DNA in Stuhlproben während der akuten Infektionsphase auch als Hinweis für einen natürlichen, klinisch nicht relevanten Ausscheidungsmechanismus des Virus verstanden werden. Auch wenn bisher keine eindeutige Evidenz für die Assoziation des HBoV mit Gastroenteritis bekannt ist, sollten bei Verdacht auf HBoV-Infektion auch Stuhlproben auf das Vorhandensein des Virus untersucht werden, zumal ein Hauptsyndrom der Bocavirusinfektionen beim Rind durch BPV die Diarrhoe des infizierten Kalbes ist [218].

3.3. $CD4^+$ Th-Zell-vermittelte Immunantworten gegen HBoV

Neben Untersuchungen der humoralen Immunantwort wurden die HBoV/VP2-VLP zur Analyse der HBoV-spezifischen zellulären Immunantwort eingesetzt. Hierzu wurde ein

ELISPOT-Testsystem etabliert, mit dem eine hohe Prävalenz von HBoV-spezifischen $CD4^+$ T-Zellen in seropositiven gesunden Probanden gezeigt werden konnte (Publikation I). Mögliche Kreuzreaktivitäten zwischen B19V- und HBoV-spezifischen $CD4^+$ T-Zellen wurden ausgeschlossen.

Bei den antigenspezifischen Th-Lymphozyten handelte es sich um Zellen des Subtyps Th1 (siehe 1.4.1.2.), welche häufig im Laufe von viralen Infekten, z.B. mit B19V oder dem Poliovirus, auftreten [212, 219]. Funktionell können diese unter anderem durch die Produktion von IFN-γ und durch die Stimulation von virusspezifischen B-Zellen zur Produktion von Antikörpern des Subtyps IgG_1 charakterisiert werden [163]. Letzteres galt auch für die HBoV-Infektion, hier war die virusspezifische humorale Antwort ausschließlich von IgG_1-Antikörpern vermittelt (Publikation II).

Analog zur Seroprävalenz deutet auch der häufige Nachweis HBoV-spezifischer zellulärer Immunantworten in gesunden Erwachsenen auf eine hohe Durchseuchung der Bevölkerung mit HBoV hin. Die protektiven und antiviralen Eigenschaften dieser Immunantworten sind hingegen noch unklar und müssen in Folgestudien analysiert werden. Obwohl Th1-Zellen eine zentrale Rolle in der antiviralen Immunabwehr spielen, werden diese auch häufig mit der Entstehung humaner Krankheiten, z.B. mit Autoimmunerkrankungen der Gelenke, assoziiert [220, 221]. Auch während Schwangerschaften, im Laufe derer es normalerweise zu einer natürlichen Reduktion der mütterlichen Immunantwort kommt, können starke Th1-vermittelte Immunreaktionen nachteilige Effekte auf den Schwangerschaftsverlauf haben [222, 223]. So ist es nicht verwunderlich, dass das zu HBoV nahverwandte B19V, welches mit einer Vielzahl entzündlicher Gelenkerkrankungen und Schwangerschaftskomplikationen assoziiert ist, ein dominantes Ziel der Th1-vermittelten Immunität darstellt [37, 63, 184, 196, 212]. Ob jedoch für HBoV ähnliche von der Immunantwort abhängige Krankheitsassoziationen existieren ist bisher nicht bekannt.

3.4. B19V-spezifische adaptive Immunantworten bei Patienten mit Herzerkrankungen und nachweisbarer B19V-DNA im Herzgewebe

Im Unterschied zu HBoV ist die adaptive, vor allem die humorale Immunantwort gegen B19V bereits gut untersucht. Jedoch ist bisher weitgehend unklar, welche Rolle patientenspezifische Unterschiede in der B19V-gerichteten Immunantwort bei der Entstehung von virusassoziierten Erkrankungen spielen.

Virale Infektionen des Herzes werden als mögliche Ursache von akuter Myokarditis und dilatativer Kardiomyopathie diskutiert. Hierbei spielen vor allem Infekte mit kardiotropen Erregern, z.B. dem Coxsackievirus B, eine zentrale Rolle [224]. In seltenen Fällen verursachen jedoch auch akute B19V-Infektionen Myokarditiden in Kindern und Erwachsenen [85-90]. Darüber hinaus werden häufig B19V-Genome in endomyokardialen Biopsien von erwachsenen Patienten mit Myokarditis oder dilatativer Kardiomyopathie ohne Anzeichen von akuten B19V-Infektionen mittels PCR nachgewiesen [56, 57]. Die klinische Relevanz dieser B19V-Genomlatenz ist bisher unverstanden, da virale DNA auch in zahlreichen weiteren Geweben in Abwesenheit klinischer Symptome oder aktiver Virusreplikation mit einer hohen Prävalenz gefunden werden kann [54, 58, 59]. So konnte B19V-DNA bereits im Knochenmark [51, 52], im Synovialgewebe [53], in der Leber [54] und in der Haut nachgewiesen werden [58, 59]. Der grundlegende Mechanismus der DNA-Latenz und sein möglicher Einfluss auf pathophysiologische und immunologische Prozesse ist unklar. Weiterhin ist nicht bekannt, ob das virale Genom spontan reaktiviert werden kann und so als mögliche endogene Quelle für die Produktion des B19V betrachtet werden muss.

In ersten durchgeführten Studien hat man eine Korrelation zwischen der Abnahme der Viruslast im Myokard und einer Verbesserung der Herzfunktionen beobachtet [92, 225]. Dieser Befund ließ sich jedoch in einer weiteren Studie nicht bestätigen [55]. Bisher wurde im Myokard von nichtvirämischen Patienten mit nachweisbarer Latenz von B19V-DNA im Herzgewebe keine lokale Virusreplikation oder Proteinexpression als Anzeichen lokaler Infektionsvorgänge mittels Immunhistologie gefunden [226]. Aufgrund dieser widersprüchlichen Ergebnisse sollte im Rahmen dieser Arbeit mit immunologischen Methoden die klinische und diagnostische Relevanz von nachweisbaren B19V-Genomen im Herzgewebe von Patienten mit Erkrankungen des Herzens analysiert werden.

Bei 20% aller untersuchten Patienten mit Myokarditis oder dilatativer Kardiomyopathie und nachweisbaren Mengen von B19V-Genomen im Herzgewebe ließen sich Anzeichen einer akuten, kürzlichen oder persistierenden B19V-Infektion nachweisen (Publikation IV). So wurde bei zwei Patienten mit Myokarditis und einer an dilatativer Kardiomyopathie erkrankten Person eine aktive Virusreplikation im Blut mittels PCR gezeigt. Zwei dieser Patienten wiesen klassische serologische Marker einer akuten bzw. persistierenden B19V-Infektion auf (Antikörper gegen lineare VP2-Epitope bzw. NS1), bei der dritten Person ähnelte die virusspezifische Antikörperantwort trotz aktiver Virämie einer abgelaufenen B19V-Infektion (Antikörper gegen konformationelle VP2-Epitope). Ob diesem Befund

eine virale Reinfektion oder eine mögliche Reaktivierung latenter Viren im Gewebe zugrunde lag konnte nicht detailliert erörtert werden. Bei einem weiteren nichtvirämischen Patienten mit Myokarditis wurden im Blut Antikörper gegen lineare Epitope des viralen Kapsidproteins VP2 nachgewiesen. Diese sind häufig in Personen mit einer kürzlichen B19V-Infektion auffindbar [96]. Da diese Antikörper jedoch auch bei 25% der untersuchten gesunden Kontrollprobanden gefunden wurden, war hier keine eindeutige Assoziation einer B19V-Infektion mit Myokarditis erkennbar.

Zelluläre Immunantworten wurden sowohl in gesunden B19V-seropositiven Kontrollprobanden als auch in Personen mit Myokarditis oder dilatativer Kardiomyopathie mittels ELISPOT detektiert (Publikation IV). Im Unterschied zu seronegativen Probanden wiesen beide Gruppen signifikant erhöhte Anzahlen IFN-γ produzierender antigenspezifischer $CD4^+$ Th-Zellen auf. Ähnliche Resultate wurden bereits in ELISA- und Proliferationsversuchen beschrieben, diese beschränkten sich aber ausschließlich auf gesunde Blutspender [194-196]. Die Stimulation von Blutzellen mit sowohl B19V/VP2- und B19V/VP12-VLP resultierte bei allen Probanden in einer ähnlichen Frequenz von antigenspezifisch reagierenden T-Zellen, was im Unterschied zur B19V-spezifischen Antikörperantwort auf eine untergeordnete Rolle der VP1u-Domäne als Zielstruktur der zellulären Immunität hindeutet. Im Vergleich zu Kontrollprobanden konnte in Patienten mit Herzerkrankungen eine leicht herabgesetzten Frequenz virusspezifischer T-Zellen beobachtet werden. Diese kann auf den allgemein schlechten Gesundheitsstatus und/oder das durchschnittlich höhere Probandenalter zurückzuführen sein (Publikation I). Zusätzliche Untersuchungen ergaben keine Korrelation zwischen der Anzahl virusspezifischer T-Zellen in Patienten mit Myokarditis oder dilatativer Kardiomyopathie und der vorliegenden B19V-DNA Last im Myokard, welche als Anzeichen für eine lokale virale Replikation aufgrund einer endogenen Reaktivierung betrachtet werden könnte. Würden virale Proteine lokal im Gewebe produziert, sollten aufgrund einer antigenspezifischen Proliferation erhöhte Frequenzen virusspezifischer T-Zellen nachgewiesen werden können. Jedoch wurden im Patientenkollektiv keine signifikanten Unterschiede hinsichtlich der Anzahl B19V-spezifischer T-Lymphozyten in Patienten mit bzw. ohne einer immunhistologisch bestätigten Kardiomyopathie beobachtet.

Zusammenfassend lässt sich aus den Ergebnissen folgern, dass in seltenen Fällen eine Beteiligung akuter B19V-Infektionen an der Entstehung und/oder Aufrechterhaltung von Herzerkrankungen, insbesondere Myokarditis, in Betracht gezogen werden muss. Jedoch kann der alleinige Nachweis von B19V-Genomen in Myokardbiopsien ohne weitere

Anzeichen für akute B19V-Infektionen nicht als ausreichend für die kausale Beziehung des Virus in der Krankheitspathogenese angesehen werden. Diese Hypothese wird zusätzlich durch die Ergebnisse einer bisher unveröffentlichten Studie unterstützt, bei der B19V-Genome mit einer hohen Prävalenz von bis zu 98% in Myokardproben von seropositiven Individuen ohne Myokarditis oder dilatativer Kardiomyopathie zu finden waren (Kuethe F., Lindner, J., *et al.*, in Vorbereitung).

4. Literaturverzeichnis

(1) Blacklow NR. Potentiation of an adenovirus-associated virus by herpes simplex virus type-2-transformed cells. J Natl Cancer Inst **1975 Jan**; 54(1):241-4.

(2) Casto BC, Atchison RW, Hammon WM. Studies on the relationship between adeno-associated virus type I (AAV-1) and adenoviruses. I. Replication of AAV-1 in certain cell cultures and its effect on helper adenovirus. Virology **1967 May**; 32(1):52-9.

(3) Brown KE, Green SW, Young NS. Goose parvovirus--an autonomous member of the dependovirus genus? Virology **1995 Jul 10**; 210(2):283-91.

(4) Zadori Z, Stefancsik R, Rauch T, Kisary J. Analysis of the complete nucleotide sequences of goose and muscovy duck parvoviruses indicates common ancestral origin with adeno-associated virus 2. Virology **1995 Oct 1**; 212(2):562-73.

(5) Anderson MJ, Lewis E, Kidd IM, Hall SM, Cohen BJ. An outbreak of erythema infectiosum associated with human parvovirus infection. J Hyg (Lond) **1984 Aug**; 93(1):85-93.

(6) Cossart YE, Field AM, Cant B, Widdows D. Parvovirus-like particles in human sera. Lancet **1975 Jan 11**; 1(7898):72-3.

(7) Cossart YE. A new particulate antigen present in serum. Dev Biol Stand **1975**; 30:444-8.

(8) Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A **2005 Sep 6**; 102(36):12891-6.

(9) Fryer JF, Kapoor A, Minor PD, Delwart E, Baylis SA. Novel parvovirus and related variant in human plasma. Emerg Infect Dis **2006 Jan**; 12(1):151-4.

(10) Fryer JF, Delwart E, Hecht FM, et al. Frequent detection of the parvoviruses, PARV4 and PARV5, in plasma from blood donors and symptomatic individuals. Transfusion **2007 Jun**; 47(6):1054-61.

(11) Cotmore SF, Tattersall P. Characterization and molecular cloning of a human parvovirus genome. Science **1984 Dec 7**; 226(4679):1161-5.

(12) Tattersall P, Ward DC. Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. Nature **1976 Sep 9**; 263(5573):106-9.

(13) Deiss V, Tratschin JD, Weitz M, Siegl G. Cloning of the human parvovirus B19 genome and structural analysis of its palindromic termini. Virology **1990 Mar**; 175(1):247-54.

(14) Shade RO, Blundell MC, Cotmore SF, Tattersall P, Astell CR. Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. J Virol **1986 Jun**; 58(3):921-36.

(15) Blundell MC, Beard C, Astell CR. In vitro identification of a B19 parvovirus promoter. Virology **1987 Apr**; 157(2):534-8.

(16) Ozawa K, Ayub J, Hao YS, Kurtzman G, Shimada T, Young N. Novel transcription map for the B19 (human) pathogenic parvovirus. J Virol **1987 Aug**; 61(8):2395-406.

(17) Zhi N, Mills IP, Lu J, Wong S, Filippone C, Brown KE. Molecular and functional analyses of a human parvovirus B19 infectious clone demonstrates essential roles for NS1, VP1, and the 11-kilodalton protein in virus replication and infectivity. J Virol **2006 Jun**; 80(12):5941-50.

(18) Blumel J, Eis-Hubinger AM, Stuhler A, Bonsch C, Gessner M, Lower J. Characterization of Parvovirus B19 genotype 2 in KU812Ep6 cells. J Virol **2005 Nov**; 79(22):14197-206.

(19) Hokynar K, Soderlund-Venermo M, Pesonen M, et al. A new parvovirus genotype persistent in human skin. Virology **2002 Oct 25**; 302(2):224-8.

(20) Nguyen QT, Wong S, Heegaard ED, Brown KE. Identification and characterization of a second novel human erythrovirus variant, A6. Virology **2002 Sep 30**; 301(2):374-80.

(21) Servant A, Laperche S, Lallemand F, et al. Genetic diversity within human erythroviruses: identification of three genotypes. J Virol **2002 Sep**; 76(18):9124-34.

(22) Gallinella G, Venturoli S, Manaresi E, Musiani M, Zerbini M. B19 virus genome diversity: epidemiological and clinical correlations. J Clin Virol **2003 Sep**; 28(1):1-13.

(23) Kaufmann B, Simpson AA, Rossmann MG. The structure of human parvovirus B19. Proc Natl Acad Sci U S A **2004 Aug 10**; 101(32):11628-33.

(24) Agbandje M, Kajigaya S, McKenna R, Young NS, Rossmann MG. The structure of human parvovirus B19 at 8 A resolution. Virology **1994 Aug 15**; 203(1):106-15.

(25) Brown CS, Van Lent JW, Vlak JM, Spaan WJ. Assembly of empty capsids by using baculovirus recombinants expressing human parvovirus B19 structural proteins. J Virol **1991 May**; 65(5):2702-6.

(26) Hurtado A, Rueda P, Nowicky J, Sarraseca J, Casal JI. Identification of domains in canine parvovirus VP2 essential for the assembly of virus-like particles. J Virol **1996 Aug**; 70(8):5422-9.

(27) Dorsch S, Liebisch G, Kaufmann B, et al. The VP1 unique region of parvovirus B19 and its constituent phospholipase A2-like activity. J Virol **2002 Feb**; 76(4):2014-8.

(28) Vihinen-Ranta M, Wang D, Weichert WS, Parrish CR. The VP1 N-terminal sequence of canine parvovirus affects nuclear transport of capsids and efficient cell infection. J Virol **2002 Feb**; 76(4):1884-91.

(29) Fu Y, Ishii KK, Munakata Y, Saitoh T, Kaku M, Sasaki T. Regulation of tumor necrosis factor alpha promoter by human parvovirus B19 NS1 through activation of AP-1 and AP-2. J Virol **2002 Jun**; 76(11):5395-403.

(30) Raab U, Beckenlehner K, Lowin T, Niller HH, Doyle S, Modrow S. NS1 protein of parvovirus B19 interacts directly with DNA sequences of the p6 promoter and with the cellular transcription factors Sp1/Sp3. Virology **2002 Feb 1**; 293(1):86-93.

(31) Gareus R, Gigler A, Hemauer A, et al. Characterization of cis-acting and NS1 protein-responsive elements in the p6 promoter of parvovirus B19. J Virol **1998 Jan**; 72(1):609-16.

(32) Moffatt S, Tanaka N, Tada K, et al. A cytotoxic nonstructural protein, NS1, of human parvovirus B19 induces activation of interleukin-6 gene expression. J Virol **1996 Dec**; 70(12):8485-91.

(33) Moffatt S, Yaegashi N, Tada K, Tanaka N, Sugamura K. Human parvovirus B19 nonstructural (NS1) protein induces apoptosis in erythroid lineage cells. J Virol **1998 Apr**; 72(4):3018-28.

(34) Rohrer C, Gartner B, Sauerbrei A, et al. Seroprevalence of parvovirus B19 in the German population. Epidemiol Infect **2008 Jan 16**;1-12.

(35) Kelly HA, Siebert D, Hammond R, Leydon J, Kiely P, Maskill W. The age-specific prevalence of human parvovirus immunity in Victoria, Australia compared with other parts of the world. Epidemiol Infect **2000 Jun**; 124(3):449-57.

(36) Cohen BJ, Buckley MM. The prevalence of antibody to human parvovirus B19 in England and Wales. J Med Microbiol **1988 Feb**; 25(2):151-3.

(37) Young NS, Brown KE. Parvovirus B19. N Engl J Med **2004 Feb 5**; 350(6):586-97.

(38) Laub R, Strengers P. Parvoviruses and blood products. Pathol Biol (Paris) **2002 Jun**; 50(5):339-48.

(39) Azzi A, Morfini M, Mannucci PM. The transfusion-associated transmission of parvovirus B19. Transfus Med Rev **1999 Jul**; 13(3):194-204.

(40) Schwarz TF, Serke S, Von Brunn A, et al. Heat stability of parvovirus B19: kinetics of inactivation. Zentralbl Bakteriol **1992 Jul**; 277(2):219-23.

(41) Brown KE, Young NS. Parvovirus B19 infection and hematopoiesis. Blood Rev **1995 Sep**; 9(3):176-82.

(42) Ozawa K, Kurtzman G, Young N. Replication of the B19 parvovirus in human bone marrow cell cultures. Science **1986 Aug 22**; 233(4766):883-6.

(43) Yaegashi N, Shiraishi H, Takeshita T, Nakamura M, Yajima A, Sugamura K. Propagation of human parvovirus B19 in primary culture of erythroid lineage cells derived from fetal liver. J Virol **1989 Jun**; 63(6):2422-6.

(44) Brown KE, Anderson SM, Young NS. Erythrocyte P antigen: cellular receptor for B19 parvovirus. Science **1993 Oct 1**; 262(5130):114-7.

(45) Munakata Y, Saito-Ito T, Kumura-Ishii K, et al. Ku80 autoantigen as a cellular coreceptor for human parvovirus B19 infection. Blood **2005 Nov 15**; 106(10):3449-56.

(46) Weigel-Kelley KA, Yoder MC, Srivastava A. Alpha5beta1 integrin as a cellular coreceptor for human parvovirus B19: requirement of functional activation of beta1 integrin for viral entry. Blood **2003 Dec 1**; 102(12):3927-33.

(47) Gallinella G, Manaresi E, Zuffi E, et al. Different patterns of restriction to B19 parvovirus replication in human blast cell lines. Virology **2000 Dec 20**; 278(2):361-7.

(48) Parker JS, Parrish CR. Cellular uptake and infection by canine parvovirus involves rapid dynamin-regulated clathrin-mediated endocytosis, followed by slower intracellular trafficking. J Virol **2000 Feb**; 74(4):1919-30.

(49) Zadori Z, Szelei J, Lacoste MC, et al. A viral phospholipase A2 is required for parvovirus infectivity. Dev Cell **2001 Aug**; 1(2):291-302.

(50) Astell CR. Terminal Hairpins of parvovirus genomes and their role in DNA replication. Handbook of parvoviruses.Boca Raton: CRC Press, **1990**:59-80.

(51) Heegaard ED, Petersen BL, Heilmann CJ, Hornsleth A. Prevalence of parvovirus B19 and parvovirus V9 DNA and antibodies in paired bone marrow and serum samples from healthy individuals. J Clin Microbiol **2002 Mar**; 40(3):933-6.

(52) Cassinotti P, Burtonboy G, Fopp M, Siegl G. Evidence for persistence of human parvovirus B19 DNA in bone marrow. J Med Virol **1997 Nov**; 53(3):229-32.

(53) Soderlund M, von Essen R, Haapasaari J, Kiistala U, Kiviluoto O, Hedman K. Persistence of parvovirus B19 DNA in synovial membranes of young patients with and without chronic arthropathy. Lancet **1997 Apr 12**; 349(9058):1063-5.

(54) Eis-Hubinger AM, Reber U, Abdul-Nour T, Glatzel U, Lauschke H, Putz U. Evidence for persistence of parvovirus B19 DNA in livers of adults. J Med Virol **2001 Oct**; 65(2):395-401.

(55) Kuethe F, Sigusch HH, Hilbig K, et al. Detection of viral genome in the myocardium: lack of prognostic and functional relevance in patients with acute dilated cardiomyopathy. Am Heart J **2007 May**; 153(5):850-8.

(56) Tschope C, Bock CT, Kasner M, et al. High prevalence of cardiac parvovirus B19 infection in patients with isolated left ventricular diastolic dysfunction. Circulation **2005 Feb 22**; 111(7):879-86.

(57) Pankuweit S, Moll R, Baandrup U, Portig I, Hufnagel G, Maisch B. Prevalence of the parvovirus B19 genome in endomyocardial biopsy specimens. Hum Pathol **2003 May**; 34(5):497-503.

(58) Norja P, Hokynar K, Aaltonen LM, et al. Bioportfolio: lifelong persistence of variant and prototypic erythrovirus DNA genomes in human tissue. Proc Natl Acad Sci U S A **2006 May 9**; 103(19):7450-3.

(59) Schwarz TF, Wiersbitzky S, Pambor M. Case report: detection of parvovirus B19 in a skin biopsy of a patient with erythema infectiosum. J Med Virol **1994 Jun**; 43(2):171-4.

(60) Noyola DE, Padilla-Ruiz ML, Obregon-Ramos MG, Zayas P, Perez-Romano B. Parvovirus B19 infection in medical students during a hospital outbreak. J Med Microbiol **2004 Feb**; 53(Pt 2):141-6.

(61) Anderson MJ, Higgins PG, Davis LR, et al. Experimental parvoviral infection in humans. J Infect Dis **1985 Aug**; 152(2):257-65.

(62) Kerr JR. Pathogenesis of human parvovirus B19 in rheumatic disease. Ann Rheum Dis **2000 Sep**; 59(9):672-83.

(63) Woolf AD, Campion GV, Chishick A, et al. Clinical manifestations of human parvovirus B19 in adults. Arch Intern Med **1989 May**; 149(5):1153-6.

(64) Reid DM, Reid TM, Brown T, Rennie JA, Eastmond CJ. Human parvovirus-associated arthritis: a clinical and laboratory description. Lancet **1985 Feb 23**; 1(8426):422-5.

(65) Heegaard ED, Brown KE. Human parvovirus B19. Clin Microbiol Rev **2002 Jul**; 15(3):485-505.

(66) Murai C, Munakata Y, Takahashi Y, et al. Rheumatoid arthritis after human parvovirus B19 infection. Ann Rheum Dis **1999 Feb**; 58(2):130-2.

(67) Taylor HG, Borg AA, Dawes PT. Human parvovirus B19 and rheumatoid arthritis. Clin Rheumatol **1992 Dec**; 11(4):548-50.

(68) Lehmann HW, Knoll A, Kuster RM, Modrow S. Frequent infection with a viral pathogen, parvovirus B19, in rheumatic diseases of childhood. Arthritis Rheum **2003 Jun**; 48(6):1631-8.

(69) Saal JG, Steidle M, Einsele H, Muller CA, Fritz P, Zacher J. Persistence of B19 parvovirus in synovial membranes of patients with rheumatoid arthritis. Rheumatol Int **1992**; 12(4):147-51.

(70) Dijkmans BA, Elsacker-Niele AM, Salimans MM, Albada-Kuipers GA, de Vries E, Weiland HT. Human parvovirus B19 DNA in synovial fluid. Arthritis Rheum **1988 Feb**; 31(2):279-81.

(71) Nikkari S, Luukkainen R, Mottonen T, et al. Does parvovirus B19 have a role in rheumatoid arthritis? Ann Rheum Dis **1994 Feb**; 53(2):106-11.

(72) Goldstein AR, Anderson MJ, Serjeant GR. Parvovirus associated aplastic crisis in homozygous sickle cell disease. Arch Dis Child **1987 Jun**; 62(6):585-8.

(73) Saarinen UM, Chorba TL, Tattersall P, et al. Human parvovirus B19-induced epidemic acute red cell aplasia in patients with hereditary hemolytic anemia. Blood **1986 May**; 67(5):1411-7.

(74) Pattison JR, Jones SE, Hodgson J, et al. Parvovirus infections and hypoplastic crisis in sickle-cell anaemia. Lancet **1981 Mar 21**; 1(8221):664-5.

(75) Seyama K, Kobayashi R, Hasle H, et al. Parvovirus B19-induced anemia as the presenting manifestation of X-linked hyper-IgM syndrome. J Infect Dis **1998 Aug**; 178(2):318-24.

(76) Frickhofen N, Abkowitz JL, Safford M, et al. Persistent B19 parvovirus infection in patients infected with human immunodeficiency virus type 1 (HIV-1): a treatable cause of anemia in AIDS. Ann Intern Med **1990 Dec 15**; 113(12):926-33.

(77) Kurtzman G, Frickhofen N, Kimball J, Jenkins DW, Nienhuis AW, Young NS. Pure red-cell aplasia of 10 years' duration due to persistent parvovirus B19 infection and its cure with immunoglobulin therapy. N Engl J Med **1989 Aug 24**; 321(8):519-23.

(78) Yaegashi N. Pathogenesis of nonimmune hydrops fetalis caused by intrauterine B19 infection. Tohoku J Exp Med **2000 Feb**; 190(2):65-82.

(79) von Kaisenberg CS, Bender G, Scheewe J, et al. A case of fetal parvovirus B19 myocarditis, terminal cardiac heart failure, and perinatal heart transplantation. Fetal Diagn Ther **2001 Nov**; 16(6):427-32.

(80) Morey AL, Keeling JW, Porter HJ, Fleming KA. Clinical and histopathological features of parvovirus B19 infection in the human fetus. Br J Obstet Gynaecol **1992 Jul**; 99(7):566-74.

(81) Tolfvenstam T, Papadogiannakis N, Norbeck O, Petersson K, Broliden K. Frequency of human parvovirus B19 infection in intrauterine fetal death. Lancet **2001 May 12**; 357(9267):1494-7.

(82) Kinney JS, Anderson LJ, Farrar J, et al. Risk of adverse outcomes of pregnancy after human parvovirus B19 infection. J Infect Dis **1988 Apr**; 157(4):663-7.

(83) Anand A, Gray ES, Brown T, Clewley JP, Cohen BJ. Human parvovirus infection in pregnancy and hydrops fetalis. N Engl J Med **1987 Jan 22**; 316(4):183-6.

(84) Brown T, Anand A, Ritchie LD, Clewley JP, Reid TM. Intrauterine parvovirus infection associated with hydrops fetalis. Lancet **1984 Nov 3**; 2(8410):1033-4.

(85) Papadogiannakis N, Tolfvenstam T, Fischler B, Norbeck O, Broliden K. Active, fulminant, lethal myocarditis associated with parvovirus B19 infection in an infant. Clin Infect Dis **2002 Nov 1**; 35(9):1027-31.

(86) Murry CE, Jerome KR, Reichenbach DD. Fatal parvovirus myocarditis in a 5-year-old girl. Hum Pathol **2001 Mar**; 32(3):342-5.

(87) Nigro G, Bastianon V, Colloridi V, et al. Human parvovirus B19 infection in infancy associated with acute and chronic lymphocytic myocarditis and high cytokine levels: report of 3 cases and review. Clin Infect Dis **2000 Jul**; 31(1):65-9.

(88) Lamparter S, Schoppet M, Pankuweit S, Maisch B. Acute parvovirus B19 infection associated with myocarditis in an immunocompetent adult. Hum Pathol **2003 Jul**; 34(7):725-8.

(89) Orth T, Herr W, Spahn T, et al. Human parvovirus B19 infection associated with severe acute perimyocarditis in a 34-year-old man. Eur Heart J **1997 Mar**; 18(3):524-5.

(90) Malm C, Fridell E, Jansson K. Heart failure after parvovirus B19 infection. Lancet **1993 May 29**; 341(8857):1408-9.

(91) Cooling LL, Koerner TA, Naides SJ. Multiple glycosphingolipids determine the tissue tropism of parvovirus B19. J Infect Dis **1995 Nov**; 172(5):1198-205.

(92) Kuhl U, Pauschinger M, Seeberg B, et al. Viral persistence in the myocardium is associated with progressive cardiac dysfunction. Circulation **2005 Sep 27**; 112(13):1965-70.

(93) Lotze U, Egerer R, Tresselt C, et al. Frequent detection of parvovirus B19 genome in the myocardium of adult patients with idiopathic dilated cardiomyopathy. Med Microbiol Immunol **2004 May**; 193(2-3):75-82.

(94) Zerbini M, Gallinella G, Cricca M, Bonvicini F, Musiani M. Diagnostic procedures in B19 infection. Pathol Biol (Paris) **2002 Jun**; 50(5):332-8.

(95) Enders M, Helbig S, Hunjet A, Pfister H, Reichhuber C, Motz M. Comparative evaluation of two commercial enzyme immunoassays for serodiagnosis of human parvovirus B19 infection. J Virol Methods **2007 Dec**; 146(1-2):409-13.

(96) Pfrepper KI, Enders M, Motz M. Human parvovirus B19 serology and avidity using a combination of recombinant antigens enables a differentiated picture of the current state of infection. J Vet Med B Infect Dis Vet Public Health **2005 Sep**; 52(7-8):362-5.

(97) Odibo AO, Campbell WA, Feldman D, et al. Resolution of human parvovirus B19-induced nonimmune hydrops after intrauterine transfusion. J Ultrasound Med **1998 Sep**; 17(9):547-50.

(98) Fairley CK, Smoleniec JS, Caul OE, Miller E. Observational study of effect of intrauterine transfusions on outcome of fetal hydrops after parvovirus B19 infection. Lancet **1995 Nov 18**; 346(8986):1335-7.

(99) Lin F, Zeng A, Yang N, et al. Quantification of human bocavirus in lower respiratory tract infections in China. Infect Agent Cancer **2007**; 2:3.

(100) Chieochansin T, Chutinimitkul S, Payungporn S, et al. Complete coding sequences and phylogenetic analysis of Human Bocavirus (HBoV). Virus Res **2007 Oct**; 129(1):54-7.

(101) Lin F, Zeng AP, Yang E, et al. [Sequence analysis of the complete genome of bocavirus WLL-1]. Bing Du Xue Bao **2007 Jan**; 23(1):57-9.

(102) Brieu N, Gay B, Segondy M, Foulongne V. Electron microscopy observation of human bocavirus (HBoV) in nasopharyngeal samples from HBoV-infected children. J Clin Microbiol **2007 Oct**; 45(10):3419-20.

(103) Lin F, Guan W, Cheng F, Yang N, Pintel D, Qiu J. ELISAs using human bocavirus VP2 virus-like particles for detection of antibodies against HBoV. J Virol Methods **2008 Apr**; 149(1):110-7.

(104) Qu XW, Liu WP, Qi ZY, et al. Phospholipase A2-like activity of human bocavirus VP1 unique region. Biochem Biophys Res Commun **2008 Jan 4**; 365(1):158-63.

(105) Lederman M, Shull BC, Stout ER, Bates RC. Bovine parvovirus DNA-binding proteins: identification by a combined DNA hybridization and immunodetection assay. J Gen Virol **1987 Jan**; 68 (Pt 1):147-57.

(106) Garcia-Garcia ML, Calvo C, Pozo F, et al. Human bocavirus detection in nasopharyngeal aspirates of children without clinical symptoms of respiratory infection. Pediatr Infect Dis J **2008 Apr**; 27(4):358-60.

(107) Fry AM, Lu X, Chittaganpitch M, et al. Human bocavirus: a novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. J Infect Dis **2007 Apr 1**; 195(7):1038-45.

(108) Regamey N, Frey U, Deffernez C, Latzin P, Kaiser L. Isolation of human bocavirus from Swiss infants with respiratory infections. Pediatr Infect Dis J **2007 Feb**; 26(2):177-9.

(109) Arden KE, McErlean P, Nissen MD, Sloots TP, Mackay IM. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J Med Virol **2006 Sep**; 78(9):1232-40.

(110) Bastien N, Chui N, Robinson JL, et al. Detection of human bocavirus in Canadian children in a 1-year study. J Clin Microbiol **2007 Feb**; 45(2):610-3.

(111) Kesebir D, Vazquez M, Weibel C, et al. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis **2006 Nov 1**; 194(9):1276-82.

(112) Catalano-Pons C, Bue M, Laude H, et al. Human bocavirus infection in hospitalized children during winter. Pediatr Infect Dis J **2007 Oct**; 26(10):959-60.

(113) Catalano-Pons C, Giraud C, Rozenberg F, Meritet JF, Lebon P, Gendrel D. Detection of human bocavirus in children with Kawasaki disease. Clin Microbiol Infect **2007 Dec**; 13(12):1220-2.

(114) Arnold JC, Singh KK, Spector SA, Sawyer MH. Human bocavirus: prevalence and clinical spectrum at a children's hospital. Clin Infect Dis **2006 Aug 1**; 43(3):283-8.

(115) Ma X, Endo R, Ishiguro N, et al. Detection of human bocavirus in Japanese children with lower respiratory tract infections. J Clin Microbiol **2006 Mar**; 44(3):1132-4.

(116) Lau SK, Yip CC, Que TL, et al. Clinical and molecular epidemiology of human bocavirus in respiratory and fecal samples from children in Hong Kong. J Infect Dis **2007 Oct 1**; 196(7):986-93.

(117) Chieochansin T, Samransamruajkit R, Chutinimitkul S, et al. Human bocavirus (HBoV) in Thailand: clinical manifestations in a hospitalized pediatric patient and molecular virus characterization. J Infect **2008 Feb**; 56(2):137-42.

(118) Vicente D, Cilla G, Montes M, Perez-Yarza EG, Perez-Trallero E. Human bocavirus, a respiratory and enteric virus. Emerg Infect Dis **2007 Apr**; 13(4):636-7.

(119) Naghipour M, Cuevas LE, Bakhshinejad T, Dove W, Hart CA. Human bocavirus in Iranian children with acute respiratory infections. J Med Virol **2007 May**; 79(5):539-43.

(120) Kleines M, Scheithauer S, Rackowitz A, Ritter K, Hausler M. High prevalence of human bocavirus detected in young children with severe acute lower respiratory tract disease by use of a standard PCR protocol and a novel real-time PCR protocol. J Clin Microbiol **2007 Mar**; 45(3):1032-4.

(121) Pozo F, Garcia-Garcia ML, Calvo C, Cuesta I, Perez-Brena P, Casas I. High incidence of human bocavirus infection in children in Spain. J Clin Virol **2007 Nov**; 40(3):224-8.

(122) Chung JY, Han TH, Kim SW, Kim CK, Hwang ES. Detection of viruses identified recently in children with acute wheezing. J Med Virol **2007 Aug**; 79(8):1238-43.

(123) Chung JY, Han TH, Kim CK, Kim SW. Bocavirus infection in hospitalized children, South Korea. Emerg Infect Dis **2006 Aug**; 12(8):1254-6.

(124) Gendrel D, Guedj R, Pons-Catalano C, et al. Human bocavirus in children with acute asthma. Clin Infect Dis **2007 Aug 1**; 45(3):404-5.

(125) Kaplan NM, Dove W, Abu-Zeid AF, Shamoon HE, Abd-Eldayem SA, Hart CA. Human bocavirus infection among children, Jordan. Emerg Infect Dis **2006 Sep**; 12(9):1418-20.

(126) Allander T, Jartti T, Gupta S, et al. Human bocavirus and acute wheezing in children. Clin Infect Dis **2007 Apr 1**; 44(7):904-10.

(127) Sloots TP, McErlean P, Speicher DJ, Arden KE, Nissen MD, Mackay IM. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol **2006 Jan**; 35(1):99-102.

(128) Bastien N, Brandt K, Dust K, Ward D, Li Y. Human Bocavirus infection, Canada. Emerg Infect Dis **2006 May**; 12(5):848-50.

(129) Foulongne V, Olejnik Y, Perez V, Elaerts S, Rodiere M, Segondy M. Human bocavirus in French children. Emerg Infect Dis **2006 Aug**; 12(8):1251-3.

(130) Weissbrich B, Neske F, Schubert J, et al. Frequent detection of bocavirus DNA in German children with respiratory tract infections. BMC Infect Dis **2006**; 6:109.

(131) Choi EH, Lee HJ, Kim SJ, et al. The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000-2005. Clin Infect Dis **2006 Sep 1** ; 43(5):585-92.

(132) Lu X, Chittaganpitch M, Olsen SJ, et al. Real-time PCR assays for detection of bocavirus in human specimens. J Clin Microbiol **2006 Sep**; 44(9):3231-5.

(133) Simon A, Groneck P, Kupfer B, et al. Detection of bocavirus DNA in nasopharyngeal aspirates of a child with bronchiolitis. J Infect **2007 Mar**; 54(3):e125-e127.

(134) Manning A, Russell V, Eastick K, et al. Epidemiological profile and clinical associations of human bocavirus and other human parvoviruses. J Infect Dis **2006 Nov 1**; 194(9):1283-90.

(135) Smuts H, Hardie D. Human bocavirus in hospitalized children, South Africa. Emerg Infect Dis **2006 Sep**; 12(9):1457-8.

(136) Khetsuriani N, Kazerouni NN, Erdman DD, et al. Prevalence of viral respiratory tract infections in children with asthma. J Allergy Clin Immunol **2007 Feb**; 119(2):314-21.

(137) Kupfer B, Vehreschild J, Cornely O, et al. Severe pneumonia and human bocavirus in adult. Emerg Infect Dis **2006 Oct**; 12(10):1614-6.

(138) Schenk T, Huck B, Forster J, Berner R, Neumann-Haefelin D, Falcone V. Human bocavirus DNA detected by quantitative real-time PCR in two children hospitalized for lower respiratory tract infection. Eur J Clin Microbiol Infect Dis **2007 Feb**; 26(2):147-9.

(139) Maggi F, Andreoli E, Pifferi M, Meschi S, Rocchi J, Bendinelli M. Human bocavirus in Italian patients with respiratory diseases. J Clin Virol **2007 Apr**; 38(4):321-5.

(140) Qu XW, Duan ZJ, Qi ZY, et al. Human bocavirus infection, People's Republic of China. Emerg Infect Dis **2007 Jan**; 13(1):165-8.

(141) Monteny M, Niesters HG, Moll HA, Berger MY. Human bocavirus in febrile children, The Netherlands. Emerg Infect Dis **2007 Jan**; 13(1):180-2.

(142) Neske F, Blessing K, Tollmann F, et al. Real-time PCR for diagnosis of human bocavirus infections and phylogenetic analysis. J Clin Microbiol **2007 Jul**; 45(7):2116-22.

(143) Terrosi C, Fabbiani M, Cellesi C, Cusi MG. Human bocavirus detection in an atopic child affected by pneumonia associated with wheezing. J Clin Virol **2007 Sep**; 40(1):43-5.

(144) Volz S, Schildgen O, Klinkenberg D, et al. Prospective study of Human Bocavirus (HBoV) infection in a pediatric university hospital in Germany 2005/2006. J Clin Virol **2007 Nov**; 40(3):229-35.

(145) Hindiyeh MY, Keller N, Mandelboim M, et al. High rate of human bocavirus and adenovirus coinfection in hospitalized Israeli children. J Clin Microbiol **2008 Jan**; 46(1):334-7.

(146) Villa L, Melon S, Suarez S, et al. Detection of human bocavirus in Asturias, Northern Spain. Eur J Clin Microbiol Infect Dis **2008 Mar**; 27(3):237-9.

(147) Christensen A, Nordbo SA, Krokstad S, Rognlien AG, Dollner H. Human bocavirus commonly involved in multiple viral airway infections. J Clin Virol **2008 Jan**; 41(1):34-7.

(148) Gerna G, Piralla A, Campanini G, Marchi A, Stronati M, Rovida F. The human bocavirus role in acute respiratory tract infections of pediatric patients as defined by viral load quantification. New Microbiol **2007 Oct**; 30(4):383-92.

(149) Redshaw N, Wood C, Rich F, Grimwood K, Kirman JR. Human bocavirus in infants, New Zealand. Emerg Infect Dis **2007 Nov**; 13(11):1797-9.

(150) Schenk T, Strahm B, Kontny U, Hufnagel M, Neumann-Haefelin D, Falcone V. Disseminated bocavirus infection after stem cell transplant. Emerg Infect Dis **2007 Sep**; 13(9):1425-7.

(151) Longtin J, Bastien M, Gilca R, et al. Human bocavirus infections in hospitalized children and adults. Emerg Infect Dis **2008 Feb**; 14(2):217-21.

(152) Esposito S, Bosis S, Niesters HG, et al. Impact of human bocavirus on children and their families. J Clin Microbiol **2008 Apr**; 46(4):1337-42.

(153) Canducci F, Debiaggi M, Sampaolo M, et al. Two-year prospective study of single infections and co-infections by respiratory syncytial virus and viruses identified recently in infants with acute respiratory disease. J Med Virol **2008 Apr**; 80(4):716-23.

(154) Smuts H, Workman L, Zar HJ. Role of human metapneumovirus, human coronavirus NL63 and human bocavirus in infants and young children with acute wheezing. J Med Virol **2008 May**; 80(5):906-12.

(155) Rihkanen H, Ronkko E, Nieminen T, et al. Respiratory viruses in laryngeal croup of young children. J Pediatr **2008 May**; 152(5):661-5.

(156) Volz S, Schildgen O, Muller A, et al. [The human bocavirus: pathogen in airway infections?]. Dtsch Med Wochenschr **2007 Jul 5**; 132(28-29):1529-33.

(157) Garbino J, Inoubli S, Mossdorf E, et al. Respiratory viruses in HIV-infected patients with suspected respiratory opportunistic infection. AIDS **2008 Mar 30**; 22(6):701-5.

(158) Manning A, Willey SJ, Bell JE, Simmonds P. Comparison of tissue distribution, persistence, and molecular epidemiology of parvovirus B19 and novel human parvoviruses PARV4 and human bocavirus. J Infect Dis **2007 May 1**; 195(9):1345-52.

(159) Allander T. Human bocavirus. J Clin Virol **2008 Jan**; 41(1):29-33.

(160) Kahn JS. Newly discovered respiratory viruses: significance and implications. Curr Opin Pharmacol **2007 Oct**; 7(5):478-83.

(161) Albuquerque MC, Rocha LN, Benati FJ, et al. Human bocavirus infection in children with gastroenteritis, Brazil. Emerg Infect Dis **2007 Nov**; 13(11):1756-8.

(162) Lee JI, Chung JY, Han TH, Song MO, Hwang ES. Detection of human bocavirus in children hospitalized because of acute gastroenteritis. J Infect Dis **2007 Oct 1**; 196(7):994-7.

(163) Murphy KM, Travers P, Walport M. Janeway´s Immunobiology. Seventh Edition ed. Taylor & Francis, **2007**.

(164) Webb T, Goodman HC. The structure and function of immunoglobulins. Mod Trends Immunol **1967**; 2:151-87.

(165) Schur PH. IgG subclasses--a review. Ann Allergy **1987 Feb**; 58(2):89-96, 99.

(166) Bird P, Calvert JE, Amlot PL. Distinctive development of IgG4 subclass antibodies in the primary and secondary responses to keyhole limpet haemocyanin in man. Immunology **1990 Mar**; 69(3):355-60.

(167) Linde A, Sundqvist VA, Mathiesen T, Wahren B. IgG subclasses to subviral components. Monogr Allergy **1988**; 23:27-32.

(168) Aalberse RC, van der GR, van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. J Immunol **1983 Feb**; 130(2):722-6.

(169) Res P, Spits H. Developmental stages in the human thymus. Semin Immunol **1999 Feb**; 11(1):39-46.

(170) Kabelitz D, Marischen L, Oberg HH, Holtmeier W, Wesch D. Epithelial defence by gamma delta T cells. Int Arch Allergy Immunol **2005 May**; 137(1):73-81.

(171) Alt FW, Oltz EM, Young F, Gorman J, Taccioli G, Chen J. VDJ recombination. Immunol Today **1992 Aug**; 13(8):306-14.

(172) Davis MM. T cell receptor gene diversity and selection. Annu Rev Biochem **1990**; 59:475-96.

(173) Stenger S, Hanson DA, Teitelbaum R, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science **1998 Oct 2**; 282(5386):121-5.

(174) Masson D, Tschopp J. Isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes. J Biol Chem **1985 Aug 5**; 260(16):9069-72.

(175) Spellberg B, Edwards JE, Jr. Type 1/Type 2 immunity in infectious diseases. Clin Infect Dis **2001 Jan**; 32(1):76-102.

(176) Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol **1997**; 15:749-95.

(177) DeKruyff RH, Rizzo LV, Umetsu DT. Induction of immunoglobulin synthesis by CD4+ T cell clones. Semin Immunol **1993 Dec**; 5(6):421-30.

(178) Rizzo LV, DeKruyff RH, Umetsu DT. Generation of B cell memory and affinity maturation. Induction with Th1 and Th2 T cell clones. J Immunol **1992 Jun 15**; 148(12):3733-9.

(179) Ohmori Y, Hamilton TA. IL-4-induced STAT6 suppresses IFN-gamma-stimulated STAT1-dependent transcription in mouse macrophages. J Immunol **1997 Dec 1**; 159(11):5474-82.

(180) D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med **1993 Sep 1**; 178(3):1041-8.

(181) Matsuda H, Sakaguchi K, Shibasaki T, Takahashi H, Kawakami Y, Furuya K. Intrauterine therapy for parvovirus B19 infected symptomatic fetus using B19 IgG-rich high titer gammaglobulin. J Perinat Med **2005**; 33(6):561-3.

(182) Erdman DD, Usher MJ, Tsou C, et al. Human parvovirus B19 specific IgG, IgA, and IgM antibodies and DNA in serum specimens from persons with erythema infectiosum. J Med Virol **1991 Oct**; 35(2):110-5.

(183) Ennis O, Corcoran A, Kavanagh K, Mahon BP, Doyle S. Baculovirus expression of parvovirus B19 (B19V) NS1: utility in confirming recent infection. J Clin Virol **2001 Aug**; 22(1):55-60.

(184) Franssila R, Soderlund M, Brown CS, Spaan WJ, Seppala I, Hedman K. IgG subclass response to human parvovirus B19 infection. Clin Diagn Virol **1996 Jun**; 6(1):41-9.

(185) Bluth MH, Norowitz KB, Chice S, et al. Detection of IgE anti-parvovirus B19 and increased CD23+ B cells in parvovirus B19 infection: relation to Th2 cytokines. Clin Immunol **2003 Aug**; 108(2):152-8.

(186) Soderlund M, Brown CS, Spaan WJ, Hedman L, Hedman K. Epitope type-specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J Infect Dis **1995 Dec**; 172(6):1431-6.

(187) Cole KS, Murphey-Corb M, Narayan O, Joag SV, Shaw GM, Montelaro RC. Common themes of antibody maturation to simian immunodeficiency virus, simian-human immunodeficiency virus, and human immunodeficiency virus type 1 infections. J Virol **1998 Oct**; 72(10):7852-9.

(188) Hammond SA, Cook SJ, Lichtenstein DL, Issel CJ, Montelaro RC. Maturation of the cellular and humoral immune responses to persistent infection in horses by equine infectious anemia virus is a complex and lengthy process. J Virol **1997 May**; 71(5):3840-52.

(189) Hemauer A, Gigler A, Searle K, et al. Seroprevalence of parvovirus B19 NS1-specific IgG in B19-infected and uninfected individuals and in infected pregnant women. J Med Virol **2000 Jan**; 60(1):48-55.

(190) von Poblotzki A, Hemauer A, Gigler A, et al. Antibodies to the nonstructural protein of parvovirus B19 in persistently infected patients: implications for pathogenesis. J Infect Dis **1995 Nov**; 172(5):1356-9.

(191) von Poblotzki A, Gigler A, Lang B, Wolf H, Modrow S. Antibodies to parvovirus B19 NS-1 protein in infected individuals. J Gen Virol **1995 Mar**; 76 (Pt 3):519-27.

(192) Venturoli S, Gallinella G, Manaresi E, Gentilomi G, Musiani M, Zerbini M. IgG response to the immunoreactive region of parvovirus B19 nonstructural protein by immunoblot assay with a recombinant antigen. J Infect Dis **1998 Dec**; 178(6):1826-9.

(193) von Poblotzki A, Gerdes C, Reischl U, Wolf H, Modrow S. Lymphoproliferative responses after infection with human parvovirus B19. J Virol **1996 Oct**; 70(10):7327-30.

(194) Franssila R, Hokynar K, Hedman K. T helper cell-mediated in vitro responses of recently and remotely infected subjects to a candidate recombinant vaccine for human parvovirus b19. J Infect Dis **2001 Mar 1**; 183(5):805-9.

(195) Franssila R, Hedman K. T-helper cell-mediated interferon-gamma, interleukin-10 and proliferation responses to a candidate recombinant vaccine for human parvovirus B19. Vaccine **2004 Sep 9**; 22(27-28):3809-15.

(196) Franssila R, Auramo J, Modrow S, et al. T helper cell-mediated interferon-gamma expression after human parvovirus B19 infection: persisting VP2-specific and transient VP1u-specific activity. Clin Exp Immunol **2005 Oct**; 142(1):53-61.

(197) Tolfvenstam T, Oxenius A, Price DA, et al. Direct ex vivo measurement of CD8(+) T-lymphocyte responses to human parvovirus B19. J Virol **2001 Jan**; 75(1):540-3.

(198) Kasprowicz V, Isa A, Jeffery K, et al. A highly restricted T-cell receptor dominates the CD8+ T-cell response to parvovirus B19 infection in HLA-A*2402-positive individuals. J Virol **2006 Jul**; 80(13):6697-701.

(199) Norbeck O, Isa A, Pohlmann C, et al. Sustained CD8+ T-cell responses induced after acute parvovirus B19 infection in humans. J Virol **2005 Sep**; 79(18):12117-21.

(200) Endo R, Ishiguro N, Kikuta H, et al. Seroepidemiology of human bocavirus in Hokkaido prefecture, Japan. J Clin Microbiol **2007 Oct**; 45(10):3218-23.

(201) Kantola K, Hedman L, Allander T, et al. Serodiagnosis of human bocavirus infection. Clin Infect Dis **2008 Feb 15**; 46(4):540-6.

(202) Fleisher TA, Oliveira JB. Functional and molecular evaluation of lymphocytes. J Allergy Clin Immunol **2004 Aug**; 114(2):227-34.

(203) Soderlund M, Brown CS, Cohen BJ, Hedman K. Accurate serodiagnosis of B19 parvovirus infections by measurement of IgG avidity. J Infect Dis **1995 Mar**; 171(3):710-3.

(204) Hutchings PR, Cambridge G, Tite JP, Meager T, Cooke A. The detection and enumeration of cytokine-secreting cells in mice and man and the clinical application of these assays. J Immunol Methods **1989 Jun 2**; 120(1):1-8.

(205) Porstmann T, Kiessig ST. Enzyme immunoassay techniques. An overview. J Immunol Methods **1992 Jun 24**; 150(1-2):5-21.

(206) Modrow S, Dorsch S. Antibody responses in parvovirus B19 infected patients. Pathol Biol (Paris) **2002 Jun**; 50(5):326-31.

(207) Maranga L, Brazao TF, Carrondo MJ. Virus-like particle production at low multiplicities of infection with the baculovirus insect cell system. Biotechnol Bioeng **2003 Oct 20**; 84(2):245-53.

(208) Saliki JT, Mizak B, Flore HP, et al. Canine parvovirus empty capsids produced by expression in a baculovirus vector: use in analysis of viral properties and immunization of dogs. J Gen Virol **1992 Feb**; 73 (Pt 2):369-74.

(209) Raulf-Heimsoth M, Sander I, Chen Z, et al. Development of a monoclonal antibody-based sandwich ELISA for detection of the latex allergen Hev b 1. Int Arch Allergy Immunol **2000 Nov**; 123(3):236-41.

(210) Mengeling WL, Paul PS. Antibodies for autonomous parvoviruses of lower animals detected in human serum. Brief report. Arch Virol **1986**; 88(1-2):127-33.

(211) Mengeling WL, Matthews PJ. Antibodies to bovine parvovirus acquired by neonatal pigs through ingestion of virus and antibody in the diet. Am J Vet Res **1990 Apr**; 51(4):632-5.

(212) Corcoran A, Doyle S, Waldron D, Nicholson A, Mahon BP. Impaired gamma interferon responses against parvovirus B19 by recently infected children. J Virol **2000 Nov**; 74(21):9903-10.

(213) Persson MA, Brown SE, Steward MW, et al. IgG subclass-associated affinity differences of specific antibodies in humans. J Immunol **1988 Jun 1**; 140(11):3875-9.

(214) Zinkernagel RM. Maternal antibodies, childhood infections, and autoimmune diseases. N Engl J Med **2001 Nov 1**; 345(18):1331-5.

(215) Kahn JS, Kesebir D, Cotmore SF, et al. Seroepidemiology of Human Bocavirus Defined Using Recombinant Virus-Like Particles. J Infect Dis **2008 May 20**.

(216) Black CP. Systematic review of the biology and medical management of respiratory syncytial virus infection. Respir Care **2003 Mar**; 48(3):209-31.

(217) Cheng WX, Jin Y, Duan ZJ, et al. Human bocavirus in children hospitalized for acute gastroenteritis: a case-control study. Clin Infect Dis **2008 Jul 15**; 47(2):161-7.

(218) Durham PJ, Lax A, Johnson RH. Pathological and virological studies of experimental parvoviral enteritis in calves. Res Vet Sci **1985 Mar**; 38(2):209-19.

(219) Mahon BP, Katrak K, Nomoto A, Macadam AJ, Minor PD, Mills KH. Poliovirus-specific CD4+ Th1 clones with both cytotoxic and helper activity mediate protective humoral immunity against a lethal poliovirus infection in transgenic mice expressing the human poliovirus receptor. J Exp Med **1995 Apr 1**; 181(4):1285-92.

(220) Skurkovich B, Skurkovich S. Inhibition of IFN-gamma as a method of treatment of various autoimmune diseases, including skin diseases. Ernst Schering Res Found Workshop **2006**;(56):1-27.

(221) van Duivenvoorde LM, Han WG, Bakker AM, et al. Immunomodulatory dendritic cells inhibit Th1 responses and arthritis via different mechanisms. J Immunol **2007 Aug 1**; 179(3):1506-15.

(222) Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today **1993 Jul**; 14(7):353-6.

(223) Dong M, He J, Wang Z, Xie X, Wang H. Placental imbalance of Th1- and Th2-type cytokines in preeclampsia. Acta Obstet Gynecol Scand **2005 Aug**; 84(8):788-93.

(224) Orthopoulos G, Triantafilou K, Triantafilou M. Coxsackie B viruses use multiple receptors to infect human cardiac cells. J Med Virol **2004 Oct**; 74(2):291-9.

(225) Caforio AL, Calabrese F, Angelini A, et al. A prospective study of biopsy-proven myocarditis: prognostic relevance of clinical and aetiopathogenetic features at diagnosis. Eur Heart J **2007 Jun**; 28(11):1326-33.

(226) Spiess K. Untersuchungen zur Genexpression von Parvovirus B19 in infizierten Zellen und Geweben mittels Immunhistochemie und in situ Hybridisierung University of Regensburg; **2005**.

5. Anhang

5.1. Publikation I: CD4+ T Helper Cell Responses Against Human Bocavirus VP2 Virus-like Particles in Healthy Adults

Journal of Infectious Diseases, 2008, Im Druck.

Juha Lindner[1], Sandra Zehentmeier[1], Rauli Franssila[2], Sascha Barabas[1], Josef Schröder[3], Ludwig Deml[1], and Susanne Modrow[1*]

Institute of Medical Microbiology and Hygiene, University of Regensburg, Germany[1]; Department of Virology, Haartman Institute and HUCH Diagnostic, University of Helsinki, Finland[2]; Institute of Pathology, University of Regensburg, Germany[3]

Running title: Immune Responses Against Human Bocavirus
Keywords: Human Bocavirus, Parvovirus B19, Immune response, T cells, Virus-like particles

*Corresponding author: Susanne Modrow, Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz-Josef-Strauss Allee 11, 93053 Regensburg, Germany. Phone: (+49)-941-9446454, Fax: (+49)-941-9446402, e-mail: susanne.modrow@klinik.uni-regensburg.de.

Abstract

Background: Human Bocavirus (HBoV) was recently described as a new member of the *Parvoviridae* and is discussed to be associated with respiratory illness in infants. Up to date, HBoV genomes have been detected in respiratory samples of children with pulmonary diseases worldwide, while only limited data are available on virus-specific immunity, mainly due to the lack of recombinant viral antigens.

Methods: HBoV virus-like particles (VLP) were produced in insect cells and characterized by electron microscopy and CsCl-gradient centrifugation. HBoV VP2-specific antibodies and $CD4^+$ T-helper cell responses were analyzed by ELISA and ELISPOT.

Results: VP2 capsid proteins of HBoV were expressed in insect cells infected with a recombinant baculovirus and the formation of icosahedral VLP (diameter: 21-25 nm, sedimentation density: 1.33 g/cm³) was demonstrated. Significantly increased VP2-specific interferon-gamma secretion was detected in peripheral blood mononuclear cell cultures of 69 healthy adults found positive for HBoV-specific IgG antibodies as compared to control stimulations. In parallel, T cell responses against identically expressed parvovirus B19 VP2-VLP were frequently observed in studied individuals without obvious cross-reactions between HBoV and parvovirus B19.

Conclusions: The data suggest a presence of HBoV-specific immune responses in adults and strongly support a high prevalence of HBoV among humans.

Introduction

Human Bocavirus (HBoV), a newly discovered member of the *Parvoviridae* family, was recently identified in children and infants with infections of the lower respiratory tract [1]. HBoV represents the second parvovirus currently discussed to cause disease in humans. Comprehensive phylogenetic analysis of the HBoV genome has revealed a close relation of HBoV to the canine minute virus and bovine parvovirus, both members of the genus *Bocavirus*. Up to date several isolates of HBoV with high sequence homology have been described [2].

Parvoviruses are small, non-enveloped viruses characterized by linear single-stranded DNA-genomes between 4 and 6 kilobases. A common feature of parvoviruses is their exceptional stability and the structural simplicity of the virions [3]. The icosahedral virus capsids generally consist of two proteins VP1 and VP2, which are sequence identical except for the amino-terminal domain of VP1, termed the VP1-*unique region*, spanning 129 and 227 amino acids in case of HBoV and parvovirus B19 (B19V), respectively [4]. Parvovirus B19 VP2 proteins have been shown to possess all features required for particle formation. Virus-like particles consisting of VP2 alone or of VP2 and VP1 may be produced in various eukaryotic expression systems, i.e. recombinant baculovirus or yeast [5, 6].

HBoV has been a target of epidemiological studies since its initial detection. Up to date, HBoV was found by PCR-based techniques not only in clinical respiratory samples but also fecal excretions of young children in Australia, North-America, Asia, Europe, Africa and the Middle-East, indicating a worldwide distribution of the virus [7-17]. The prevalence of HBoV-DNA in samples of children and infants with symptoms of respiratory illness has been found to range between 1.5-18.3% and may vary depending on seasonal fluctuation and the age of studied patients. Since the majority of epidemiological studies have been performed retrospectively, HBoV has not been clearly identified as a sole infectious agent responsible for respiratory diseases. In fact HBoV tends to be linked with a high rate of co-infections with other respiratory viruses in children, e.g. picornaviruses, adenoviruses and respiratory syncytial virus. Clinical symptoms associated with HBoV infections include cough, fever, pneumonia and bronchitis. So far, only single reported cases of HBoV infections have been observed in mainly immunocompromized adult individuals [18, 19].

First immunological studies have described the use of recombinant HBoV VP1 protein in the detection of virus-specific immunoglobulin (Ig) G responses in the Japanese population

[20]. Thereby, ubiquitous VP1-specific antibody-responses with a seroprevalence of up to 100% were detected in adult individuals and in children (age above 6 years), whereas seronegative individuals were predominantly found in small children at the age of one year. No data are currently available on HBoV-specific T cell immunity. Numerous studies on parvovirus B19 however describe the viral VP proteins as immunodominant targets of the cellular and humoral immune response [21-24], suggesting a possibly similar role for the HBoV capsid proteins in virus-specific immunity.
In the present study we describe the recombinant expression and characterization of HBoV VP2-VLP and their use in the detection of cellular immune responses against HBoV in healthy adults.

Materials and Methods

Study subjects and serology. A total of 69 healthy caucasians from the South-Eastern part of Bavaria, Germany (39 male, 30 female, age range: 23-73 years, mean age: 39 years) were studied for HBoV and B19V-specific T cell responses (Table 1) and for the presence of virus-specific antibodies by ELISA. For HBoV, 100 ng of HBoV VP2-VLP were coated on Nunc-Immuno^{TM} MediSorp^{TM} plates (Nunc GmbH, Wiesbaden, Germany) in phosphate buffered saline (PBS) overnight at 4°C, washed six times with washing buffer (PBS, 0.05% Tween 20) and blocked with dilution buffer (PBS, 2% Tween 20, 3% FCS) for 1 h at 37°C. After incubation with serum samples (1:100 in dilution buffer) for 2 h at 37°C, the plates were washed and an anti-human IgG-specific HRP-coupled secondary antibody (Dako Deutschland GmbH, Hamburg, Germany) (1:6000 in dilution buffer) was added for 1 h at 37°C. Development was performed using the BD OptEIA^{TM} Substrate (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Parvovirus B19-specific IgG and IgM antibodies were detected in donor sera using a standardized enzyme-linked immunoassay (ELISA) (Biotrin International, Dublin, Ireland) and RecomBlot analysis (Mikrogen GmbH, Neuried, Germany).

Generation of a recombinant baculovirus for the expression of HBoV VP2 antigens. A recombinant baculovirus encoding the VP2 gene of HBoV ST2 isolate was generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Karlsruhe, Germany). Briefly, the VP2 gene was amplified from a pCR®4-TOPO® vector (Invitrogen) including the genome sequence of HBoV ST2 (Accession number: NC_007455), generously provided to us by Dr. Tobias Allander (Karolinska Institute, Stockholm, Sweden) using the primers 5'-

GAGGAGCGGCCGCATGTCTGACACTGACATTC and 3'-CCGCCCTCGAGTTACAACACTTTATTGATG with the Phusion High-Fidelity DNA Polymerase (Finnzymes OY, Espoo, Finland). The amplicon was introduced into the pFastBac1 vector (Invitrogen) by *Xho*I and *Not*I restriction sites. The generation of a recombinant HBoV VP2 bacmid pFastBac/VP2bac and its following transfection into Sf9 insect cells was performed according to the manufacturer's instructions. The resulting virus bacVP2/HBoV was amplified in three consecutive passages of infection and subsequently used for protein production. For the expression of parvovirus B19 VP2 virus-like particles, the previously established and described recombinant baculovirus bacVP2/B19V was used [25].

Study subjects	Number		HIV control [a]	HBoV VP2 [a]	B19 VP2 [a]			
					B19 seronegative [b]	Number	B19 seropositive [b]	Number
total	69	median:	3 ± 4	20 ± 20	6 ± 8	22	38 ± 32	47
		range:	0 - 18	1 - 70	1 - 29		1 - 159	
		p-value[c]:		<0.0001			<0.0001	
gender								
male	39	median:	4 ± 4	17 ± 17	4 ± 9	12	33 ± 25	27
		range:	0 - 11	1 - 66	1 - 29		1 - 124	
		p-value[c]:		<0.0001			0.0004	
female	30	median:	3 ± 4	35 ± 22	12 ± 7	10	51 ± 35	20
		range:	0 - 18	6 - 70	2 - 20		15 - 159	
		p-value[c]:		<0.0001			<0.0001	
year of birth								
1980 - 1989	19	median:	4 ± 2	35 ± 19	12 ± 7	5	38 ± 27	14
		range:	1 - 9	8 - 67	4 - 21		5 - 115	
		p-value[c]:		<0.0001			0.0095	
1970 - 1979	21	median:	3 ± 3	31 ± 22	6 ± 5	11	40 ± 14	10
		range:	1 - 11	6 - 70	1 - 17		20 - 62	
		p-value[c]:		<0.0001			<0.0001	
1960 - 1969	9	median:	2 ± 2	10 ± 23	3 ± 1	3	26 ± 39	6
		range:	2 - 8	1 - 66	2 - 4		2 - 106	
		p-value[c]:		0.004			0.2619	
1950 - 1959	12	median:	5 ± 5	16 ± 14	16 ± 1	2	55 ± 49	10
		range:	1 - 18	3 - 51	26 - 29		16 - 159	
		p-value[c]:		0.0056			0.6061	
1940 - 1949	5	median:	8 ± 5	20 ± 10	n.d.	0	40 ± 16	5
		range:	0 - 12	8 - 30			18 - 58	
		p-value[c]:		<0.0001			n.d.	
1930 - 1939	3	median:	6 ± 2	17 ± 4	15	1	27 ± 20	2
		range:	6 - 10	11 - 19			13 - 41	
		p-value[c]:		<0.0001			n.d.	

Table 1. Antigen-specific IFN-γ secretion in HBoV seropositive healthy adults. [a] The data shown represent median values of spot forming cells (SFC) per $2x10^5$ PBMC ± standard deviation. [b] Parvovirus B19 serology refers to the presence of virus-specific IgG antibodies in donor serum. [c] Statistical analysis of HBoV and B19V-specific T cell responses was performed in relation to the HIV peptide or B19V VP2-VLP negative controls, respectively. n.d., not done.

Production and purification of recombinant HBoV virus-like particles. Production of both HBoV and parvovirus B19 VLP was performed in High-5 insect cells. Cells were cultured

in Insect-Xpress Medium (Cambrex Bio Science Walkersville Inc., Walkersville, USA), infected with bacVP2/HBoV or bacVP2/B19V at a multiplicity of infection of 3 and harvested 72 h post infection. Recombinant VLP were purified by CsCl cushion centrifugation. A total of $6x10^7$ cells were lysed in 5 ml buffer containing 10 mM Tris/HCl (pH 7.4), 10 mM NaCl, 15 mM $MgCl_2$, 0.5% Triton X-100 with 40 µl/ml Complete Protease Inhibitor Cocktail (Roche Applied Science, Mannheim, Germany) and subjected to repeated freeze-thaw cycles. The precleared lysates were loaded on double cushions consisting of CsCl-solutions with densities of 1.52 g/cm^3 and 1.22 g/cm^3 in TE-buffer (10 mM Tris/HCl pH 8.7, 1 mM EDTA, 0.5% Triton X-100). Ultracentrifugation was performed at 100.000xg for 4 h at 10ºC without brakes. After centrifugation protein samples were collected in fractions and subjected to SDS-PAGE and Western blot analysis. Endotoxin contaminations of antigen-containing fractions were excluded using the QCL-1000 Chromogenic LAL Endpoint Assay (Lonza GmbH, Wuppertal, Germany) and determined to be 1.57 EU/mg and 8.2 EU/mg for HBoV and B19V VP2-VLP, respectively. Characterized antigens were dialyzed against PBS and stored in aliquots at -80ºC. Freeze-thaw cycles of the purified proteins were avoided.

Isolation and culture of human PBMC. Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood samples by Pancoll centrifugation (PAN Biotech GmbH, Aidenbach, Germany) (800xg, 30 min) using Leucosep tubes (Greiner Bio-One, Frickenhausen, Germany). Cells were washed twice in PBS and cultured in RPMI-1640 medium (PAN Biotech GmbH) containing 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% heat-inactivated human AB serum (PAA Laboratories, Linz, Austria) in an atmosphere with 5% CO_2 at 37ºC.

ELISPOT assays. $2x10^5$ PBMC were seeded in four replicate wells on Mahan 4550 MultiScreen® ELISPOT-plates (Millipore, Bedford, MA, USA) previously coated with 5 µg/ml of anti-human interferon-gamma (IFN-γ) monoclonal antibody D1K (Mabtech, Nacka Strand, Sweden), stimulated with 5 µg/ml VP2-VLP of either HBoV or B19V and incubated at 37ºC for 60 h. As negative and positive controls, cells were stimulated with 5 µg/ml of human immunodeficiency virus (HIV) p24-derived murine e10F-peptide [26] and 5 ng/ml staphylococcus enterotoxin B (Sigma Aldrich, Hannover, Germany), respectively. For development, the plates were incubated for 2 h with 1 µg/ml of biotinylated anti-IFN-γ 7-B6-1-Biotin antibody (Mabtech) and 1 h with streptavidin-alkaline phosphatase (diluted

1:1000 in PBS). Staining was performed using the NBT/BCIP Stock Solution (Roche Diagnostics GmbH, Mannheim, Germany) in buffer containing 0.1 M NaCl, 0.1 M Tris/HCl pH 9.5, 0.05 M $MgCl_2$. The number of spot-forming cells (SFC) was counted under magnification using a Bioreader 2000 (BioSys, Karben, Germany).

T cell depletion assays. Depletion of $CD4^+$ or $CD8^+$ T cells from donor PBMC cultures was performed using CD4 and CD8 Dynabeads® (Dynal Biotech ASA, Oslo, Norway). For control, the purity of depleted cell populations was assessed by staining with the CytoStat® Multicolor Reagent CD45-FITC / CD4-PE / CD8-ECD / CD3-PC5 (Beckman Coulter, Krefeld, Germany) and an anti-CD14-PE antibody (BD Biosciences, Heidelberg, Germany) (data not shown). For analysis, a FACS Epics® XL-MCL flow cytometer (Beckman Coulter) was used. All experiments were conducted according to the manufacturer's instructions.

Statistical data analysis. Serological and cellular responses were statistically evaluated using the Mann-Whitney U-test for independent samples.

Results

Generation of recombinant baculovirus bacVP2/HBoV for the expression of HBoV VP2 proteins in insect cells. The recombinant baculovirus bacVP2/HBoV encoding the VP2 gene of the HBoV Stockholm 2 isolate (nts 3443-5071) was generated and used for infection of High-5 cells. In parallel, cells were infected with a previously established recombinant baculovirus bacVP2/B19V encoding the VP2 protein of parvovirus B19. SDS-PAGE studies of bacVP2/HBoV and bacVP2/B19V-infected cells lysed 72 hours after infection revealed a high-yield expression of the VP2 proteins with an apparent molecular weight of approximately 62 kDa after coomassie staining (Figure 1A and B, lanes 1). The identity of both HBoV and parvovirus B19 VP2 proteins was demonstrated by Western blot analysis using sera from HBoV or B19V IgG seropositive individuals (Figure 1A and B, lanes 2).

Purification and characterization of recombinant HBoV VP2 virus-like particles. To analyze whether the expressed HBoV VP2 proteins posses the capacity to spontaneously aggregate to virus-like particles as shown for the VP2 proteins of numerous parvoviruses, bacVP2/HBoV-infected High-5 cells were submitted to a procedure previously established

for the purification of VP2-VLP of human parvovirus B19 [25]. Separation of proteins in precleared lysates of High-5 cells expressing either HBoV or B19V VP2 by CsCl ultracentrifugation resulted in pure preparations of VP2 proteins, as shown by SDS-PAGE and subsequent coomassie staining (Figure 1A and B, lanes 3).

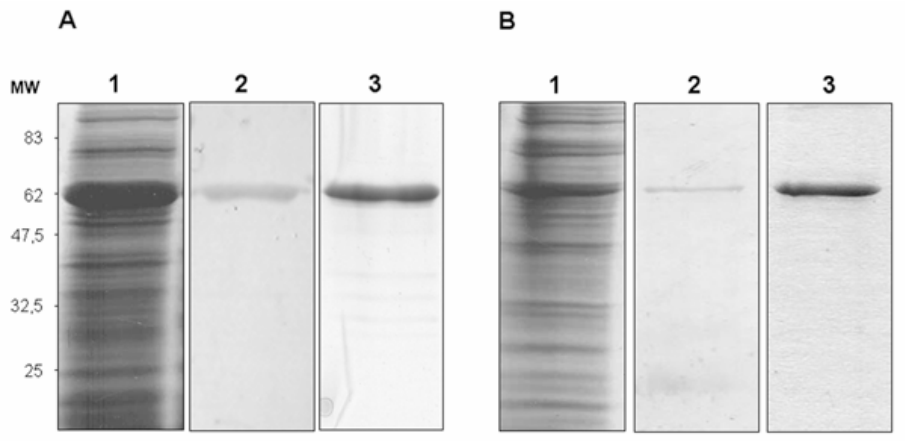

Figure 1. *Production of VP2 proteins of HBoV and parvovirus B19 and the purification of virus-like particles.* SDS-PAGE analysis of lysates of High-5 cells infected with bacVP2/HBoV (A) or bacVP2/B19V (B), stained with coomassie blue (lanes 1) or analyzed by Western blotting with IgG derived from HBoV or parvovirus B19 seropositive individuals (lanes 2). Coomassie blue stained SDS-PAGE of HBoV and B19V VP2 proteins after purification via CsCl ultracentrifugation (lanes 3).

BacVP2/HBoV infected High-5 cells and purified preparations of HBoV VP2 proteins were submitted to electron microscopy. Analysis of cross sections prepared from bacVP2/HBoV-infected High-5 cells revealed a high number of particulate structures in the cytoplasm, suggesting spontaneous formation of HBoV virus-like particles in VP2-expressing cells (Figure 2A). This could be confirmed by electron microscopic analysis of CsCl gradient fractions in which VP2 proteins could be detected by SDS-PAGE and Western blot. Here distinct icosahedral capsid structures with an approximate diameter of 21–25 nm (Figure 2B) were detectable. The density of HBoV VP2-VLP was assessed by ultracentrifugation in continuous CsCl gradients (1.29 g/cm^3 to 1.4 g/cm^3) for 48 h at 150.000xg and revealed a value of approximately 1.33 g/cm^3.

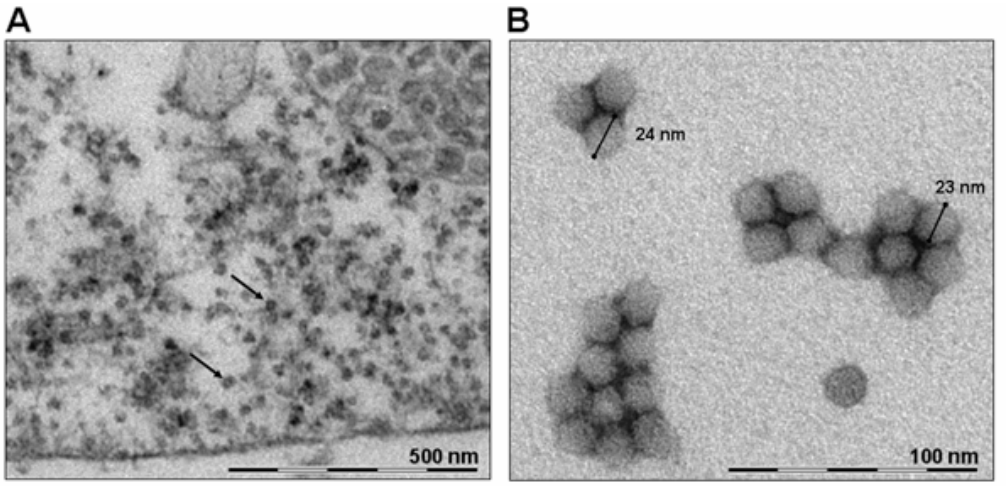

Figure 2. *Electron microscopic analysis of HBoV VP2-VLP.* (A) High-5 insect cells were infected with the recombinant baculovirus bacVP2/HBoV and harvested 72 h post-infection. Ultrathin sections of the infected cells were examined by electron microscopy. Particulate structures, representatively marked by arrows, were observed in the cytoplasm. (B) HBoV VP2-VLP purified from lysates of High-5 cells infected with bacVP2/HBoV by CsCl ultracentrifugation. Fractions containing VP2 proteins were prepared for electron microscopy by negative staining with 2% phosphotungstic acid (PTA).

Detection of HBoV- and B19V-specific antibodies. In total, 69 adults were included in the study and determined to exhibit strong IgG-mediated humoral immune responses against HBoV VP2-VLP by ELISA (OD_{450} range: 0.248–1.124, median: 0.863; Figure 3). Since HBoV seronegative individuals have been described and were found predominantly in infants and young children (≤ 2 years) [20, 27, Lindner *et al.*, in preparation], sera of 10 healthy infants (mean age: 11.8 months, age range: 6 – 45 months) without detectable virus-specific antibodies (OD_{450} range: 0.017-0.104, median: 0.056) were selected and representatively included in the study to ensure the specificity of performed serological analysis. With respect to parvovirus B19, 47 out of 69 volunteers displayed IgG-antibodies against the VP1/VP2 proteins indicating past B19V infection (Table 1). B19V-specific IgM antibodies were not detectable in any of the sera.

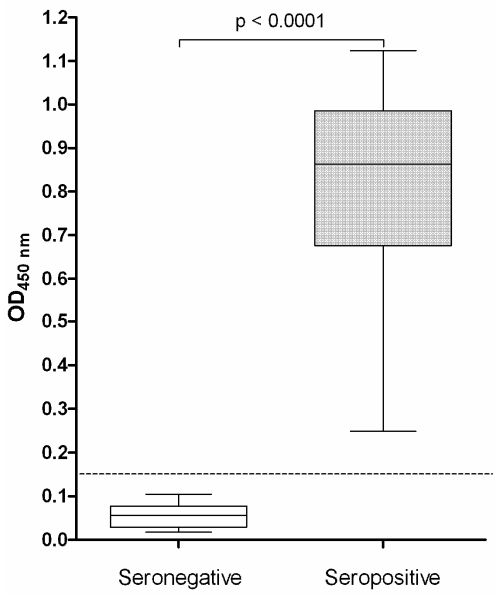

Figure 3. *Detection of HBoV-specific IgG antibodies in healthy adults.* Using VP2-VLP, all 69 studied healthy adult individuals were found positive for HBoV-specific IgG-antibodies by ELISA (Seropositive). Since seronegative individuals have been predominantly described and could only be found in young children and infants, a total of 10 representative children (mean age: 11.8 months) were included to ensure the specificity of performed serological analysis (Seronegative). The cutoff for positive results (dashed line) was defined as the 2.5-fold of the median optical density (drawn horizontal line) of analyzed infants without HBoV-specific IgG-reactions.

IFN-γ mediated CD4$^+$ T-helper cell responses against HBoV VP2-VLP in healthy adult individuals. Since sufficient volumes of blood samples could not be obtained from very young children, only HBoV seropositive adults were analyzed for HBoV- and B19V-specific cellular immune responses using the ELISPOT assay. Using PBMC stimulated with 5 µg/ml of either HBoV or parvovirus B19 VP2-VLP, virus-specific *ex vivo* IFN-γ immune responses with median values of 20 and 38 SFC/2x10^5 PBMC were detected in HBoV and B19V seropositive individuals, respectively (Figure 4). The correlation of the number of HBoV-specific IFN-γ secreting cells with anti-HBoV IgG responses did not reveal any statistical significance (p=0.5809). For this analysis HBoV seropositive individuals were subdivided in two groups, characterized by OD_{450} values either below or above the median OD_{450}, respectively. Both groups displayed median numbers of 20 SFC/2x10^5 PBMC.

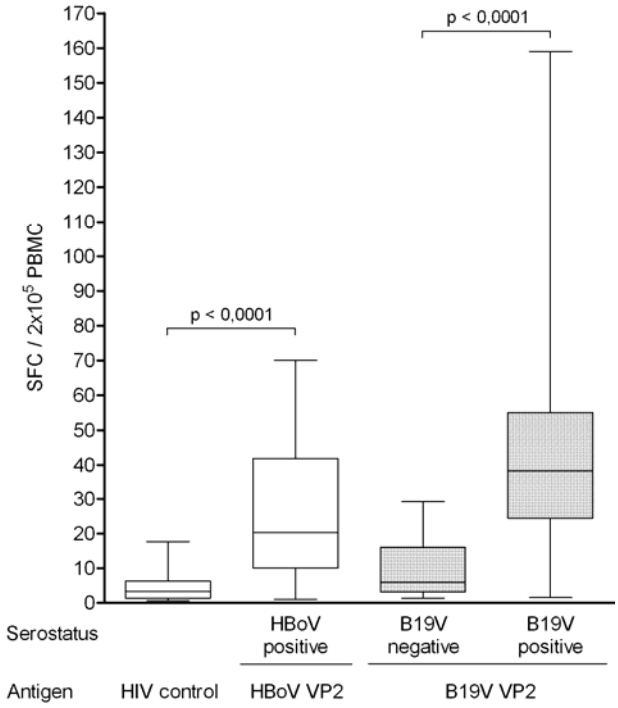

Figure 4. *IFN-γ secretion in PBMC of adult individuals upon stimulation with HBoV and parvovirus B19 VP2 capsid antigens.* PBMC isolated from blood of 69 HBoV seropositive, parvovirus B19 seropositive (n=47) or seronegative (n=22) donors were subjected to stimulation with either HBoV or parvovirus B19 VP2-VLP for 60 h. The HIV p24-derived peptide e10F served as negative control for all studied HBoV seropositive individuals (HIV control). The data show the median (drawn horizontal line) and the range of IFN-γ SFC values per $2x10^5$ PBMC as detected by the ELISPOT assay.

No significant IFN-γ responses (median value: 3 SFC/$2x10^5$ PBMC) were measured against the synthetic HIV peptide e10F, which was used as control for HBoV stimulations. Control stimulations of parvovirus B19 seronegative individuals using B19V VP2-VLP resulted in only background levels of IFN-γ secretion (median value: 6 SFC/$2x10^5$ PBMC). To exclude the possibility that cross-reactions between VP2 proteins of HBoV and parvovirus B19 contributed to the positive test values, HBoV-specific immune responses were analyzed in dependency of B19V serology. Thereby, no statistically significant correlation (p=0.4237) was observed as median values of 17 and 26 SFC/$2x10^5$ PBMC were measured upon HBoV VP2-VLP stimulation in B19V seronegative and seropositive individuals, respectively.

To associate the HBoV VP2-specific cytokine responses to specific cell populations, stimulations of PBMC cultures depleted from either $CD4^+$ or $CD8^+$ T cells were performed

(Figure 5). In PBMC cultures of five tested HBoV seropositive individuals a substantial reduction of the median number of IFN-γ secreting cells from 21 to 2 SFC/2x10^5 PBMC (p=0.0079) was observed after depletion of $CD4^+$ T helper cells, while HBoV-specific T cell responses remained detectable (median value: 18 SFC/2x10^5 PBMC) after depletion of $CD8^+$ cytotoxic T lymphocytes (p=0.8413). This suggests a pivotal role of the $CD4^+$ lymphocyte subset in the cellular immune response against VP2 proteins of HBoV.

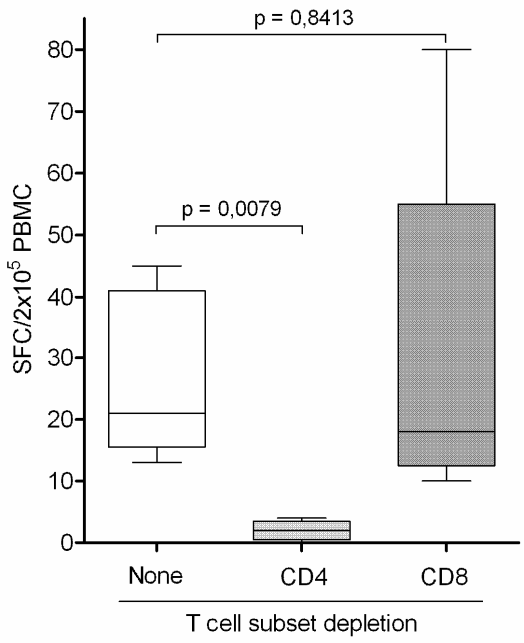

Figure 5. *HBoV VP2-specific IFN-γ secretion in HBoV seropositive individuals after T cell subset depletion.* PBMC of HBoV seropositive individuals were depleted from either $CD4^+$ or $CD8^+$ T cells and subjected to stimulation with HBoV VP2-VLP. IFN-γ secretion was assessed by the ELISPOT assay. Values shown represent the median (drawn horizontal line) and the range of SFC values per 2x10^5 PBMC of independent stimulations performed on five study subjects.

Correlation of the HBoV VP2-specific immune responses with the age of the study subjects revealed an age-dependent decrease of the median number of IFN-γ secreting cells from 35 SFC/2x10^5 PBMC in young individuals born between 1980 and 1989 (age 18-27 years) to 17 SFC/2x10^5 PBMC in elderly adults born between 1930 and 1939 (age 68-77 years) (Figure 6). In general, females displayed elevated or equal $CD4^+$ T cell responses against HBoV VP2-VLP as compared to males in all age groups. This observation was most prominent in individuals born between 1970 and 1979 (age 37-28 years) as revealed by median values of 25 SFC/2x10^5 PBMC for male and 51 SFC/2x10^5 PBMC for female individuals (p=0.0667).

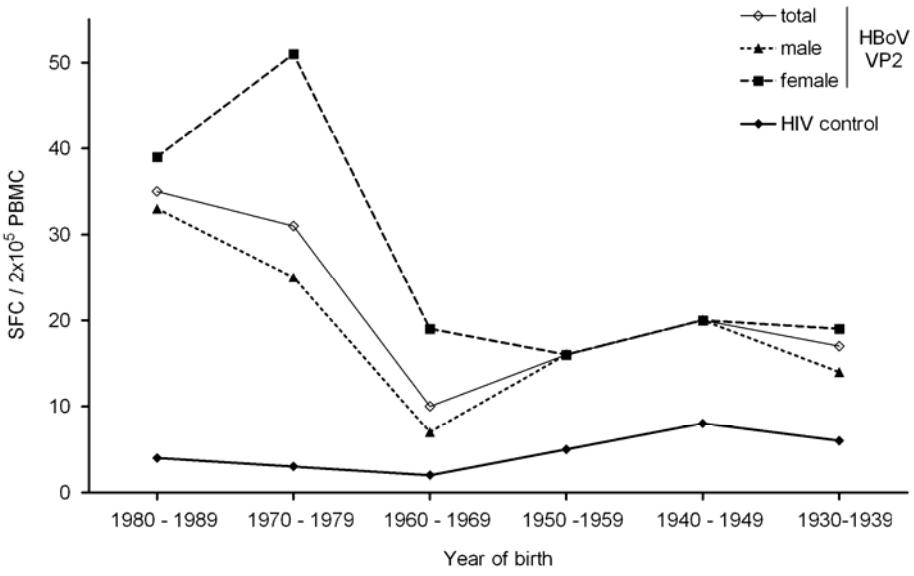

Figure 6. *Age dependent decrease of HBoV VP2-specific IFN-γ secreting cells in HBoV seropositive individuals.* HBoV VP2-specific IFN-γ responses of all studied seropositive adult study subjects were correlated with the year of birth and gender of the respective individuals. Data shown represent median IFN-γ SFC values per $2x10^5$ PBMC of study subjects grouped according to their year of birth.

Discussion

HBoV virus-like particles consisting of VP2 proteins can be produced using the baculovirus expression system. Similar capacities have been described previously for other parvoviruses such as parvovirus B19, the canine and the porcine parvovirus [5, 28, 29]. Using electron microscopy and CsCl gradient centrifugation, the HBoV VP2-VLP were demonstrated to possess a comparable diameter to native HBoV virions [30] and similar sedimentation densities to capsids of the canine parvovirus [31].

Recombinant VLP represent potent reagents for virological and clinical research and have greatly contributed to the improvement of sensitive diagnostic tests as well as vaccine development against many viruses, e.g. the human papillomavirus and hepatitis B virus [32, 33]. Recombinant VP2 particles are known to be crucial in diagnosis of parvovirus B19 infections as virus-specific antibodies time-dependently lose their affinity against linear antigenic regions and get replaced by humoral responses against conformational epitopes [34]. If similar applications can be established for HBoV VP2-VLP needs to be assessed in further studies.

Using HBoV VLP we observed frequent VP2-specific humoral immune responses in healthy adults, while seronegative individuals were detected predominantly amongst small children (≤ 2 years of age) [27, Lindner *et. al*, in preparation]. This finding is furthermore strongly supported by recent epidemiological data, which describe the seroprevalence of HBoV-specific IgG-antibodies to reach up to 100% already during early childhood [20]. Due to the very young age of seronegative individuals it was not possible to obtain sufficient volumes of blood samples for the analysis of HBoV-specific T-cell immunity.

In seropositive adults, frequent cellular immune responses were observed upon HBoV VP2-VLP stimulation. Thereby, the median number of IFN-γ secreting cells against HBoV was shown to be slightly lower compared to that observed against VP2 particles of parvovirus B19, which have been previously shown to serve as targets for IFN-γ and IL-10 mediated $CD4^+$ T helper cell responses [35, 36]. However, this observation was mainly based on four study subjects exhibiting high cytokine responses against the parvovirus B19 VP2 proteins. These single high responses might be explained by possible recent B19V infections or viral restimulation, as the seroprevalence of parvovirus B19 in the German adult population averages at 77% and therefore acute infections may occur in adults [37].

Compared to synthetic peptide controls, slightly elevated IFN-γ responses against B19V VP2-VLP were observed in seronegative individuals. This suggests that minor unspecific cytokine secretion may be induced in blood cells by residual pyrogens, e.g. endotoxin, included in recombinant antigen but not in synthetic peptide preparations [38]. Yet, HBoV VP2-specific T cell responses were not detected in all studied individuals, as for many donors only low or undetectable IFN-γ secretion was observed, suggesting an antigen-specificity of the detected T cell responses.

In the study group an age-dependent decrease of HBoV-specific T cell responses was observed. This might be due to an age-related decrease and impairment of the amount and functions of virus-specific T cell populations, like it has been described for other viral infections, e.g. for those with adenovirus [39]. It is furthermore known that active T cell responses against pathogens undergo confinement to specific memory populations with increasing time after primary infection in the absence of exogenous restimulation events or endogenous reactivations of latent viral infections. As all data currently published on the epidemiology of HBoV strongly suggest that primary infection occurs in the very early years of childhood [9-17, 20], the time-dependent reduction of virus-specific T cell subsets might be explained by a lack of frequent pathogen contact. This may be considered as additional proof for the early time-point of HBoV infection during childhood. Independent

of age, female individuals showed higher numbers of IFN-γ secreting cells than male study subjects especially in younger individuals, possibly indicating a more frequent spontaneous viral restimulation, e.g. by increased contact to acutely infected children, resulting in an overall elevated HBoV-specific cellular immune response. This hypothesis is further supported by the fact that the most prominent difference in gender-specific cellular responses was observed in individuals born between 1970 and 1979, an age-group in which women presumably have most frequent contact to small children. Yet, as no information on the family status of the study subjects is available, these data will have to be confirmed by additional epidemiological investigations.

Acknowledgements

The authors thank Dr. Tobias Allander and colleagues from the Karolinska Institute, Stockholm, Sweden, for the generous gift of the pCR®4-TOPO® vector including the genome sequence of HBoV ST2. We would like to furthermore thank Prof. Klaus Hedman and colleagues, University of Helsinki, for experimental and theoretical support. The excellent technical assistance of Julia Mischner and Richard Klar as well as the critical proof reading of the manuscript by Dr. Valeria Runza is appreciated.

Footnote

The work was supported by the German Research Foundation (DFG), grant Mo620/7-1, and the DAAD (Deutscher Akademischer Auslandsdienst) grant PPP-Finnland D/04/04483.

The authors do not declare any conflict of interest.

References

1. Allander, T., M. T. Tammi, M. Eriksson, A. Bjerkner, A. Tiveljung-Lindell, and B. Andersson. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc. Natl. Acad. Sci. U. S. A **2005**; 102:12891-12896.

2. Chieochansin, T., S. Chutinimitkul, S. Payungporn, T. Hiranras, R. Samransamruajkit, A. Theamboolers, and Y. Poovorawan. Complete coding sequences and phylogenetic analysis of Human Bocavirus (HBoV). Virus Res. **2007**; 129:54-7.

3. Boschetti, N., K. Wyss, A. Mischler, T. Hostettler, and C. Kempf. Stability of minute virus of mice against temperature and sodium hydroxide. Biologicals **2003**; 31:181-185.

4. Dorsch, S., G. Liebisch, B. Kaufmann, P. Von Landenberg, J. H. Hoffmann, W. Drobnik, and S. Modrow. The VP1 unique region of parvovirus B19 and its constituent phospholipase A2-like activity. J. Virol. **2002**; 76:2014-2018.

5. Brown, C. S., J. W. Van Lent, J. M. Vlak, and W. J. Spaan. Assembly of empty capsids by using baculovirus recombinants expressing human parvovirus B19 structural proteins. J. Virol. **1991**; 65:2702-2706.

6. Lowin, T., U. Raab, J. Schroeder, R. Franssila, and S. Modrow. Parvovirus B19 VP2-proteins produced in Saccharomyces cerevisiae: comparison with VP2-particles produced by baculovirus-derived vectors. J. Vet. Med. B Infect. Dis. Vet. Public Health **2005**; 52:348-352.

7. Bastien, N., K. Brandt, K. Dust, D. Ward, and Y. Li. Human Bocavirus infection, Canada. Emerg. Infect. Dis. **2006**; 12:848-850.

8. Kaplan, N. M., W. Dove, A. F. Abu-Zeid, H. E. Shamoon, S. A. Abd-Eldayem, and C. A. Hart. Human bocavirus infection among children, Jordan. Emerg. Infect. Dis. **2006**; 12:1418-1420.

9. Kesebir, D., M. Vazquez, C. Weibel, E. D. Shapiro, D. Ferguson, M. L. Landry, and J. S. Kahn. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J. Infect. Dis. **2006**; 194:1276-1282.

10. Ma, X., R. Endo, N. Ishiguro, T. Ebihara, H. Ishiko, T. Ariga, and H. Kikuta. Detection of human bocavirus in Japanese children with lower respiratory tract infections. J. Clin. Microbiol. **2006**; 44:1132-1134.

11. Sloots, T. P., P. McErlean, D. J. Speicher, K. E. Arden, M. D. Nissen, and I. M. Mackay. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J. Clin. Virol. **2006**; 35:99-102.

12. Kleines, M., S. Scheithauer, A. Rackowitz, K. Ritter, and M. Hausler. High prevalence of human bocavirus detected in young children with severe acute lower respiratory tract disease by use of a standard PCR protocol and a novel real-time PCR protocol. J. Clin. Microbiol. **2007**; 45:1032-1034.

13. Maggi, F., E. Andreoli, M. Pifferi, S. Meschi, J. Rocchi, and M. Bendinelli. Human bocavirus in Italian patients with respiratory diseases. J. Clin. Virol. **2007**; 38:321-325.

14. Naghipour, M., L. E. Cuevas, T. Bakhshinejad, W. Dove, and C. A. Hart. Human bocavirus in Iranian children with acute respiratory infections. J. Med. Virol. **2007**; 79:539-543.

15. Qu, X. W., Z. J. Duan, Z. Y. Qi, Z. P. Xie, H. C. Gao, W. P. Liu, C. P. Huang, F. W. Peng, L. S. Zheng, and Y. D. Hou. Human bocavirus infection, People's Republic of China. Emerg. Infect. Dis. **2007**; 13:165-168.

16. Vicente, D., G. Cilla, M. Montes, E. G. Perez-Yarza, and E. Perez-Trallero. Human bocavirus, a respiratory and enteric virus. Emerg. Infect. Dis. **2007**; 13:636-637.

17. Arden, K. E., P. McErlean, M. D. Nissen, T. P. Sloots, and I. M. Mackay. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J. Med. Virol. **2006**; 78:1232-1240.

18. Kupfer, B., J. Vehreschild, O. Cornely, R. Kaiser, G. Plum, S. Viazov, C. Franzen, R. L. Tillmann, A. Simon, A. Muller, and O. Schildgen. Severe pneumonia and human bocavirus in adult. Emerg. Infect. Dis. **2006**; 12:1614-1616.

19. Manning, A., V. Russell, K. Eastick, G.H. Leadbetter, N. Hallam, K. Templeton, P. Simmonds. Epidemiological profile and clinical associations of human bocavirus and other human parvoviruses. J. Infect. Dis. **2006**; 194:1283-1290.

20. Endo, R., N. Ishiguro, H. Kikuta, S. Teramoto, R. Shirkoohi, X. Ma, T. Ebihara, H. Ishiko, and T. Ariga. Seroepidemiology of human bocavirus in hokkaido prefecture, Japan. J. Clin. Microbiol. **2007**; 45:3218-3223.

21. Manaresi, E., G. Gallinella, M. Zerbini, S. Venturoli, G. Gentilomi, and M. Musiani. IgG immune response to B19 parvovirus VP1 and VP2 linear epitopes by immunoblot assay. J. Med. Virol. **1999**; 57:174-178.

22. Franssila, R., K. Hokynar, and K. Hedman. T helper cell-mediated in vitro responses of recently and remotely infected subjects to a candidate recombinant vaccine for human parvovirus b19. J. Infect. Dis. **2001**; 183: 805-809.

23. Corcoran, A., B. P. Mahon, and S. Doyle. B cell memory is directed toward conformational epitopes of parvovirus B19 capsid proteins and the unique region of VP1. J. Infect. Dis. **2004**; 189:1873-1880.

24. Lindner, J., S. Barabas, K. Saar, D. Altmann, A. Pfister, M. Fleck, L. Deml, and S. Modrow. CD4(+) T-cell responses against the VP1-unique region in individuals with recent and persistent parvovirus B19 infection. J. Vet. Med. B Infect. Dis. Vet. Public Health **2005**; 52:356-361.

25. Kaufmann, B., U. Baxa, P. R. Chipman, M. G. Rossmann, S. Modrow, and R. Seckler. Parvovirus B19 does not bind to membrane-associated globoside in vitro. Virology **2005**; 332:189-198.

26. Wild, J., A. Bojak, L. Deml, and R. Wagner. Influence of polypeptide size and intracellular sorting on the induction of epitope-specific CTL responses by DNA vaccines in a mouse model. Vaccine **2004**; 22:1732-1743.

27. Kantola, K., Hedman, L., Allander, T., Jartti, T., Lehtinen, P., Ruuskanen, O., Hedman, K., and Söderlund-Venermo, M. (2007). Serdiagnosis of human bocavirus infections. J. Inf. Dis. **2007**; In press.

28. Hurtado, A., P. Rueda, J. Nowicky, J. Sarraseca, and J. I. Casal. Identification of domains in canine parvovirus VP2 essential for the assembly of virus-like particles. J. Virol. **1996**; 70:5422-5429.

29. Maranga, L., T. F. Brazao, and M. J. Carrondo. Virus-like particle production at low multiplicities of infection with the baculovirus insect cell system. Biotechnol. Bioeng. **2003**; 84:245-253.

30. Brieu, N., B. Gay, M. Segondy, and V. Foulongne. Electron microscopy observation of human bocavirus (HBoV) in nasopharyngeal samples from HBoV-infected children. J. Clin. Microbiol **2007**; 45:3419-20.

31. Saliki, J. T., B. Mizak, H. P. Flore, R. R. Gettig, J. P. Burand, L. E. Carmichael, H. A. Wood, and C. R. Parrish. Canine parvovirus empty capsids produced by expression in a baculovirus vector: use in analysis of viral properties and immunization of dogs. J. Gen. Virol. **1992**; 73 (Pt 2):369-374.

32. Joura, E. A., S. Leodolter, M. Hernandez-Avila, C. M. Wheeler, G. Perez, L. A. Koutsky, S. M. Garland, D. M. Harper, G. W. Tang, D. & other authors. Efficacy of a quadrivalent prophylactic human papillomavirus (types 6, 11, 16, and 18) L1 virus-like-particle vaccine against high-grade vulval and vaginal lesions: a combined analysis of three randomised clinical trials. Lancet. **2007**; 369:1693-1702.

33. Shouval, D. Hepatitis B vaccines. J. Hepatol. **2003**; 39 Suppl 1:S70-S76.

34. Soderlund, M., C. S. Brown, W. J. Spaan, L. Hedman, and K. Hedman. Epitope type-specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J. Infect. Dis. **1995**; 172:1431-1436.

35. Franssila, R. and K. Hedman. T-helper cell-mediated interferon-gamma, interleukin-10 and proliferation responses to a candidate recombinant vaccine for human parvovirus B19. Vaccine **2004**; 22:3809-3815.

36. Franssila, R., J. Auramo, S. Modrow, M. Mobs, C. Oker-Blom, P. Kapyla, M. Soderlund-Venermo, and K. Hedman. T helper cell-mediated interferon-gamma expression after human parvovirus B19 infection: persisting VP2-specific and transient VP1u-specific activity. Clin. Exp. Immunol. **2005**; 142:53-61.

37. Eis-Hubinger, A. M., J. Oldenburg, H. H. Brackmann, B. Matz, and K. E. Schneweis. The prevalence of antibody to parvovirus B19 in hemophiliacs and in the general population. Zentralbl. Bakteriol **1996**; 284:232-240.

38. Ulmer, A. J., H. Flad, T. Rietschel, and T. Mattern. Induction of proliferation and cytokine production in human T lymphocytes by lipopolysaccharide (LPS). Toxicology **2000**; 152: 37-45.

39. Sester, M., U. Sester, S. S. Alarcon, G. Heine, S. Lipfert, M. Girndt, B. Gartner, and H. Kohler. Age-related decrease in adenovirus-specific T cell responses. J. Infect. Dis. **2002**; 185:1379-1387.

5.2. Publikation II: Humoral Immune Response Against Human Bocavirus VP2 Virus-like Particles

Viral Immunology, 2008, Im Druck.

Juha Lindner[1], Lüdya Karalar[1], Sandra Zehentmeier[1], Annelie Plentz[1], Heiko Pfister[2], Wilhelm Struff[3], Michael Kertai[4], Hugo Segerer[4], and Susanne Modrow[1*]

[1]Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz- Josef-Strauss Allee 11, 93053 Regensburg, Germany
[2]Mikrogen GmbH, Floriansbogen 2-4, 82061 Neuried, Germany
[3]DRK Blutspendedienst West, Zentrum für Transfusionsmedizin Münster, Sperlichstrasse 15, 48151 Münster, Germany
[4]Children's Hospital St. Hedwig, Steinmetzstraße 1-3, 93049 Regensburg, Germany

Keywords: Human Bocavirus, Seroprevalence, Epidemiology, ELISA, Virus-like particles
Running title: Human Bocavirus Seroepidemiology

*Corresponding author: Susanne Modrow, Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz-Josef-Strauss Allee 11, 93053 Regensburg, Germany. Phone: (+49)-941-9446454, Fax: (+49)-941-9446402, e-mail: susanne.modrow@klinik.uni-regensburg.de

Abstract

Human bocavirus (HBoV) was recently detected in samples of children and infants with infections of the respiratory tract. Here we analyze the prevalence of IgG- and IgM-antibodies against HBoV virus-like VP2-particles in healthy adult blood donors and children using a newly established standardized enzyme-linked immunosorbent assay.

Virus-specific IgG-antibodies were frequently detected in infants with active viremia and respiratory illness (10/24, 42%) and in young children without detectable HBoV genomes in blood (27/52, 52%). In sera obtained from healthy adults, ubiquitous VP2-specific antibodies were found in 280/299 (94%) cases.

HBoV-specific IgM-antibodies were detected in 10/24 (42%) sera obtained from HBoV DNA positive children, 6/24 sera (25%) displayed equivocal responses. In contrast, VP2-specific IgM were not detectable in samples obtained from 52 children without detectable amounts of HBoV genomes in blood. Only 2/299 sera from healthy adult blood donors were found to be IgM positive (1%), equivocal IgM-responses were observed in 9/299 (3%) individuals.

In conclusion a high IgG seroprevalence of HBoV in the adult population was observed, whereas the presence of virus-specific IgM was associated with viremia. These data show that ELISA test systems for the detection of HBoV-specific antibodies provide a valuable tool for serological diagnosis of this new emerging pathogen.

Introduction

Human bocavirus (HBoV) was recently described as a new member of the *Parvoviridae* (3) and currently represents the second parvovirus associated with human disease. Since it's detection in 2005 in Sweden, HBoV has quickly advanced to claim its position as one of the most common and widespread pathogens found in small children and infants with symptoms of the lower respiratory tract (4,24). The symptoms currently associated with HBoV infection include wheezing, pneumonia and bronchitis (2). The virus has been additionally found in fecal samples of children suffering from gastric diseases, yet a significant association of HBoV with gastroenteritis remains to be proven (1,20,21). In total, HBoV tends to be associated with a high rate of co-infections (13), e.g. with respiratory syncytial virus, and thus still needs to be clearly identified as sole infectious agent of human disease.

Up to date, the diagnosis of HBoV widely relies on the detection of viral DNA by PCR-based techniques. Thereby, HBoV genomes have been detected mainly in respiratory but also fecal samples of young children and infants worldwide (5,16-18,24,27,28,32,35,37). The reported prevalence of viral DNA in these studies differs considerably and has been described to range between 1.5-18.3% in children suffering from respiratory disease.

As recombinant HBoV antigens have been recently become available, the detection of HBoV-specific humoral and cellular immune responses moves into the close focus of immunological and viral research. A high prevalence of antibodies directed against HBoV capsid proteins VP1 and VP2 has been recently detected in up to 94% of adult individuals and children older than 2 to 3 years, while seronegative individuals were mainly observed in infants at an age of one year (10,22). Recent data furthermore describe the frequent detection of both IgG- and IgM-antibodies in 73% and 49% of children with HBoV viremia by Western blot analysis, respectively (15).

We have recently described the generation of recombinant HBoV virus-like particles (VLP) consisting of VP2 capsid proteins (25). These could be shown to display similar diameters as compared to native HBoV and to VLP from related parvoviruses, such as parvovirus B19, canine and porcine parvovirus (6,7,14,29). Using HBoV-VLP, we observed the frequent presence of VP2-specific $CD4^+$ T helper cell responses in healthy adults, supporting a high prevalence of HBoV-specific immunity in the adult population.

All current data on HBoV epidemiology indicate that viral infection occurs very early in life and allow the assumption that this novel virus is broadly distributed in the human population. Yet, as only limited data is currently available on the prevalence of HBoV-

specific IgM- and IgG-antibodies, the aim of our study was to establish a standardized enzyme-linked immunosorbent assay (ELISA) for the analysis of virus-specific antibodies in the German population.

Materials and Methods

Study subjects

A total of 76 serum samples obtained from children were included in the study. 52/76 samples had been collected from children (33 male, 19 female, mean age: 38.02 months, age range: 1 – 123 months) who had presented as inpatients at the Children´s Hospital St. Hedwig, University of Regensburg, due to non-infectious diseases (i.e. accidents, bone fractures) not associated with HBoV infection. In these sera, no viral genomes were detected by PCR. An additional 24 samples were derived from children (12 male, 12 female, mean age: 27.67 months, age range: 4 – 87 months) with mainly respiratory tract infections who were determined positive for HBoV DNA (viral load range: $1x10^2$-$1.2x10^6$ geq/ml) (Karalar *et al.*, submitted). Furthermore, 299 serum samples obtained from healthy adult blood donors in the region of Münster, Germany (195 male, 104 female, mean age: 42 years, age range: 19-78 years) were included in the study.

Ethics

The study was approved by the ethical committee of the University of Regensburg (nr. 06/85). Written patient consent was obtained prior to sample processing.

Detection of human bocavirus DNA

Viral nucleic acid was isolated from 200 µl serum using the QiaAmp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For real time TaqMan PCR, the following primers and probe were used for amplification of HBoV genome sequences: Forward primer 5'-CCA CCT ATC GTC TTG CAC TGC-3' (nts 2586-2606), reverse primer 5'-TTT TCC CCG ATG TAC TCT CCC-3' (nts 2619-2639), probe FAM-5'-TCG AAG ACC TCA GAC CAA GTG ATG AAG ACG-3'-TAMRA (nts 2608-2637), positions according to Genbank accession number DQ000496.1. Duplicate PCR reactions were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Weiterstadt, Germany) with 300 nM forward primer, 300 nM reverse primer, 150 nM probe and 200 nM dNTP. An initial denaturation phase of 10 min at 95°C was followed by 45 cycles at 95°C (15 sec) and 60°C (1 min). In each run, tenfold serial

dilutions of plasmid DNA (HBoV ST2 isolate (3)) and a negative serum were amplified as controls and quantification standard. For healthy adults, PCR analysis of HBoV DNA was performed in pools of each 5 serum samples.

Human bocavirus VP2 virus-like particles

HBoV VP2-VLP were produced as previously described (25). Briefly, High-5 insect cells were infected with recombinant bacVP2/HBoV baculovirus encoding the HBoV VP2 gene at a multiplicity of infection of 3 and cultured for 72 h in Insect-Xpress Medium (Cambrex Bio Science Walkersville Inc., Walkersville, USA) at 26°C. Afterwards cells were lysed in buffer (10 mM Tris/HCl, 10 mM NaCl, 15 mM $MgCl_2$, 0.5% Triton X-100, pH 7.4) containing 40 µl/ml Complete Protease Inhibitor Coctail (Roche Applied Science, Mannheim, Germany) and the lysate was subjected to CsCl ultracentrifugation at 100.000xg for 4 h at 10ºC without brakes. After centrifugation samples containing VP2-protein samples were collected in fractions and dialyzed against phosphate-buffered saline (PBS) prior to storage at -80°C. Freeze-thaw cycles were avoided.

Detection of HBoV- and B19V-specific antibodies by ELISA

For detection of HBoV VP2-specific IgG and IgM, 100 ng of purified HBoV VP2-VLP were coated onto Nunc-Immuno^{TM} MediSorp^{TM} plates (Nunc GmbH, Wiesbaden, Germany) in coating buffer (0.2 M Na_2CO_3, 0.2 M $NaHCO_3$, pH 9.5) overnight at 4°C, washed six times with washing buffer (PBS, 0.05% Tween 20) and blocked with dilution buffer (PBS, 2% Tween 20, 3% FCS) for 1 h at 37°C. After incubation with respective serum samples for 2 h at 37°C, the plates were washed and rabbit anti-human IgG- or IgM-specific HRP-coupled secondary antibodies were added for 1 h at 37°C (1:6000 and 1:1000 in dilution buffer, respectively; both Dako Deutschland GmbH, Hamburg, Germany). For analysis of HBoV-specific IgG-subclass responses, HRP-coupled secondary antibodies specific for IgG-subclass 1-4 (Invitrogen, Karlsruhe, Germany; all 1:100 in dilution buffer) were used. Development was performed using the BD OptEIA^{TM} Substrate (BD Biosciences, Heidelberg, Germany) according to the manufacturer´s instructions.

As an international IgG standard for HBoV is not yet available, sera of a healthy adult male (age: 28 years) and of a boy (age: 22 months) both exhibiting strong HBoV-specific IgG- and IgM-responses, respectively, were introduced for internal reference in all performed experiments. Serial dilutions of both reference sera were included in duplicates on each ELISA plate and appointed a relative unit (RU) value ranging from 4000 RU

(dilution 1:25) to 3.91 RU (dilution 1:125600). Optical densities obtained for each dilution were plotted against the natural logarithm of RU, whereupon values in the range between 1000-125 RU reproducibly resulted in a linear plot and were used to define the detection range for each ELISA. Samples diluted 1:100 that resulted in values exceeding the upper detection limit (1000 RU) were re-diluted (1:200-1:1000) and the resulting RU values were extrapolated. Sera with background values below the lower cut-off (125 RU) were considered negative. Additionally, the 2.5-fold of the median RU value (225 RU for both IgG- and IgM-ELISA) of all sera considered as negative for HBoV-specific antibodies was defined as cut-off for equivocal samples.

Parvovirus B19-specific IgG and IgM were detected in representative donor sera by a standardized enzyme-linked immunoassay (Mikrogen GmbH, Neuried, Germany).

Detection of HBoV-specific antibodies by Western blot

For the detection of HBoV VP2-specific IgG and IgM by Western blot, aliquots of solutions containing recombinant HBoV VP2-protein were separated by 12.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (SERVA Electrophoresis GmbH, Heidelberg, Germany). The membranes were blocked overnight at 4°C with incubation buffer (PBS containing 5% dry milk and 0.2% Triton-X 100) and subsequently incubated for 2 h with serum samples diluted 1:50 in incubation buffer. After intensive washing with PBS containing 0.05% Tween-20, alkaline phosphatase-coupled anti-human-IgG or -IgM antibodies were added (both Dako Deutschland GmbH, Hamburg, Germany; 1:1000 in incubation buffer) for 2 h. Development was performed using the NBT/BCIP Stock Solution (Roche Diagnostics GmbH, Mannheim, Germany) in buffer containing 0.1 M NaCl, 0.1 M Tris/HCl pH 9.5, 0.05 M $MgCl_2$.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U-test for independent samples.

Results

Validation of the HBoV VP2 ELISA

In order to asses the reproducibility of results obtained by the HBoV-specific ELISA, representative sera were tested in five replicates on the same (intra-assay) or on independent plates (inter-assay). The results were subsequently analyzed for their

coefficient of variation (CV) by division of the respective standard deviations (SD) by the replicate RU responses and subsequently multiplied by 100. With regard to the IgG-ELISA, the intra- and inter-assay variations were determined to be 9% and 11% for 10 positive (median: 839 RU, range: 237 - 1833 RU) and 1% and 10% for 10 negative samples (median: 94 RU, range: 53 - 123 RU), respectively. The intra- and inter-assay variations of samples tested for HBoV VP2-specific IgM averaged at 4% and 3% for 5 positive (median: 863 RU, range: 340 - 6416 RU) and at 0.2% and 4% for 10 negative samples (median: 91 RU, range: 53 - 113 RU), respectively. No significant correlation was observed between CV-values and detectable titers of HBoV-specific antibodies. In all replicate experiments performed, the analyzed serum samples were appointed to the same HBoV IgM- and IgG-serostatus with CV variations below 20%, demonstrating a high reproducibility of the established ELISA (33).

To exclude possible cross-reactions of HBoV-specific antibodies, parvovirus B19 VP2-specific IgG were determined for a representative sub-group of adults (n=56, mean age: 39 years, age range: 19-70) and correlated with HBoV-specific humoral immune responses (Figure 1).

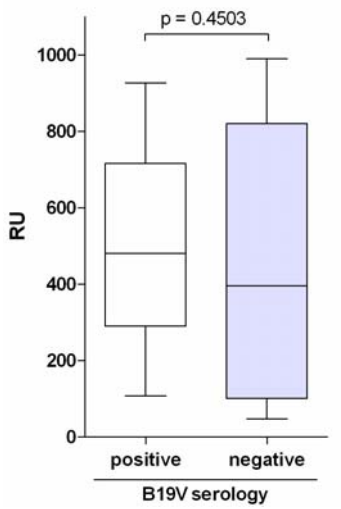

FIGURE 1. *HBoV-specific antibody-responses in dependence of parvovirus B19 serology.* In order to exclude possible cross-reactions between antibodies directed against the VP2 proteins of HBoV and parvovirus B19 (B19V), HBoV-specific IgG were analyzed in representative 22 B19V seronegative and 34 seropositive individuals. The data show the median (drawn horizontal line) and the range of relative units (RU) as determined by ELISA.

Thereby, no significant IgG cross-reactions between HBoV and B19V VP2-proteins were observed, as B19V-seronegative (n=22) and seropositive (n=34) individuals exhibited

HBoV-specific IgG-responses to similar degrees with median values of 394 RU and 478 RU, respectively. All children who were included in the study did not display IgM-antibodies against B19V, irrespective of the presence of HBoV-specific IgM. This indicates that HBoV-specific IgM-antibodies do not cross-react with the VP2-protein of parvovirus B19. The validity of the established ELISA was confirmed by Western blot analysis using representative sera previously determined positive, equivocal or negative for HBoV VP2-specific antibodies (Figure 2). Thereby, 10 IgG and 5 IgM-positive sera with low titers close to the respective cut-off values were confirmed to contain HBoV VP2-specific antibodies, while 10 seronegative sera did not react with the viral antigen in Western blot analysis. Faint VP2-protein bands and/or background reactivity were observed in 6 of 10 representatively analyzed equivocal samples.

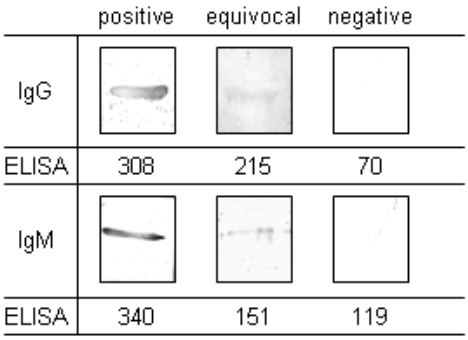

FIGURE 2. *Validation of the established HBoV-ELISA by Western blot.* Sera which were previously determined positive, equivocal or negative for HBoV-specific IgG- and IgM-antibodies by ELISA were used for the detection of the viral VP2 protein by Western blot. The antibody titers of the shown representative sera are indicated in relative units (RU).

Seroprevalence of HBoV-specific antibodies in adults and children

HBoV-specific humoral immune responses were analyzed in sera from children, either with or without detectable amounts of HBoV genomes in blood, and from HBoV DNA negative healthy adults (Table 1). In the group of control children which were treated due to symptoms not associated with infectious disease, VP2-specific IgG were detected in 27/52 (52%) sera, 4/52 samples were tested equivocal (8%). A total of 21/52 (40%) children did not display IgG-reactivities against HBoV. 10/24 (42%) and 2/24 (8%) sera with detectable amounts of HBoV DNA that were derived from children suffering from respiratory tract disease exhibited positive and equivocal IgG-responses against HBoV VP2-VLP, respectively. In 12/24 (50%) serum samples HBoV-specific antibodies could not be observed.

	Children ≤ 10 years						Adults		
	HBoV DNA negative [a]			HBoV DNA positive [a]			HBoV DNA negative [a]		
	Negative	Equivocal	Positive	Negative	Equivocal	Positive	Negative	Equivocal	Positive
	IgG ELISA								
N	21/52	4/52	27/52	12/24	2/24	10/24	5/299	14/299	280/299
%	40	8	52	50	8	42	2	4	94
Mean Age [b]	24	40	48.6	26.3	31.5	28.6	37	45	42
Age Range [b]	1 - 123	7 - 90	4 – 120	4 - 54	29 - 34	8 - 87	21 - 63	19 - 70	19 - 76
Median RU	88 ± 17	203 ± 17	541 ± 377	72 ± 23	176 ± 24	446 ± 186	106 ± 12	174 ± 29	914 ± 461
RU range	53 - 119	189 - 225	232 – 1777	47 - 123	152 - 199	234 - 834	88 - 123	129 - 219	237 - 1995
	IgM ELISA								
N	52/52	0/52	0/52	8/24	6/24	10/24	288/299	9/299	2/299
%	100	0	0	33	25	42	96	3	1
Mean Age [b]	38	n.d.	n.d.	27.4	19.8	32.6	42	47	67.5
Age Range [b]	1 - 123			7 - 45	12 - 30	13 - 54	19 - 76	29 - 69	67 - 68
Median RU	94 ± 7	n.d.	n.d.	102 ± 12	151 ± 31	1170 ± 1798	93 ± 18	160 ± 26	1046 ± 183
RU range	81 - 120			89 - 123	134 - 216	340 - 6416	53 - 125	128 - 207	863 - 1229

TABLE 1. *Seroprevalence of HBoV-specific IgG- and IgM-antibodies.* [a]Serum was tested for the presence of HBoV DNA. [b]Age-specification in months for children and years for adults.

IgG-negative individuals were predominantly detected in children at an age of one to 3 years, 66% of all seronegative individuals were found in this age-group (Figure 3). The presence of viral genomes was most frequent in children at an age between 2 and 3 years. In contrast, ubiquitous IgG-responses against HBoV VP2 were detected in 280/299 (94%) healthy adults. In adults, equivocal and negative results were obtained for only 14/299 (4%) and 5/299 (2%) serum samples, respectively. Significantly elevated titers of HBoV-specific IgG-antibodies (median: 914 RU, range: 237 – 1995 RU) were observed in healthy adults as compared to children with (median: 446 RU, range: 234 – 834 RU; p=0.0001) or without (median: 541 RU, range: 232 – 1777 RU; p=0.0001) HBoV DNA in serum.

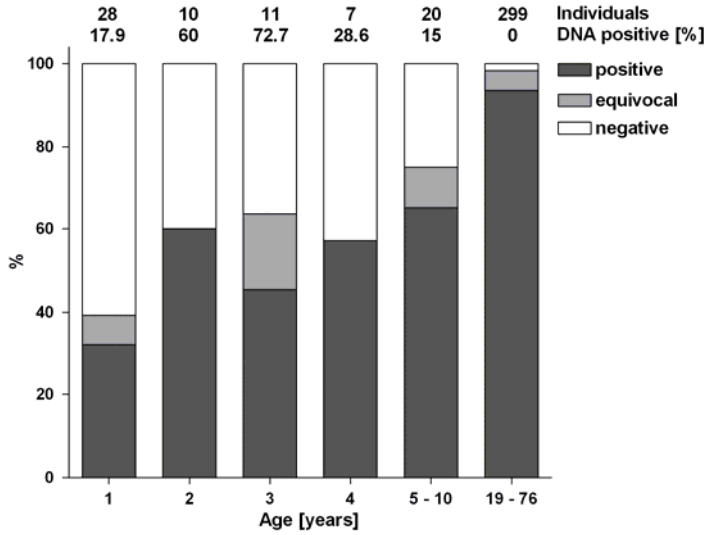

FIGURE 3. *Age-dependent prevalence of HBoV-specific IgG-antibodies.* The data show the relative percentages of individuals with positive, negative and equivocal IgG-antibody responses against HBoV VP2. The percentage of individuals with detectable HBoV genomes in serum in each respective age group is displayed.

Studies using sera from each four representative healthy adults (mean age: 33.5 years, age range: 19 – 65 years) and children (mean age: 47 months, age range: 1-123 months), which were selected due to their elevated HBoV-specific IgG responses, revealed that the humoral response to HBoV VP2 is predominantly mediated by IgG-antibodies of subclass 1 (IgG_1) ($p<0.0001$ as compared to respective IgG-subclass responses of seronegative controls) (Figure 4).

IgM-antibodies were detected predominantly in infants with HBoV viremia. Thereby, positive and equivocal IgM-responses were observed in 10/24 (42%) and 6/24 (25%) samples, respectively, whereas no HBoV-specific IgM were observed in control children

(0/52) (Table 1). With respect to adults, 288/299 (96%) individuals were found to be IgM-negative, while 2/299 (1%) and 9/299 (3%) serum samples presented positive or equivocal results, respectively.

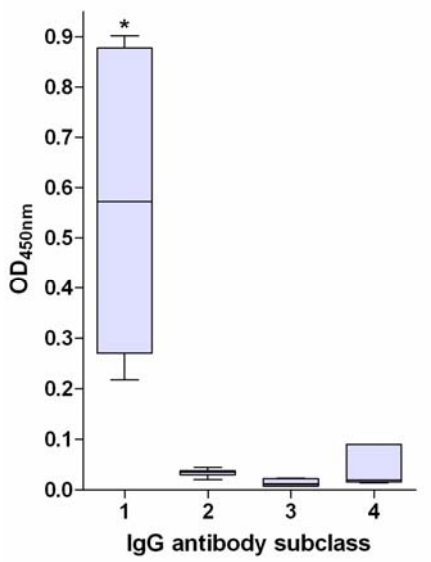

FIGURE 4. *Analysis of the IgG-subclass response against HBoV VP2-VLP.* Representative sera of four adults and four children previously found positive for HBoV-specific IgG-antibodies were analyzed for IgG-subclass responses. Exclusive IgG_1-antibodies against HBoV were detected in all analyzed individuals ($p<0.0001$) as compared to analysis performed on seronegative children (data not shown). No significant responses were observed for IgG-subclasses 2, 3 and 4. The data show the median (drawn horizontal line) and the range of the optical densities (OD) as detected by ELISA.

Discussion

The VP2 capsid proteins of parvoviruses have been shown to represent a major target for the humoral immune response. Furthermore, VP2-VLP of parvovirus B19, a related human parvovirus, are known to include conformational epitopes that are recognized by B19-specific antibodies (34). First data describe the detection of HBoV-specific antibodies by Western blot and immunofluorescence assays (10,15). In the present work we established an ELISA for the detection of HBoV-specific IgG and IgM in human sera based on the use of HBoV VP2-VLP as coating antigen. The results demonstrated a high reproducibility and validity and fulfilled the general standards recommended for the establishment of serological tests (33).

HBoV VP2-specific antibodies did not recognize epitopes present in B19V VP2 virus-like particles, excluding cross-reactions between these two parvoviruses infecting humans. Potential cross-reactions between HBoV and the closely related bovine parvovirus (BPV) were not analyzed in the present study. BPV-specific IgG have been previously detected in adults and are thought to be acquired by ingestion of BPV contaminated milk (30). As children at an age between one and two years often consume bovine milk products, the observation that HBoV-seronegative individuals are frequent in this age group argues against the possibility that BPV-specific IgG may have contributed to the positive values measured in the ELISA. This assumption is furthermore supported by recent data, which describe no cross-reactivity between BPV- and HBoV-specific antibodies in ELISA experiments (22).

In total, IgG-antibodies against HBoV were found in a high percentage (94%) of adult healthy blood donors, suggesting a ubiquitous prevalence of the virus in the population. Only a limited number of sera were found positive or equivocal for HBoV-specific IgM in individuals without detectable numbers of HBoV genomes in the blood. Whether these rare IgM responses are transiently induced in the course of re-exposures to HBoV, possibly via the contact to infected children, or represent primary HBoV infections in adulthood remains to be solved.

Seronegative individuals were detected predominantly in children between 1 and 3 years. A high seroprevalence of 65% was observed in children between 5 to 10 years of age. These data strongly support previous findings that HBoV infection takes place during the early childhood (10). They furthermore resemble findings on other respiratory viruses that cause disease preferentially in infants, i.e. respiratory syncytial virus and metapneumovirus (19,26). HBoV genomes were detected predominantly in sera of children aged between 2 and 3 years, frequently in combination with virus-specific IgM, suggesting an association of HBoV-infection with respiratory disease. This is in accordance to previous data that report the preferential detection of HBoV DNA in children at an age of 2 years (31,38) and of HBoV-specific IgM in DNA positive individuals (15).

The virus-specific humoral immune response was shown to be mediated by IgG_1-subclass antibodies in adults and children without detectable amounts of HBoV genomes in serum. This finding is consistent with data describing VP2-specific IgG_1-isotypes as main mediators of the long-term immune response against parvovirus B19 (12). This finding furthermore supports our previous data on HBoV VP2 serving as a major target for T

helper cell responses (25), as high levels of IgG_1 antibodies against parvovirus B19 capsid proteins are associated with antigen-specific $CD4^+$ T lymphocyte reactions (8,11,23).

When tested by Western blot analysis, recent data describe a decrease in the prevalence of HBoV-specific IgG from 43% to 29% in children aged between 2 and 5 years (15). The difference to the data presented here may be explained by the assumption that during the time period of up to six months following acute infection virus-specific IgG may preferentially recognize linear epitopes present in denatured VP2-proteins. Subsequently these antibodies may be replaced by IgG recognizing conformational epitopes present in particulate VLP. This phenomenon has been described in detail using VP2-VLP and denatured VP2 antigens of the related parvovirus B19 for the detection of B19V-specific IgG (9,36). Similar effects in HBoV humoral immunity might render the use of linear antigens or Western blot analysis unsuitable for the detection of virus-specific past immunity and furthermore highlight the importance of recombinant virus-like particles in HBoV-diagnostics. Yet, whether similar effects can be observed for HBoV needs to be assessed in further comparative serological studies with denatured and native viral antigens.

Acknowledgements

The work was supported by the German Research Foundation (DFG), grant Mo620/7-1., and the DAAD (Deutscher Akademischer Auslandsdienst) grant PPP-Finnland D/04/04483. The excellent technical assistance of Julia Mischner, Irene Nebeja and Anette Rohrhofer is highly appreciated.

References

1. Albuquerque MC, Rocha LN, Benati FJ et al. Human bocavirus infection in children with gastroenteritis, Brazil. Emerg Infect Dis 2007; 13:1756-8.

2. Allander T, Jartti T, Gupta S et al. Human bocavirus and acute wheezing in children. Clin Infect Dis 2007; 44:904-10.

3. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, and Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A 2005; 102:12891-6.

4. Arden KE, McErlean P, Nissen MD, Sloots TP, and Mackay IM. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J Med Virol 2006; 78:1232-40.

5. Bastien N, Brandt K, Dust K, Ward D, and Li Y. Human Bocavirus infection, Canada. Emerg Infect Dis 2006; 12:848-50.

6. Brieu N, Gay B, Segondy M, and Foulongne V. Electron microscopy observation of human bocavirus (HBoV) in nasopharyngeal samples from HBoV-infected children. J Clin Microbiol 2007.

7. Brown CS, Van Lent JW, Vlak JM, and Spaan WJ. Assembly of empty capsids by using baculovirus recombinants expressing human parvovirus B19 structural proteins. J Virol 1991; 65:2702-6.

8. Corcoran A, Doyle S, Waldron D, Nicholson A, and Mahon BP. Impaired gamma interferon responses against parvovirus B19 by recently infected children. J Virol 2000; 74:9903-10.

9. Corcoran A, Mahon BP, and Doyle S. B cell memory is directed toward conformational epitopes of parvovirus B19 capsid proteins and the unique region of VP1. J Infect Dis 2004; 189:1873-80.

10. Endo R, Ishiguro N, Kikuta H et al. Seroepidemiology of human bocavirus in hokkaido prefecture, Japan. J Clin Microbiol 2007; 45:3218-23.

11. Franssila R and Hedman K. T-helper cell-mediated interferon-gamma, interleukin-10 and proliferation responses to a candidate recombinant vaccine for human parvovirus B19. Vaccine 2004; 22:3809-15.

12. Franssila R, Soderlund M, Brown CS, Spaan WJ, Seppala I, and Hedman K. IgG subclass response to human parvovirus B19 infection. Clin Diagn Virol 1996; 6:41-9.

13. Fry AM, Lu X, Chittaganpitch M et al. Human bocavirus: a novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. J Infect Dis 2007; 195:1038-45.

14. Hurtado A, Rueda P, Nowicky J, Sarraseca J, and Casal JI. Identification of domains in canine parvovirus VP2 essential for the assembly of virus-like particles. J Virol 1996; 70:5422-9.

15. Kantola K, Hedman L, Allander T et al. Serodiagnosis of human bocavirus infection. Clin Infect Dis 2008; 46:540-6.

16. Kaplan NM, Dove W, Abu-Zeid AF, Shamoon HE, Abd-Eldayem SA, and Hart CA. Human bocavirus infection among children, Jordan. Emerg Infect Dis 2006; 12:1418-20.

17. Kesebir D, Vazquez M, Weibel C et al. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis 2006; 194:1276-82.

18. Kleines M, Scheithauer S, Rackowitz A, Ritter K, and Hausler M. High prevalence of human bocavirus detected in young children with severe acute lower respiratory tract disease by use of a standard PCR protocol and a novel real-time PCR protocol. J Clin Microbiol 2007; 45:1032-4.

19. Kotaniemi-Syrjanen A, Laatikainen A, Waris M, Reijonen TM, Vainionpaa R, and Korppi M. Respiratory syncytial virus infection in children hospitalized for wheezing: virus-specific studies from infancy to preschool years. Acta Paediatr 2005; 94:159-65.

20. Lau SK, Yip CC, Que TL et al. Clinical and molecular epidemiology of human bocavirus in respiratory and fecal samples from children in Hong Kong. J Infect Dis 2007; 196:986-93.

21. Lee JI, Chung JY, Han TH, Song MO, and Hwang ES. Detection of human bocavirus in children hospitalized because of acute gastroenteritis. J Infect Dis 2007; 196:994-7.

22. Lin F, Guan W, Cheng F, Yang N, Pintel D, and Qiu J. ELISAs using human bocavirus VP2 virus-like particles for detection of antibodies against HBoV. J Virol Methods 2008; 149:110-7.

23. Lindner J, Barabas S, Saar K et al. CD4(+) T-cell responses against the VP1-unique region in individuals with recent and persistent parvovirus B19 infection. J Vet Med B Infect Dis Vet Public Health 2005; 52:356-61.

24. Lindner J and Modrow S. Human Bocavirus – a novel parvovirus to infect humans. Intervirology 2008; 51(2):116-122.

25. Lindner J, Zehentmeier S, Franssila R et al. CD4+ T Helper Cell Responses Against Human Bocavirus VP2 Virus-like Particles in Healthy Adults. J Infect Dis 2008. In press.

26. Liu L, Bastien N, Sidaway F, Chan E, and Li Y. Seroprevalence of human metapneumovirus (hMPV) in the Canadian province of Saskatchewan analyzed by a recombinant nucleocapsid protein-based enzyme-linked immunosorbent assay. J Med Virol 2007; 79:308-13.

27. Ma X, Endo R, Ishiguro N et al. Detection of human bocavirus in Japanese children with lower respiratory tract infections. J Clin Microbiol 2006; 44:1132-4.

28. Maggi F, Andreoli E, Pifferi M, Meschi S, Rocchi J, and Bendinelli M. Human bocavirus in Italian patients with respiratory diseases. J Clin Virol 2007; 38:321-5.

29. Maranga L, Brazao TF, and Carrondo MJ. Virus-like particle production at low multiplicities of infection with the baculovirus insect cell system. Biotechnol Bioeng 2003; 84:245-53.

30. Mengeling WL and Paul PS. Antibodies for autonomous parvoviruses of lower animals detected in human serum. Brief report. Arch Virol 1986; 88:127-33.

31. Naghipour M, Cuevas LE, Bakhshinejad T, Dove W, and Hart CA. Human bocavirus in Iranian children with acute respiratory infections. J Med Virol 2007; 79:539-43.

32. Qu XW, Duan ZJ, Qi ZY et al. Human bocavirus infection, People's Republic of China. Emerg Infect Dis 2007; 13:165-8.

33. Raulf-Heimsoth M, Sander I, Chen Z et al. Development of a monoclonal antibody-based sandwich ELISA for detection of the latex allergen Hev b 1. Int Arch Allergy Immunol 2000; 123:236-41.

34. Salimans MM, van Bussel MJ, Brown CS, and Spaan WJ. Recombinant parvovirus B19 capsids as a new substrate for detection of B19-specific IgG and IgM antibodies by an enzyme-linked immunosorbent assay. J Virol Methods 1992; 39:247-58.

35. Sloots TP, McErlean P, Speicher DJ, Arden KE, Nissen MD, and Mackay IM. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol 2006; 35:99-102.

36. Soderlund M, Brown CS, Spaan WJ, Hedman L, and Hedman K. Epitope type-specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J Infect Dis 1995; 172:1431-6.

37. Vicente D, Cilla G, Montes M, Perez-Yarza EG, and Perez-Trallero E. Human bocavirus, a respiratory and enteric virus. Emerg Infect Dis 2007; 13:636-7.

38. Weissbrich B, Neske F, Schubert J et al. Frequent detection of bocavirus DNA in German children with respiratory tract infections. BMC Infect Dis 2006; 6:109.

5.3. Publikation III: Prevalence and Clinical Aspects of Human Bocavirus Infection in Children

Eingereichtes Manuskript.

Lüdya Karalar[1#], Juha Lindner[1#], Sven Schimanski[1], Michael Kertai[2], Hugo Segerer[2] and Susanne Modrow[1*]

[#]Both authors contributed equally to this work.

[1]Institute for Medical Microbiology and Hygiene, University of Regensburg, Germany; [2]Children´s Hospital St. Hedwig, Regensburg, Germany.

Keywords: Human Bocavirus, respiratory disease, gastrointestinal disease, immune response, seroprevalence
Running title: Clinical Aspects of Human Bocavirus Infection

[*]Corresponding author: Susanne Modrow, Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz-Josef-Strauss Allee 11, 93053 Regensburg, Germany. Phone: (+49)-941-9446454, Fax: (+49)-941-9446402, e-mail: susanne.modrow@klinik.uni-regensburg.de

Abstract

Background: Human Bocavirus (HBoV) was recently described as a new member of the *Parvoviridae* and suggested to be associated with respiratory and gastric disease in infants and young children.

Methods: Sera of 357 paediatric patients hospitalized with infectious and non-infectious diseases were analyzed for the presence of HBoV-DNA and virus-specific antibodies by quantitative PCR and ELISA.

Results: HBoV seroprevalence was determined to range between 25% in infants younger than one year of age and 93% in children older than three years. Viral loads ranging between $1x10^2$–$1.2x10^6$ geq/ml were observed in 6.7% (20/297) of serum samples obtained from preferentially young children suffering from infectious diseases. HBoV genomes were furthermore detected in 5% (3/60) of sera collected from individuals with non-infectious illnesses.

HBoV-DNA was most frequent in patients with respiratory disease (9.6%). Whereas only 5.2% patients with upper respiratory tract disease were viremic, HBoV-DNA was found in 14.6% and 10.0% of patients with lower respiratory tract illness and pneumonia. Acute HBoV infections were also observed in 7.5% of patients with gastroenteritis and in one child with inflammatory bowel disease. None of 77 patients hospitalized for various other infectious diseases (e.g. rash, urinary tract, meningitis) displayed viremia. In 60.9% and 47.8% of DNA-positive children HBoV-specific IgM and IgG was observed, respectively.

Conclusions: The presented prospective study provides comprehensive data on the clinical association of acute HBoV-infection with respiratory illness and on the seroprevalence of virus-specific antibodies in children.

Introduction

Human Bocavirus (HBoV), a recently discovered member of the *Parvoviridae* family, was identified in children with infections of the lower respiratory tract and represents the second parvovirus that causes disease in humans [1, 2]. Comprehensive phylogenetic analysis of the viral genome has revealed a close relation of HBoV to the canine minute virus and bovine parvovirus, both members of the genus *Bocavirus*. Several isolates of HBoV with high sequence homology have been described [3]. HBoV was detected by PCR-based techniques not only in clinical respiratory samples but also fecal excretions of young children in Australia, North-America, Asia, Europe, Africa and the Middle-East, indicating a worldwide distribution of the virus [4-19]. The prevalence of HBoV-DNA in samples of children with symptoms of respiratory illness has been found to range between 1.5-18.3% and may vary depending on seasonal fluctuation and the age of the patients. In children HBoV tends to be linked with a high rate of co-infections with other respiratory viruses, e.g. with picornaviruses, adenoviruses, respiratory syncytial virus and human metapneumovirus [20]. A recent study describes HBoV as the fourth most prevalent single virus after RSV, rhino- and adenovirus in children hospitalized with respiratory disease [21]. As most of the studies did not include control samples from children without symptoms indicating the involvement of infections, HBoV has not been clearly identified as an infectious agent responsible for respiratory diseases.

First studies on the seroprevalence of HBoV have described the use of recombinant VP1 and VP2 proteins and VP2 virus-like particles (VP2-VLP) in the detection of HBoV-specific IgG in the Japanese, Chinese, Finnish and German population, respectively [22-25]. Thereby, ubiquitous antibody-responses with a seroprevalence of up to 94%-100% were detected in adults and in children aged above 6 years, whereas seronegative individuals were predominantly found in small children around the age of one to two years. In the current study we present a comprehensive overview on the frequency and impact of acute HBoV infection in 297 children and adolescents hospitalized with symptoms indicating the involvement of infectious diseases. In addition, serum samples derived from 60 children were included that were admitted due to various diseases not associated with infections (e.g. planned surgery, bone fractures, accidents).

Materials and Methods

Patients

Serum samples were collected from 297 patients (160 male, 137 female, age range: 1-190 months, mean age: 57.4 months) who were admitted to the Children´s Hospital St. Hedwig, University of Regensburg, during the time period between December 2006 and May 2007. Follow-up samples were available from 6 children. All individuals were immunocompetent caucasians with domicile in the city of Regensburg or the surrounding regions located in the South-Eastern part of Bavaria, Germany.

Controls

Sera from 60 children (38 male, 22 female, age range: 1-194 months, mean age: 77.9 months) were obtained (December 2006 to May 2007). These individuals were inpatients of Children´s Hospital St. Hedwig, University of Regensburg, due to non-infectious conditions, i.e. planned surgery, accidents, fractures, growth retardation.

Ethics

The study was approved by the ethical committee of the University of Regensburg (nr. 06/85). Only blood samples that were drawn for diagnostic and/or clinical reasons were included in the study. Parental consent was obtained prior to sample processing.

Detection of HBoV genomes

Viral nucleic acid was isolated from 200 µl serum using the QiaAmp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For real time TaqMan PCR, the following primers and probe were used for amplification of HBoV genome sequences: Forward primer 5'-CCA CCT ATC GTC TTG CAC TGC-3' (nts 2586-2606), reverse primer 5'-TTT TCC CCG ATG TAC TCT CCC-3' (nts 2619-2639), probe FAM-5'-TCG AAG ACC TCA GAC CAA GTG ATG AAG ACG-3'-TAMRA (nts 2608-2637), positions according to Genbank number DQ000496.1. Duplicate PCR reactions were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Weiterstadt, Germany) with 300 nM forward primer, 300 nM reverse primer, 150 nM probe and 200 nM dNTP. An initial denaturation phase of 10 min at 95°C was followed by 45 cycles at 95°C (15 sec) and 60°C (1 min). Tenfold serial dilutions of plasmid DNA (HBoV ST2 isolate, [1]) and a negative serum were amplified as controls and quantification standard.

Detection of HBoV-specific IgM and IgG antibodies

HBoV VP2-VLP were produced and purified as described elsewhere [26]. For the detection of HBoV-specific antibodies a recently established ELISA assay based on the use of HBoV VP2-VLP was used [22]. Briefly, 100 ng of purified HBoV VP2-VLP were coated on Nunc-Immuno^{TM} MediSorp^{TM} plates (Nunc GmbH, Wiesbaden, Germany) in coating buffer (0.2 M Na_2CO_3, 0.2 M $NaHCO_3$; pH 9.5) overnight at 4°C, washed six times with washing buffer (PBS, 0.05% Tween 20) and blocked with dilution buffer (PBS, 2% Tween 20, 3% FCS) for 1 h at 37°C. After incubation with respective serum samples for 2 h at 37°C, the plates were washed and rabbit anti-human IgG- or IgM-specific HRP-coupled secondary antibodies (1:6000 and 1:1000 in dilution buffer, respectively; both Dako Deutschland GmbH, Hamburg, Germany) were added for 1 h at 37°C. Development was performed using the BD OptEIA^{TM} Substrate (BD Biosciences, Heidelberg, Germany) according to the manufacturer´s instructions.

Laboratory analysis of infectious diseases

All patients were routinely tested for infectious agents causing respiratory tract infection (diagnostic bacterial culture techniques, PCR and serology for diagnosis of respiratory syncytial virus, Epstein-Barr virus, influenza virus, adenovirus, rotavirus).

Statistical data analysis

All data were statistically evaluated using the Chi-square and the Mann-Whitney U-test for independent samples.

Results

Description of patients included in the study

297 children and adolescents admitted to a children´s hospital due to symptoms indicating acute infectious diseases were included in the study. With respect to their clinical diagnosis, the children were divided into three groups (Table 1): Group A comprised 156/297 patients (52.5%) with diseases of the respiratory tract which were subdivided into 58 patients with symptoms involving the upper respiratory tract (i.e. rhinitis, otitis media, tracheitis, tonsillitis, cough), 48 patients with symptoms involving the lower respiratory tract (i.e. bronchitis, bronchiolitis), and 50 patients with pneumonia. 64/297 patients (21.5%, group B) presented with gastrointestinal symptoms (i.e. diarrhea, nausea,

vomiting). 11 of these were diagnosed with chronic inflammatory bowel disease. The third patient group (group C) enclosed 77 patients (77/297, 25.9%) with a rather broad range of infections involving neither the respiratory nor the gastrointestinal tract, e.g. fever, febrile convulsion, meningitis, encephalitis, urinary tract infection and rash. As respiratory aspirates were available only from a small subgroup of patients, analysis was focused on serum samples that were drawn at the time points the patients were hospitalized and tested for the presence of HBoV-specific humoral immune reactions and viral DNA. Furthermore, 60 children that presented with diseases not associated with infections were included into the study.

Age-related prevalence of HBoV-DNA and of HBoV-specific IgM and IgG

For all tested children a seroprevalence of 71.4% (255/357 patients) was observed ranging from 23% at an age of 7-9 months to more than 90% in children of 31-36 months and older (Figure 1A). Significant differences between girls and boys were not evident (not shown). A reduction of seroprevalence from 78% to 23% was observed in children up to an age of 7-9 months. In parallel, the median IgG titers declined from 741 relative units (RU) to 65 RU (Figure 1B). As in these age-groups acute HBoV infections were only occasionally observed (Figure 1C), the detected IgG mainly represent maternal antibodies. Starting with an age of 25 months rising values of both seroprevalence and IgG-titers were observed as a consequence of the accumulating numbers of acute HBoV infections occurring in children aged between 13 to 30 months (Figure 1A, C).

HBoV genomes were observed preferentially in sera from patients younger than 30 months. In older patients only sporadic HBoV-infections were observed (Figure 1C). Whereas the mean age of all patients was 67.7 months, patients with acute HBoV infection displayed a lower mean age of 24.9 months (Table 2).

Viral genomes and HBoV-specific IgM and IgG antibodies in patients and controls

Viral genomes indicating acute HBoV-infection were detected in 20 of 297 patients (6.7%) and 3 of 60 controls (5.0%) (Table 1). While these overall percentages were rather similar, differences became evident after correlating patient groups A, B and C with markers for acute HBoV infection: In the serum samples derived from 15/156 (9.6%) patients of group A (patients 1-15) presenting with symptoms of respiratory tract disease, HBoV genomes were detectable with virus loads varying between 10^2-10^3 geq/ml and $1.2x10^6$ geq/ml (Tables 1, 3).

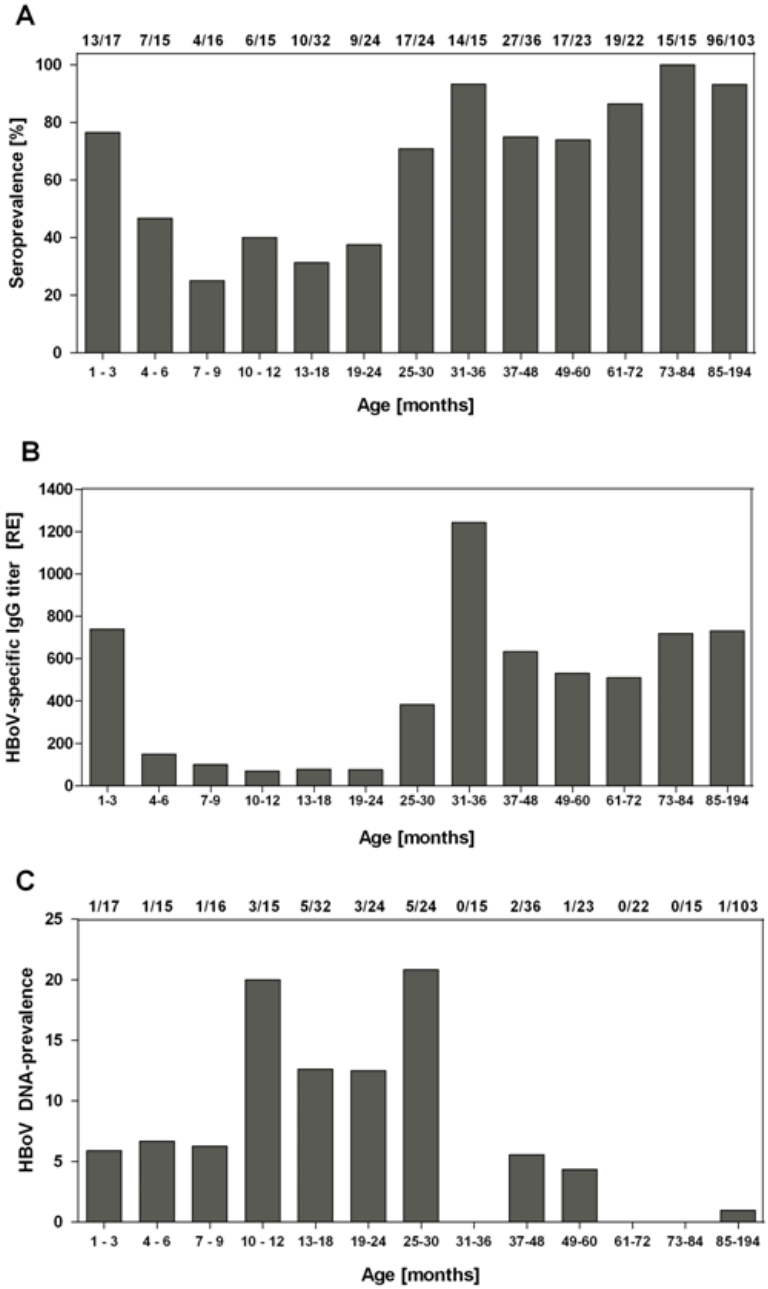

Figure 1: Seroprevalence (A), median HBoV IgG-titers (B) and DNA-prevalence (C) in children and adolescents. The numbers given above the columns representing the data obtained for various age-groups in parts A and C indicate the number of individuals with positive results in relation to that of all individuals tested.

Follow-up samples were available for patient 10, a girl suffering from bronchitis. The reduction of the virus load from $1.9x10^5$ to $4.6x10^3$ during a time period of three days was observed in combination with rising amounts of VP2-specific IgM antibodies (Table 3). The prevalence of HBoV-DNA in serum was highest in children suffering from diseases of the lower respiratory tract (7/48, 14.6%) and from pneumonia (5/50, 10.0%) as compared to upper respiratory tract disease (3/58, 5.2%) (Table 1).

Clinical course/ symptoms	**Number of patients**	**Mean age (months)**	**Age range (months)**	**HBoV VP2-specific IgG**	**HBoV-DNA**
Infectious diseases	**297**	**57.4**	**1-190**	**209/297 70.3%**	**20/297 6.7%**
A. Respiratory tract	**156**	**40.5**	**1-178**	**95/156 60.9%**	**15/156 9.6%**
upper respiratory tract (rhinitis, otitis media, tracheitis, tonsillitis)	58	48.3	1-178	36/58 62.1%	3/58 5.2%
lower respiratory tract (bronchitis, broncheolitis)	48	20.1	1-95	24/48 50.0%	7/48 14.6%
pneumonia	50	51.8	5-167	35/50 70.0%	5/50 10.0%
B. Gastrointestinal tract	**64**	**68.5**	**1-173**	**49/64 76.6%**	**5/64 7.8%**
diarrhea, nausea, vomiting	53	58.4	1-161	38/53 71.7%	4/53 7.5%
chronic inflammatory bowel disease	11	117.5	41-173	11/11 100.0%	1/11 9.1%
C. Others (fever, febrile convulsion urinary tract, exanthema, meningitis, encephalitis)	**77**	**82.7**	**4-190**	**65/77 84.4%**	**0/77 0.0%**
Non-infectious diseases (bone fracture, growth retardation, planned surgery)	**60**	**77.9**	**1-194**	**44/60 73.3%**	**3/60 5.0%**
Total	**357**	**67.7**	**1-194**	**255/357 71.4%**	**23/357 6.4%**

Table 1: HBoV-specific IgG-antibodies and viral DNA present in serum samples of patients and controls. he number of patients in which specific antibodies or viral DNA could be detected is shown for each subgroup.

With respect to patients of group C (other infections, 0/77) and those with non-infectious diseases (3/60, 5.0%) the frequency of acute HBoV infection was significantly elevated in patients with lower respiratory tract disease and pneumonia (Table 4). Co-infections with other pathogens were observed in patient 4 (respiratory syncytial virus) and patient 12 (Streptococcus A). Acute HBoV infection with rather low virus loads of $1x10^2$ to $3.3x10^3$ geq/ml was also observed in 5/64 patients (7.8%) with gastroenteritis, one of these cases was diagnosed as chronic inflammatory bowel disease. In patients 16-18 with gastroenteritis, rotavirus infection was diagnosed in addition to HBoV.

With the exception for patients with lower respiratory tract disease, the mean age of the HBoV-DNA positive patients was lower as compared to that of all patients of the individual groups (Table 2). This tendency was most evident in children with gastroenteritis (group B) with a mean age of 58.4 months for all patients and of 15.5 months for HBoV-infected patients. While young children preferentially presented with gastroenteritis or respiratory tract symptoms, particularly pneumonia was the lead diagnosis of children infected at an age of 22 months and above.

Patients	**Mean age of all patients (months)**	**Mean age of HBoV DNA-positive patients (months)**
Total	67.7	24.9
Infectious diseases	57.4	22.5
A. Respiratory tract	40.5	22.1
Upper respiratory tract	48.3	20.6
Lower respiratory tract	20.1	18.7
Pneumonia	51.8	31.0
B. Gastrointestinal tract	68.5	20.6
Gastroenteritis	58.4	15.5
Non-infectious diseases	77.9	40.3

Table 2: Mean age of the studied patient groups.

In children with non-infectious diseases, HBoV genomes were detectable in three patients: one newborn boy (patient 21) suffering from hematemesis, one girl (patient 22) presenting with atonic seizures and a boy with a submandibular gland abscess (patient 23). Respiratory or gastrointestinal symptoms that possibly took place in the weeks before were not reported.

Patient	Age (months) /Sex	Clinical course	HBoV serology IgG	IgM	HBoV-DNA (geq/ml)	Co-infection
Group A						
1	18/f	fever, rhinitis, cough	positive	weakly pos.	10^2-10^3	---
2	22/f	acute tracheitis	positive	weakly pos.	$1.8x10^3$	---
3	22/m	otitis media	positive	positive	$1.6x10^3$	---
4	4/m	acute bronchitis	negative	negative	$1.0x10^2$	RSV
5	12/m	bronchitis, otitis media	negative	weakly pos.	$1.9x10^5$	---
6	12/m	bronchitis	negative	negative	$3.1x10^5$	---
7	17/m	spastic bronchitis	negative	positive	$1.2x10^6$	---
8	27/m	bronchitis	positive	positive	$1.8x10^3$	---
9	29/m	peribronchitis, otitis media	negative	negative	$2.3x10^4$	---
10/	30/f 0*	bronchitis	negative	weakly pos.	$1.9x10^5$	---
	30 +2*	bronchitis	negative	positive	$1.1x10^5$	---
	30 +3*	bronchitis	negative	positive	$4.6x10^3$	---
11	15/f	bronchopneumonia	negative	negative	$5.6x10^4$	---
12	18/f	pneumonia, respiratory insufficiency	negative	weakly pos.	$2.2x10^5$	---
13	29/f	bronchopneumonia	weakly pos.	negative	10^2-10^3	---
14	42/f	pneumonia	negative	positive	$1.0x10^3$	Strep A
15	51/f	atypical pneumonia	negative	positive	$1.1x10^5$	---
Group B						
16	8/f	gastroenteritis	positive	negative	10^2-10^3	rotavirus
17	12/m	gastroenteritis	positive	weakly pos.	10^2-10^3	rotavirus
18	13/m	gastroenteritis	positive	positive	10^2-10^3	rotavirus
19	29/m	gastroenteritis, diarrhea	positive	positive	$3.3x10^3$	---
20	41/m	inflammatory bowel disease	positive	positive	10^2-10^3	---

Table 3: Individual data of patients with acute HBoV infection. * Follow-up samples of patient 10 were taken 2 and 3 days after the first sample, respectively. RSV: respiratory syncytial virus.

HBoV-specific antibodies

IgG against HBoV VP2-VLP were detected in all patient groups irrespective of the disease pattern. Overall 255/357 children were IgG positive: 209/297 (70.3%) of patients with infections and in 44/60 (73.3%) of patients with non-infectious diseases (Table 1). The seroprevalence ranged from 50% in patients with lower respiratory tract disease to 84.4% in patient group C.

The majority of HBoV DNA-positive patients (14/23, 60.7%) produced virus-specific IgM antibodies as an additional marker for acute infection: 9/23 patients (39.1%: nrs. 3, 7, 8, 10, 14, 15, 18, 19, 20) were tested positive for VP2-specific IgM; furthermore 5/23 patients (21.7%: nrs. 1, 2, 5, 12, 17) displayed weakly positive values for IgM (Table 3). VP2-specific IgG was observed in 11/23 patients with acute infection (47.8%, nrs. 1-3, 4, 16-21, 23), borderline results were obtained in 2/23 patients (nrs. 13, 23). In 8/23 patients (34.8%) IgG was detected in combination with positive or borderline IgM values (nrs. 1, 2, 3, 8, 7-20).

Patients (group C)	**Patients with Other infectious disease**	**non-infectious diseases**
Patients (group A)		
Respiratory tract disease	p=0.00491	p=0.27165
Upper respiratory tract	p=0.04357	p=0.96600
Lower respiratory tract	p=0.00056	p=0.08776
Pneumonia	p=0.00699	p=0.38904
Patients (group B)		
Gastrointestinal tract disease	p=0.01251	p=0.52407
Diarrhea/nausea	p=0.01434	p=0.57511

Table 4: p-values describing the significancies of acute HBoV infection in patients (group A and B) as compared to patients of group C and patients with non-infectious conditions.

Discussion

HBoV genomes have been detected in respiratory tract samples of children worldwide mainly in retrospective analyses [7, 9, 13, 15, 27]. Until recently the serodiagnosis of HBoV infection was not possible due to the lack of suitable antigen preparations. Based on recombinant HBoV VP2-like particles HBoV-specific T-helper cell reactions have been

detected in adults [26]. Furthermore, an ELISA was established based on HBoV VP2-VLP that resulted in the detection of HBoV-specific IgG and IgM in 94% and 1% of adult healthy blood donors in Germany, respectively [22]. These data point to the ubiquitous prevalence of HBoV infection in the population and to a seroconversion in childhood.

In contrast to others we did not use respiratory aspirates for analyzing the association of acute HBoV-infection with disease. This decision was based on two major reasons: (I) HBoV is closely related to bovine parvovirus (BPV) and canine minute virus (CaMV). In general, acute BPV and CaMV infections are known to be associated with viremia; the respective viruses have been shown to be present in blood and in various organs and tissues in infected calves and dogs [28-30]. (II) Respiratory aspirates were not routinely obtained from all patients with respiratory disease; they were not available at all from children suffering from non-respiratory disease. Therefore we preferred the analysis of serum samples derived from 357 children and adolescents which presented with symptoms of various infectious and non-infectious diseases for HBoV-specific IgG and IgM indicative for acute and past HBoV-infection in combination with a PCR assay for the quantification of HBoV genomes. In this cohort 88% of children aged between 30 and 194 months displayed HBoV VP2-specific IgG as a serological marker for previous HBoV infection. This value is consistent with data observed by others in German and Japanese adults [22, 25] and indicates that the incidence of HBoV infection is highest among young children. This assumption was confirmed when analyzing serum samples from children aged between 10 and 30 months by PCR: 16 out of a total of 23 DNA positive patients (69.6%) belonged to this age group. As HBoV-specific IgM could be detected in almost 70% of the DNA-positive patients, these individuals display the characteristic markers for acute viral infections. In newborns and young children up to an age of 9 months HBoV-DNA was detectable only in three patients. This low incidence is probably due to the high prevalence of maternal IgG protective against acute HBoV infection. Together with declining concentrations of maternal IgG a rising incidence of HBoV infection was observed. Children at an age between 31 and 36 months displayed high titers of HBoV-specific IgG (Figure 1B). This is consistent with data from the related parvovirus B19, where antiviral IgG reaches highest values in the weeks and months directly following the acute infection and declines afterwards [31, 32]. Also for HBoV declining titers were found in the age-groups between 30 and 194 months. As a consequence of the increasing values of IgG titers and seroprevalence acute HBoV infections were rare in children older

than 30 months: Only 4 of 217 (1.8%) individuals showed markers of acute HBoV infection.

With respect to distinct patient groups we observed the highest rate of acute HBoV infections in children presenting with symptoms of lower respiratory tract infection and pneumonia. The samples were collected at the time point of the children's admission to the hospital irrespective of the different stages of acute HBoV-infection. This procedure may explain that HBoV-specific IgM and/or IgG were not detectable in all samples from DNA-positive children likewise. As compared to patients with other infectious diseases (group C) this enhanced rate of acute HBoV infection was highly significant. In contrast to other reports co-infectious agents were only observed in two HBoV DNA-positive patients: RSV infection was diagnosed in a 4 month old boy with acute bronchitis and streptococci A infection in a girl suffering from pneumonia (Table 3). This is particularly surprising as RSV-infections were the main cause for bronchitis and pneumonia in patients belonging to group A (data not shown). The low rate of observed co-infections may be due to the focus of our study on the exclusive analysis of serum samples that were drawn at the time point of the patients' admission to the clinic. In the case of pathogens causing systemic infections associated with respiratory tract disease testing of sera for the presence of virus and of antiviral immune reactions represents a more reliable diagnostic marker as compared to the analysis of respiratory aspirates. The use of highly sensitive PCR methods for the detection of viral and/or bacterial nucleic acids in respiratory samples from hospitalized children may render positive results due to aerogenic contaminations or nosocomially transmitted co-infections. These are frequent in paediatric wards, particularly during epidemic phases of respiratory infections in winter and early spring time. Belonging to the *Parvoviridae,* HBoV has to be assumed as an extremely stable and resistant pathogen against disinfectants and heat treatment [33-35]. This property may facilitate nosocomial transmissions between children.

In addition to patients suffering from respiratory tract illness, acute HBoV infection was observed young children presenting with gastroenteritis (mean age 15.5 months). In 3 out of four children, rotavirus co-infection was observed together with HBoV. A recent publication describes the occurrence of HBoV in stool samples of children with acute gastroenteritis [36]. Similarly to our results a high rate of co-infections with rotavirus was reported. Therefore causative role of HBoV with acute gastroenteritis remains unclear.

Acute HBoV infection (IgM/IgG positive, DNA positive) was also observed in a boy presenting with an acute episode of an underlying chronic inflammatory bowel disease.

Markers for acute HBoV infection were not observed in any of the children presenting with symptoms for other infectious diseases (group C). When analyzing children with non-infectious diseases low amounts of HBoV genomes were detectable in 3 patients. Symptoms neither for respiratory nor for gastrointestinal tract diseases were recallable in any of these cases. Together with the high HBoV seroprevalence in older children and adults this observation indicates that HBoV infections may be frequently associated without illness or with mild symptoms not requiring hospitalization.

In conclusion our data show that acute HBoV infection is most frequent in young children at an age between 10 and 30 months. The acute infection may be associated with lower respiratory tract disease but also with diarrhea and gastroenteritis. Together with reports from other groups our data indicate HBoV as new infectious agent in the growing groups of viruses and bacteria causing respiratory and gastrointestinal tract disease in young children.

Acknowledgements

The authors thank Dr. Tobias Allander and colleagues from the Karolinska Institute, Stockholm, Sweden, for the generous gift of the pCR®4-TOPO® vector including the genome sequence of HBoV ST2.

References

1. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A **2005**; 102(36):12891-6.

2. Allander T, Jartti T, Gupta S, et al. Human bocavirus and acute wheezing in children. Clin Infect Dis **2007**; 44(7):904-10.

3. Chieochansin T, Chutinimitkul S, Payungporn S, et al. Complete coding sequences and phylogenetic analysis of Human Bocavirus (HBoV). Virus Res **2007**; 129(1):54-7.

4. Lindner J, Modrow S. Human Bocavirus - A Novel Parvovirus to Infect Humans. Intervirology **2008**; 51(2):116-22.

5. Albuquerque MC, Rocha LN, Benati FJ, et al. Human bocavirus infection in children with gastroenteritis, Brazil. Emerg Infect Dis **2007**; 13(11):1756-8.

6. Kleines M, Scheithauer S, Rackowitz A, Ritter K, Hausler M. High prevalence of human bocavirus detected in young children with severe acute lower respiratory tract disease by use of a standard PCR protocol and a novel real-time PCR protocol. J Clin Microbiol **2007**; 45(3):1032-4.

7. Pozo F, Garcia-Garcia ML, Calvo C, Cuesta I, Perez-Brena P, Casas I. High incidence of human bocavirus infection in children in Spain. J Clin Virol **2007**; 40(3):224-8.

8. Qu XW, Duan ZJ, Qi ZY, et al. Human bocavirus infection, People's Republic of China. Emerg Infect Dis **2007**; 13(1):165-8.

9. Volz S, Schildgen O, Klinkenberg D, et al. Prospective study of Human Bocavirus (HBoV) infection in a pediatric university hospital in Germany 2005/2006. J Clin Virol **2007**; 40(3):229-35.

10. Kaplan NM, Dove W, Abu-Zeid AF, Shamoon HE, Abd-Eldayem SA, Hart CA. Human bocavirus infection among children, Jordan. Emerg Infect Dis **2006**; 12(9):1418-20.

11. Naghipour M, Cuevas LE, Bakhshinejad T, Dove W, Hart CA. Human bocavirus in Iranian children with acute respiratory infections. J Med Virol **2007**; 79(5):539-43.

12. Vicente D, Cilla G, Montes M, Perez-Yarza EG, Perez-Trallero E. Human bocavirus, a respiratory and enteric virus. Emerg Infect Dis **2007**; 13(4):636-7.

13. Lau SK, Yip CC, Que TL, et al. Clinical and molecular epidemiology of human bocavirus in respiratory and fecal samples from children in Hong Kong. J Infect Dis **2007**; 196(7):986-93.

14. Bastien N, Brandt K, Dust K, Ward D, Li Y. Human Bocavirus infection, Canada. Emerg Infect Dis **2006**; 12(5):848-50.

15. Smuts H, Hardie D. Human bocavirus in hospitalized children, South Africa. Emerg Infect Dis **2006**; 12(9):1457-8.

16. Ma X, Endo R, Ishiguro N, et al. Detection of human bocavirus in Japanese children with lower respiratory tract infections. J Clin Microbiol **2006**; 44(3):1132-4.

17. Kesebir D, Vazquez M, Weibel C, et al. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis **2006**; 194(9):1276-82.

18. Maggi F, Andreoli E, Pifferi M, Meschi S, Rocchi J, Bendinelli M. Human bocavirus in Italian patients with respiratory diseases. J Clin Virol **2007**; 38(4):321-5.

19. Sloots TP, McErlean P, Speicher DJ, Arden KE, Nissen MD, Mackay IM. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol **2006**; 35(1):99-102.

20. Arden KE, McErlean P, Nissen MD, Sloots TP, Mackay IM. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J Med Virol **2006**; 78(9):1232-40.

21. Calvo C, Garcia-Garcia ML, Pozo F, Carvajal O, Perez-Brena P, Casas I. Clinical Characteristics of Human Bocavirus Infections Compared With Other Respiratory Viruses in Spanish Children. Pediatr Infect Dis J **2008**.

22. Lindner J, Karalar L, Zehentmeier S, et al. Humoral Immune Responses Against Human Bocavirus VP2 Virus-like Particles. Viral Immunol. [in press] **2008**.

23. Lin F, Guan W, Cheng F, Yang N, Pintel D, Qiu J. ELISAs using human bocavirus VP2 virus-like particles for detection of antibodies against HBoV. J Virol Methods **2008**; 149(1):110-7.

24. Kantola K, Hedman L, Allander T, et al. Serodiagnosis of human bocavirus infection. Clin Infect Dis **2008**; 46(4):540-6.

25. Endo R, Ishiguro N, Kikuta H, et al. Seroepidemiology of human bocavirus in hokkaido prefecture, Japan. J Clin Microbiol **2007**; 45(10):3218-23.

26. Lindner J, Zehentmeier S, Franssila R, et al. CD4+ T Helper Cell Responses Against Human Bocavirus VP2 Virus-like Particles in Healthy Adults. J Infect Dis. [in press] **2008**.

27. Kesebir D, Vazquez M, Weibel C, et al. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis **2006**; 194(9):1276-82.

28. Bates RC, Storz J, Reed DE. Isolation and comparison of bovine parvoviruses. J Infect Dis **1972**; 126(5):531-6.

29 Durham PJ, Lax A, Johnson RH. Pathological and virological studies of experimental parvoviral enteritis in calves. Res Vet Sci **1985**; 38(2):209-19.

30. Pollock R, Carmichael LE. The canine parvoviruses. In: Tijssen P, ed. The Handbook of Parvoviruses, Vol. II.Bota Raton, USA: CRC Press Inc, **1990**:113-34.

31. Rohrer C, Gartner B, Sauerbrei A, et al. Seroprevalence of parvovirus B19 in the German population. Epidemiol Infect **2008**;1-12.

32. Modrow S, Dorsch S. Antibody responses in parvovirus B19 infected patients. Pathol Biol (Paris) **2002**; 50(5):326-31.

33. Boschetti N, Wyss K, Mischler A, Hostettler T, Kempf C. Stability of minute virus of mice against temperature and sodium hydroxide. Biologicals **2003**; 31(3):181-5.

34. Prowse C, Ludlam CA, Yap PL. Human parvovirus B19 and blood products. Vox Sang **1997**; 72(1):1-10.

35. Santagostino E, Mannucci PM, Gringeri A, et al. Transmission of parvovirus B19 by coagulation factor concentrates exposed to 100 degrees C heat after lyophilization. Transfusion **1997**; 37(5):517-22.

36. Cheng WX, Jin Y, Duan ZJ, et al. Human bocavirus in children hospitalized for acute gastroenteritis: a case-control study. Clin Infect Dis **2008**; 47(2):161-7.

5.4. Publikation IV: Adaptive Immune Responses Against Parvovirus B19 in Patients with Myocardial Disease

Eingereichtes Manuskript.

Juha Lindner[1#], Michel Noutsias[2#], Dirk Lassner[3], Jürgen Wenzel[1], Heinz-Peter Schultheiss[2], Uwe Kuehl[2], and Susanne Modrow[1*]

1: Institute of Medical Microbiology and Hygiene, University of Regensburg, Germany
2: Charité Centrum 11, Cardiovascular Medicine, Campus Benjamin Franklin, Charité - Universitätsmedizin Berlin, Germany
3: Institute of Cardiac Diagnostic and Therapy, Berlin, Germany
#: Both authors contributed equally to this work.

Keywords: parvovirus B19, cellular immune response, humoral immune response, cardiomyopathy, myocarditis

Running title: Parvovirus B19 Adaptive Immunity in Heart Disease

*Corresponding author: Prof. Dr. Susanne Modrow, Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz-Josef-Strauss Allee 11, 93053 Regensburg, Germany. Phone: (+49)-941-9446454, Fax: (+49)-941-9446402, e-mail: susanne.modrow@klinik.uni-regensburg.de.

Abstract

Background: Parvovirus B19 (B19V) genomes are frequently detected in endomyocardial biopsies (EMBs) from patients with acute myocarditis (AMC) and dilated cardiomyopathy (DCM), but also in various healthy tissues. The clinical relevance of these genomes is unclear.

Objectives: To investigate potential pathogenic influences of B19V-DNA in EMBs, we comparatively analyzed B19V-specific adaptive immune responses in AMC/DCM patients and healthy controls.

Study design: 15 AMC/DCM patients and 51 controls were analyzed by ELISA, Western blot, qPCR and ELISpot-assays.

Results: B19V-DNA in EMBs ranged between <100 and $1.2x10^5$ geq/µg genomic DNA. Acute B19V-infection was determined in 3 patients. In patients, slightly lower levels of B19V-specific T-cells were observed as compared to the controls, no differences were observed in virus-specific serology. Viral DNA-load in EMBs could not be correlated to the number of B19V-specific T-cells. No differences in T-cell response, viremia and/or serological markers indicative for viral pathogenesis were observed in patients with inflammatory cardiomyopathy.

Conclusions: Discrepancies in B19V-specific adaptive immunity were not observed in AMC/DCM patients as compared to healthy controls. The data indicate that the exclusive detection of B19V-DNA in EMBs is not sufficient to associate B19V with AMC/DCM but should be complemented with additional virological and immunological parameters in further studies.

Introduction

Viral infections of the heart are considered as likely causes of acute myocarditis (AMC) and dilated cardiomyopathy (DCM), since viral genomes can frequently be detected in endomyocardial biopsies (EMBs) [1-7]. Parvovirus B19 (B19V) genomes are frequently detected in various tissues and organs of healthy and diseased individuals, e.g. in the bone marrow, synovium, liver, and skin [8-11]. In myocardial tissues, B19V-DNA loads ranging between 10^2 and 10^5 genome equivalents (geq) per microgram genomic DNA have been reported in patients with heart disease [12,13], often in combination with genomes of further cardiotropic pathogens, e.g. enteroviruses [3,4,13,14]. The biological and clinical relevance of the presence of B19V-DNA in EMBs remains incompletely understood. It has been linked to diastolic and endothelial dysfunction in heart failure patients and to progressive cardiac dysfunction in chronic DCM patients [3,13,15], however, its prognostic value is controversially discussed [16].

B19V-infections are common with a seroprevalence of up to 80% in the elderly [17]. Associated symptoms include the childhood disease *erythema infectiosum* and a broad range of illnesses in adults, including transient arthritis, hepatitis, encephalitis [18,19].

B19V-specific adaptive immune reactions have been characterized in patients with various diseases, but not AMC or DCM. In acutely infected individuals, IgM and IgG against the structural proteins VP1 and VP2 are frequently observed, with IgG preferentially recognizing linear epitopes as present in denatured VP2-proteins [20]. With increasing time following acute infection, these antibodies are replaced by IgG directed against conformational epitopes present in VP2 virus-like particles (VLP). Antibodies against the NS1-protein have been frequently detected in individuals with persistent B19V-infections [21,22]. Current knowledge on T-cell mediated immune responses against B19V is limited. In individuals with past B19V-infections $CD4^+$ and $CD8^+$ T-cell responses against the viral structural and non-structural proteins have been described [23-26]. Yet, only limited data are available on the impact of these responses on viral persistence and the pathogenesis of B19V-induced diseases.

The aims of this study were to investigate whether adaptive immune responses in AMC/DCM patients with detectable B19V-genomes in EMBs are different from those observed in healthy individuals, and whether the strength and type of the reaction correlate with B19V viral loads in EMBs.

Materials and Methods

Study subjects and clinical diagnosis.

15 patients (1 female, 14 males, age range: 25-70 years, mean age: 53.2±12.1 years) presenting with AMC or DCM and detectable amounts of B19V-DNA in EMBs were investigated (Table 1). Patients with further detectable viral genomes in EMBs, i.e. enterovirus, human herpes virus type 6, adenovirus, Epstein-Barr virus, were excluded from this study. None of the patients were under immunomodulatory treatment.

AMC was clinically suspected in 8 individuals (1 female, 7 males; age range: 24-65 years; mean age: 49.6±12.8 years), while DCM was clinically suspected in 7 patients (all males; age range: 42-69 years; mean age: 57.4±10.6 years) [4, 5, 27-30]. AMC patients had a significantly shorter clinical history duration (1.4±2.1 months before EMBs obtainment) as compared to DCM patients (14.2±11.9 months; $p=0.01$).

The clinically suspected diagnosis of DCM was based on significantly impaired LVEF (LVEF≤45%) and/or dilated LV in association with heart failure symptoms. LVEF was significantly more depressed in DCM (36.1+8.4%) as compared to AMC (48.6+10.7%) patients assessed by LV angiography ($p<0.05$) and by echocardiography using the modified Simpson's approach (DCM: 36.7±8.8%; AMC: 49.5±12.1%; $p<0.05$). Furthermore, DCM patients had significantly more pronounced echocardiographic LV dilatation parameters (LV end-diastolic diameter: DCM: 64.6±8.5; AMC: 54.4±6.0 mm; LV end-systolic diameter: DCM: 47.9±9.7; AMC: 36.3±4.9 mm; $p<0.05$).

Controls

Serum and blood samples from 51 healthy Caucasian adults (27 females, 24 males, age range: 23-73 years, mean age: 35±10.2 years) served as controls. No EMBs were available from control individuals due to obvious ethical restrictions.

EMBs obtainment and evaluation

EMBs obtainment and detection of B19V-genomes in sera and EMBs were performed as described previously [3,28,31-33]. The histological evaluation of EMBs did not confirm active or borderline myocarditis according to the Dallas criteria [34]. The immunohistological detection of intramyocardial inflammation (DCMi) was conducted as described elsewhere [28,35].

Patient	Sex	Age (years)	CSD	EMBs Diagnosis	Chief complaint	Cardiac decompensation	Antecedent infection	Time between disease and EMB (months)	Increased Troponin T	ECG alterations	LVEF (%)	LVEDD (mm)
P1	male	44	AMC-ACS	DCMi (+)	AP	No	no	0.03	Yes	SR, STElev I, aVL, V2-V5	37	47
P2	male	24	AMC-ACS	DCM (-)	AP	No	RTI	0.03	Yes	SR, STElev I, aVL, V4-V6	59	53
P3	male	42	DCM	DCMi (+)	D	No	no	26.7	No	SR, normal	37	60
P4	male	55	DCM	DCMi (+)	D, P	Yes	no	13.7	No	Afib	26	79
P5	male	69	DCM	DCMi (+)	D	No	no	27.3	No	Afib, negTW II, III, aVF	43	56
P6	female	49	AMC-ACS	DCMi (+)	AP	No	RTI	0.03	Yes	SR, STElev I, II, V5-V6	61	52
P7	male	55	DCM	DCMi (+)	D	No	no	24.2	No	SR, negTW II, III	35	62
P8	male	59	AMC-DLVF	DCMi (+)	D, P	Yes	no	2.17	No	Afib	35	54
P9	male	69	DCM	DCM (-)	D, P	Yes	no	2.0	No	Afib, LBBB	24	73
P10	male	64	DCM	DCMi (+)	D	No	no	0.6	No	SR, LBBB	43	57
P11	male	47	DCM	DCM (-)	D	Yes	no	4.9	No	SR, normal	45	65
P12	male	65	AMC-ACS	DCM (-)	AP	No	GITI	6.1	Yes	SR, STDep II, III, aVF	62	47
P13	male	53	AMC-ACS	DCM (-)	AP, D	No	no	2.0	Yes	SR, LBBB	47	60
P14	male	58	AMC-DLVF	DCMi (+)	D, P	No	GITI	0.5	No	SR, VTach	43	58
P15	male	44	AMC-DLVF	DCMi (+)	D	No	RTI	0.23	No	SR, STDep II, III, aVF	45	64

Table 1. *Clinical characterization of patients presenting with AMC and DCM.* CSD: clinically suspected diagnosis (AMC: acute myocarditis; DCM: dilated cardiomyopathy). ACS: acute coronary syndrome. DLVF: depressed left ventricular function. AP: angina pectoris. D: dyspnea. P: palpitations. DCM (+/-): immunohistologically detected inflammatory DCM. RTI: respiratory tract infection. GITI: gastrointestinal tract infection. ECG: electrocardiography. SR: sinus rhythm, STElev: ST-segment elevation. STDep: ST-segment depression. negTW: negative T-waves. LBBB: left bundle branch block. Afib: atrial fibrillation. VTach: ventricular tachycardia. LVEF: left ventricular ejection fraction, determined by LV angiography. LVEDD: left ventricular enddiastolic diameter, determined by echocardiography.

Serological analyses

Sera were analyzed for B19V-specific antibodies by ELISA (Biotrin International, Dublin, Ireland) and recomLine® (Mikrogen GmbH, Neuried, Germany) [36].

Detection of B19V-specific $CD4^+$ T helper cell responses

B19V-like particles consisting of VP2 proteins (VP2-VLP) or both VP1 and VP2 proteins (VP1/2-VLP) were produced and purified as described previously [23,37]. The detection of B19V-specific IFN-γ mediated T-helper cell responses was performed by ELISpot [23]. Briefly, $2x10^5$ peripheral blood mononuclear cells (PBMC) were seeded on ELISPOT-plates (Millipore, Bedford, MA, USA) previously coated with 5 µg/ml of anti-human-IFN-γ monoclonal antibody D1K (Mabtech, Nacka Strand, Sweden) and stimulated with 5 µg/ml of VP2-VLP and VP1/2-VLP. As controls, cells were stimulated with 5 µg/ml of an irrelevant human immunodeficiency virus peptide and 5 ng/ml staphylococcus enterotoxin B (Sigma Aldrich, Hannover, Germany) [38]. For development, the plates were incubated with 1 µg/ml of biotinylated anti-IFN-γ 7-B6-1 antibody (Mabtech) and with streptavidin-alkaline phosphatase. Staining was performed using the NBT/BCIP Stock Solution (Roche Diagnostics GmbH, Mannheim, Germany).

Ethical consent

These investigations were approved by the ethics committees of the Charité (Universitätsmedizin Berlin, Sonderforschungsbereich TR19) and the University of Regensburg, Germany (Nr. 05/152). Informed consent was obtained from all patients.

Statistical analysis

Statistical analysis was performed using the Students-t test, the Mann-Whitney U-test for independent samples and the Pearson's r-test. A probability value of $p<0.05$ was considered statistically significant.

Results

Detection and quantification of B19V-DNA

B19V viral loads ranging from below 100 to $1.2x10^5$ geq/µg genomic DNA (median: 622 geq) were observed in the patients EMBs. In sera obtained from two AMC patients (P14, P15) and one DCM patient (P11) viral DNA loads ranging between 10^2-10^3 geq/ml were detected at initial clinical presentation (Table 2). In follow-up samples, B19V-DNA was detected in sera of P14 ($6.3x10^2$ geq/ml) and P15 ($4.4x10^3$ geq/ml). B19V-DNA was not detectable in sera obtained from healthy seropositive adults.

Patients		B19V-DNA			B19V-specific serology						IFN-γ secretion[2]	
N	Diagnosis	EMBs[1]	Serum		IgG ELISA [IU]		IgG-specificity		IgM-ELISA		VP2	VP1/2
			Initial	Follow-up	Initial	Follow-up	Initial	Follow-up	Initial	Follow-up		
P1	AMC	1,578	negative	negative	223	165	VP2p	VP2p	negative	negative	2	10
P2	AMC	193	negative	negative	202	156	VP2p, VP2l	VP2p, VP2l	negative	negative	5	9
P3	DCM	206	negative	negative	164	93	VP2p	n.p.	negative	negative	2	2
P4	DCM	622	negative	negative	67	31	VP2p	VP2p	negative	negative	16	18
P5	DCM	773	n.p.	negative	n.p.	48	n.p.	VP2p	n.p.	negative	41	36
P6	AMC	561	negative	negative	192	170	VP2p	VP2p	negative	negative	63	51
P7	DCM	4,044	negative	negative	211	198	VP2p	VP2p	negative	negative	25	30
P8	AMC	255	negative	negative	219	134	VP2p	VP2p	negative	negative	25	15
P9	DCM	127	negative	negative	196	215	VP2p	VP2p	negative	negative	13	15
P10	DCM	126	negative	negative	176	201	VP2p	VP2p	negative	negative	18	9
P11	DCM	59	10^2-10^3	negative	220	206	VP2p, NS1	VP2p, NS1	negative	negative	44	49
P12	AMC	447	n.p.	negative	n.p.	154	n.p.	VP2p	n.p.	negative	26	18
P13	AMC	2,048	negative	negative	79	34	VP2p	n.p.	negative	negative	21	18
P14	AMC	2,096	10^2-10^3	$4{,}4x10^3$	218	179	VP2p	VP2p	negative	negative	33	33
P15	AMC	115,091	10^2-10^3	10^2-10^3	146	274	VP2p, VP2l	VP2p, VP2l	positive	negative	27	30

Table 2. *Results of EMBs diagnostics and of B19V-specific adaptive immune responses.* Quantification of B19V genomes in EMBs and serum, and B19V specific humoral and T-cell mediated response. [1]B19V-DNA in EMBs and in serum as indicated in geq/µg genomic DNA and geq/ml, respectively. B19V IgG titers are presented in international units (IU). [2]B19V antigen-specific IFN-γ secretion indicated in spot-forming cells/$2x10^5$ PBMC. n.p.: not performed. IFN-γ: interferon-gamma. VP2p/l: IgG-specificity to conformational (VP2p) and linear (VP2l) epitopes of the B19V capsid proteins.

B19V serology

59% (30/51) of healthy individuals were tested positive for B19V-specific IgG by ELISA, all were IgM negative. IgG directed against conformational epitopes present in VP2 particles (VP2p) were detectable in all seropositive individuals, whereas antibodies recognizing linear VP2 epitopes (VP2l) and the NS1-protein were observed in 25% (7/28) and 11% (3/28) of samples, respectively. The median IgG titer was 224 IU (range: 65-286 IU).

Both the initial and the follow-up serum samples obtained from all AMC/DCM patients were positive for anti-B19V IgG with median titers of 196 IU (range: 67-223 IU) and 165 IU (range: 31-274 IU), respectively. Statistically significant differences between the patients and healthy controls were not evident. Furthermore, differences of median anti-B19V IgG-titers in AMC (initial sera: 202 IU; follow-up sera: 161 IU) and DCM patients (initial sera: 186 IU, follow-up sera: 198 IU) were not observed. IgG against VP2p were detected in all initial (13/13) and follow-up (13/13) samples. From two patients (P3, P13), follow-up serum volumes were not sufficient for recomLine® analysis. VP2l-specific IgG were observed in both initial and follow-up samples of AMC patients P2 and P15 (15%, 2/13). NS1-specific IgG were present in sera obtained from patient P11 (DCM; 7.7%, 1/13). All AMC/DCM patients except of P15 at initial presentation were anti-B19V-IgM negative (Table 2). No statistical significance was observed for LVEF (p=0.2256) and LVEDD (p=0.5714) when comparing AMC/DCM patients with or without indications for acute or persistent B19V-infection.

Analysis of B19V-specific T-cell mediated immune responses

IFN-γ immune responses with median values of 3 and 4 spot-forming cells (SFC)/2x10^5 PBMC were detected in seronegative healthy adults upon VP2-VLP and VP1/2-VLP stimulation, respectively (Figure 1). In seropositive controls, significantly increased respective median values of 32.5 (VP2-VLP) and 40 SFC/2x10^5 PBMC (VP1/2-VLP) were observed (both $p<0.0001$). At the time point of follow-up, increased antigen-specific IFN-γ secretion as compared to seronegative healthy controls was detectable in AMC patients with median values of 18 SFC/2x10^5 PBMC for both VP2-VLP and VP1/2-VLP (both $p<0.05$). In individuals suffering from DCM, median values of 25.5 (VP2-VLP, $p<0.05$) and 18 (VP1/2-VLP, p=0.0001) SFC/2x10^5 PBMC were obtained.

No correlation was observed between the B19V-DNA load determined in EMBs and the number of antigen-specific IFN-γ secreting cells after VP2-VLP (r=0.0479, p=0.8655) and

VP1/2-VLP (r=0.1387, p=0.6219) stimulation. In B19V-viremic patients, significantly increased values of IFN-γ secreting cells upon VP1/2 (p=0.4417) or VP2 (p=0.3429) stimulation were not evident as compared to non-viremic individuals.

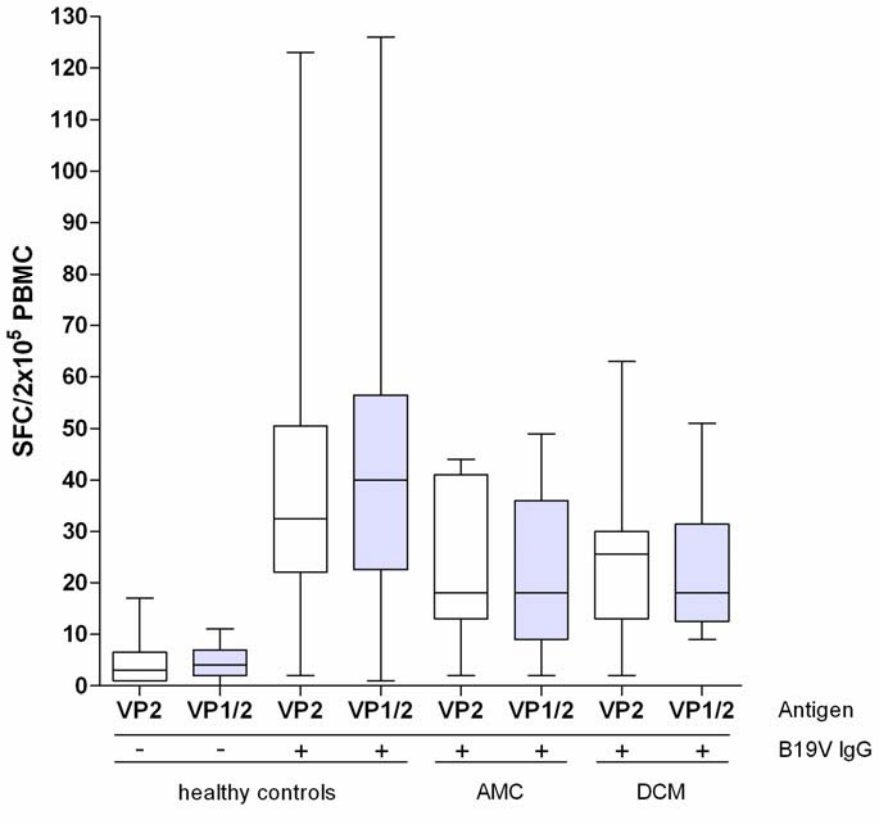

Figure 1. *T-cell responses against B19V capsid proteins VP2 and VP1.* PBMC isolated from blood from 21 and 30 B19V seronegative and -positive healthy individuals and of 15 AMC/DCM patients were subjected to stimulation with either VP2 or VP1/2 B19V-like particles for 60 h. The data show the median (drawn horizontal line) and the range of net IFN-γ spot-forming cell (SFC) values per $2x10^5$ PBMC as detected by the ELISPOT assay. B19V IgG: Anti-B19V IgG as detected by ELISA.

B19V-specific immune responses in immunohistologically confirmed DCMi patients with B19V genomes in EMBs

Immunohistological evaluation of EMBs confirmed DCMi in 10/15 (67%) of the investigated patients. The frequency of DCMi markers was statistically not different in AMC (63%) and DCM patients (71%). Virtually all investigated infiltrate phenotypes and CAMs showed significantly higher density and expression in EMBs with immunohistologically confirmed DCMi versus the remaining patients (Table 3).

Significant differences in T-cell responses upon stimulation with VP2-VLP and VP1/2-VLP, respectively, were not observed between patients with (25 vs. 21 SFC/2x10^5 PBMC; p=0.7233) or without DCMi (24 vs. 18 SFC/2x10^5 PBMC; p=0.7679).

Phenotype / CAMs	DCMi (-) (n=5 / 33%)	DCMi (+) (n=10 / 67%)	p-value
CD3/mm^2	3.3±2.5	11.5±4.8	0.0035
CD11a/LFA-1/mm^2	4.2±2.8	31.4±10.9	<0.0001
CD45R0/mm^2	5.3±4.1	21.6±8.0	0.001
CD11b/Mac-1/mm^2	20.2±14.8	42.8±14.9	0.0156
HLA class I AF	3.6±2.2	7.8±1.2	0.0003
CD54/ICAM-1 AF	0.7±0.4	2.6±0.9	0.0008
CD106/VCAM-1 AF	0.02±0.01	0.07±0.04	0.0185

Table 3. *Immunohistological detection of DCMi in EMBs.* Mean cell numbers/mm^2 in EMBs tissue and CAMs expression evaluation (unit: area fraction/AF) by digital image analysis in EMBs with immunohistological proof of DCMi (DCMi (+)) versus DCMi-negative (DCMi (-)) EMBs. The values are given as means±SD.

Discussion

B19V-genomes have been shown to persist in various human tissues over long periods of time in the absence of clinical symptoms and productive viral replication [8,9,11,39]. The biological and clinical relevance of B19V-DNA latency in myocardial tissue remains incompletely understood. Controversial results exist on the prognostic relevance of detectable amounts of B19V-genomes in EMBs of AMC/DCM [3,15,16]. Despite these data, the presence of B19V-DNA in myocardial tissue is frequently used as diagnostic parameter for the diagnosis of virus-associated heart disease. In order to investigate the role of B19V in AMC/DCM in more detail, we analyzed B19V-specific adaptive immune responses in healthy individuals and patients with detectable B19V-genomes in EMBs [23,36].

In 12 of 15 AMC/DCM patients (80%), no indications for acute B19V-infection were observed. In two AMC patients (P14, P15) and one DCM patient (P11) viremia was evident in initial and/or follow-up sera, pointing to a potential involvement of the virus in heart disease in these cases [40-43]. In patient P15, acute B19V-infection was confirmed by the additional presence of virus-specific IgM and IgG directed against linear VP2-epitopes

in sera. In patient P11, NS1-specific IgG suggesting prolonged or persistent B19V-infection, whereas only IgG directed against conformational VP2 epitopes indicating past immunity were present in P14. Together with the finding that viral DNA loads in EMBs of these patients were significantly lower as compared to P15, persistent rather than acute infections was considered [21,22].

The analysis of cellular immune responses against viral VP2- and VP1/2-VLP demonstrated the presence of antigen-specific T-cells in both seropositive controls and AMC/DCM patients. Despite detectable amounts of B19V-DNA in EMBs, the number of IFN-γ secreting PBMC was slightly lower in patients as compared to healthy adults. This may indicate a possible impairment of B19V-specific cellular immunity in AMC/DCM patients possibly contributing to an increased B19V susceptibility or favor B19V reactivations and/or re-infections in the myocardium (Figure 1, Table 2). Yet, AMC/DCM patients might also exhibit decreased cellular immune reactions against pathogens due to their generally impaired health status or due to their relatively high age. Similar features have been previously described for $CD4^+$ T-cell responses against the closely related human bocavirus [23].

In case detectable amounts of viral genomes in EMBs might reflect local virus replication and protein synthesis, a boost of antiviral adaptive immune reactions should be expected. However, a correlation between the B19V-DNA loads in EMBs and increased humoral or T-cell mediated immune responses was not observed. Signs for inflammatory reactions (DCMi) were observed in patients P14 and P15 by the immunohistological detection of $CD3^+$ cells infiltrating in cardiac tissue, yet, increased frequencies of B19V-specific T-cells were not observed.

In summary, we were not able to observe distinct differences in B19V-specific adaptive immune responses in patients with AMC/DCM as compared to healthy controls. Whereas viral genomes in EMBs that are combined with productive B19V-infections and viremia may rarely be associated with acute myocarditis, the data imply that the sole presence of latent B19V-DNA in myocardial tissues may be clinically irrelevant and therefore may possess insufficient diagnostic relevance for the association of B19V with AMC/DCM.

Acknowledgements

The work was supported by the German Research Foundation (DFG), grant Mo620/7-1, the DAAD (Deutscher Akademischer Auslandsdienst) grant PPP-Finnland D/04/04483 to SM, and the DFG through the Sonderforschungsbereich Transregio 19 “Inflammatory

Cardiomyopathy", projects B2 to MN and Z1 to UK and HPS. The excellent technical assistance of Irene Nebeja, Ingeborg Held and Anette Rohrhofer is appreciated.

References

[1] Bowles NE, Richardson PJ, Olsen EG, Archard LC. Detection of Coxsackie-B-virus-specific RNA sequences in myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy. Lancet 1986 May 17; 1(8490):1120-3.

[2] Dettmeyer R, Kandolf R, Baasner A, Banaschak S, Eis-Hubinger AM, Madea B. Fatal parvovirus B19 myocarditis in an 8-year-old boy. J Forensic Sci 2003 Jan; 48(1):183-6.

[3] Kuhl U, Pauschinger M, Seeberg B, Lassner D, Noutsias M, Poller W, et al. Viral persistence in the myocardium is associated with progressive cardiac dysfunction. Circulation 2005 Sep 27; 112(13):1965-70.

[4] Kuhl U, Pauschinger M, Schwimmbeck PL, Seeberg B, Lober C, Noutsias M, et al. Interferon-beta treatment eliminates cardiotropic viruses and improves left ventricular function in patients with myocardial persistence of viral genomes and left ventricular dysfunction. Circulation 2003 Jun 10; 107(22):2793-8.

[5] D'Ambrosio A, Patti G, Manzoli A, Sinagra G, Di Lenarda A, Silvestri F, et al. The fate of acute myocarditis between spontaneous improvement and evolution to dilated cardiomyopathy: a review. Heart 2001 May; 85(5):499-504.

[6] Noutsias M, Pauschinger M, Poller WC, Schultheiss HP, Kuhl U. Immunomodulatory treatment strategies in inflammatory cardiomyopathy: current status and future perspectives. Expert Rev Cardiovasc Ther 2004 Jan; 2(1):37-51.

[7] Mahrholdt H, Goedecke C, Wagner A, Meinhardt G, Athanasiadis A, Vogelsberg H, et al. Cardiovascular magnetic resonance assessment of human myocarditis: a comparison to histology and molecular pathology. Circulation 2004 Mar 16; 109(10):1250-8.

[8] Cassinotti P, Burtonboy G, Fopp M, Siegl G. Evidence for persistence of human parvovirus B19 DNA in bone marrow. J Med Virol 1997 Nov; 53(3):229-32.

[9] Soderlund M, von Essen R, Haapasaari J, Kiistala U, Kiviluoto O, Hedman K. Persistence of parvovirus B19 DNA in synovial membranes of young patients with and without chronic arthropathy. Lancet 1997 Apr 12; 349(9058):1063-5.

[10] Eis-Hubinger AM, Reber U, Abdul-Nour T, Glatzel U, Lauschke H, Putz U. Evidence for persistence of parvovirus B19 DNA in livers of adults. J Med Virol 2001 Oct; 65(2):395-401.

[11] Norja P, Hokynar K, Aaltonen LM, Chen R, Ranki A, Partio EK, et al. Bioportfolio: lifelong persistence of variant and prototypic erythrovirus DNA genomes in human tissue. Proc Natl Acad Sci U S A 2006 May 9; 103(19):7450-3.

[12] Bultmann BD, Klingel K, Nabauer M, Wallwiener D, Kandolf R. High prevalence of viral genomes and inflammation in peripartum cardiomyopathy. Am J Obstet Gynecol 2005 Aug; 193(2):363-5.

[13] Tschope C, Bock CT, Kasner M, Noutsias M, Westermann D, Schwimmbeck PL, et al. High prevalence of cardiac parvovirus B19 infection in patients with isolated left ventricular diastolic dysfunction. Circulation 2005 Feb 22; 111(7):879-86.

[14] Mahrholdt H, Wagner A, Deluigi CC, Kispert E, Hager S, Meinhardt G, et al. Presentation, patterns of myocardial damage, and clinical course of viral myocarditis. Circulation 2006 Oct 10; 114(15):1581-90.

[15] Caforio AL, Calabrese F, Angelini A, Tona F, Vinci A, Bottaro S, et al. A prospective study of biopsy-proven myocarditis: prognostic relevance of clinical and aetiopathogenetic features at diagnosis. Eur Heart J 2007 Jun; 28(11):1326-33.

[16] Kuethe F, Sigusch HH, Hilbig K, Tresselt C, Gluck B, Egerer R, et al. Detection of viral genome in the myocardium: lack of prognostic and functional relevance in patients with acute dilated cardiomyopathy. Am Heart J 2007 May; 153(5):850-8.

[17] Rohrer C, Gartner B, Sauerbrei A, Bohm S, Hottentrager B, Raab U, et al. Seroprevalence of parvovirus B19 in the German population. Epidemiol Infect 2008 Jan 16;1-12.

[18] Anderson MJ, Lewis E, Kidd IM, Hall SM, Cohen BJ. An outbreak of erythema infectiosum associated with human parvovirus infection. J Hyg (Lond) 1984 Aug; 93(1):85-93.

[19] Young NS, Brown KE. Parvovirus B19. N Engl J Med 2004 Feb 5; 350(6):586-97.

[20] Soderlund M, Brown CS, Spaan WJ, Hedman L, Hedman K. Epitope type-specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J Infect Dis 1995 Dec; 172(6):1431-6.

[21] Hemauer A, Gigler A, Searle K, Beckenlehner K, Raab U, Broliden K, et al. Seroprevalence of parvovirus B19 NS1-specific IgG in B19-infected and uninfected individuals and in infected pregnant women. J Med Virol 2000 Jan; 60(1):48-55.

[22] von Poblotzki A, Hemauer A, Gigler A, Puchhammer-Stockl E, Heinz FX, Pont J, et al. Antibodies to the nonstructural protein of parvovirus B19 in persistently infected patients: implications for pathogenesis. J Infect Dis 1995 Nov; 172(5):1356-9.

[23] Lindner J, Zehentmeier S, Franssila R, Schroder J, Barabas S, Deml L, et al. CD4+ T Helper Cell Responses Against Human Bocavirus VP2 Virus-like Particles in Healthy Adults. J Infect Dis 2008. In press.

[24] Franssila R, Auramo J, Modrow S, Mobs M, Oker-Blom C, Kapyla P, et al. T helper cell-mediated interferon-gamma expression after human parvovirus B19 infection: persisting VP2-specific and transient VP1u-specific activity. Clin Exp Immunol 2005 Oct; 142(1):53-61.

[25] Lindner J, Barabas S, Saar K, Altmann D, Pfister A, Fleck M, et al. CD4(+) T-cell responses against the VP1-unique region in individuals with recent and persistent parvovirus B19 infection. J Vet Med B Infect Dis Vet Public Health 2005 Sep; 52(7-8):356-61.

[26] Tolfvenstam T, Oxenius A, Price DA, Shacklett BL, Spiegel HM, Hedman K, et al. Direct ex vivo measurement of CD8(+) T-lymphocyte responses to human parvovirus B19. J Virol 2001 Jan; 75(1):540-3.

[27] Escher F, Modrow S, Sabi T, Kuhl U, Lassner D, Schultheiss HP, et al. Parvovirus B19 profiles in patients presenting with acute myocarditis and chronic dilated cardiomyopathy. Med Sci Monit. 2008. In press.

[28] Noutsias M, Seeberg B, Schultheiss HP, Kuhl U. Expression of cell adhesion molecules in dilated cardiomyopathy: evidence for endothelial activation in inflammatory cardiomyopathy. Circulation 1999 Apr 27; 99(16):2124-31.

[29] Angelini A, Calzolari V, Calabrese F, Boffa GM, Maddalena F, Chioin R, et al. Myocarditis mimicking acute myocardial infarction: role of endomyocardial biopsy in the differential diagnosis. Heart 2000 Sep; 84(3):245-50.

[30] Wojnicz R, Nowalany-Kozielska E, Wojciechowska C, Glanowska G, Wilczewski P, Niklewski T, et al. Randomized, placebo-controlled study for immunosuppressive treatment of inflammatory dilated cardiomyopathy: two-year follow-up results. Circulation 2001 Jul 3; 104(1):39-45.

[31] Kuhl U, Pauschinger M, Bock T, Klingel K, Schwimmbeck CP, Seeberg B, et al. Parvovirus B19 infection mimicking acute myocardial infarction. Circulation 2003 Aug 26; 108(8):945-50.

[32] Liefeldt L, Plentz A, Klempa B, Kershaw O, Endres AS, Raab U, et al. Recurrent high level parvovirus B19/genotype 2 viremia in a renal transplant recipient analyzed by real-time PCR for simultaneous detection of genotypes 1 to 3. J Med Virol 2005 Jan; 75(1):161-9.

[33] Knoll A, Louwen F, Kochanowski B, Plentz A, Stussel J, Beckenlehner K, et al. Parvovirus B19 infection in pregnancy: quantitative viral DNA analysis using a kinetic fluorescence detection system (TaqMan PCR). J Med Virol 2002 Jun; 67(2):259-66.

[34] Aretz HT. Myocarditis: the Dallas criteria. Hum Pathol 1987 Jun; 18(6):619-24.

[35]Noutsias M, Pauschinger M, Ostermann K, Escher F, Blohm JH, Schultheiss HP, et al. Digital image analysis system for the quantification of infiltrates and cell adhesion molecules in inflammatory cardiomyopathy. Med Sci Monit 2002; 8:MT59-MT71.

[36] Pfrepper KI, Enders M, Motz M. Human parvovirus B19 serology and avidity using a combination of recombinant antigens enables a differentiated picture of the current state of infection. J Vet Med B Infect Dis Vet Public Health 2005 Sep; 52(7-8):362-5.

[37] Kaufmann B, Baxa U, Chipman PR, Rossmann MG, Modrow S, Seckler R. Parvovirus B19 does not bind to membrane-associated globoside in vitro. Virology 2005 Feb 5; 332(1):189-98.

[38] Wild J, Bojak A, Deml L, Wagner R. Influence of polypeptide size and intracellular sorting on the induction of epitope-specific CTL responses by DNA vaccines in a mouse model. Vaccine 2004 Apr 16; 22(13-14):1732-43.

[39] Donoso MO, Meyer R, Prosch S, Nitsche A, Leitmeyer K, Kallies R, et al. High prevalence of cardiotropic viruses in myocardial tissue from explanted hearts of heart transplant recipients and heart donors: a 3-year retrospective study from a German patients' pool. J Heart Lung Transplant 2005 Oct; 24(10):1632-8.

[40] Jonetzko P, Graziadei I, Nachbaur K, Vogel W, Pankuweit S, Zwick R, et al. Fatal course of parvovirus B19-associated myocarditis in a female liver transplant recipient. Liver Transpl 2005 Apr; 11(4):463-6.

[41] Gutersohn A, Zimmermann U, Bartel T, Erbel R. A rare case of acute 'infective' myocardial infarction triggered by acute parvovirus B19 myocarditis. Nat Clin Pract Cardiovasc Med 2005 Mar; 2(3):167-71.

[42] Lamparter S, Schoppet M, Pankuweit S, Maisch B. Acute parvovirus B19 infection associated with myocarditis in an immunocompetent adult. Hum Pathol 2003 Jul; 34(7):725-8.

[43] Enders G, Dotsch J, Bauer J, Nutzenadel W, Hengel H, Haffner D, et al. Life-threatening parvovirus B19-associated myocarditis and cardiac transplantation as possible therapy: two case reports. Clin Infect Dis 1998 Feb; 26(2):355-8.

5.5. Publikation VII: Human Bocavirus – A Novel Parvovirus to Infect Humans

Review

Intervirology

Intervirology 2008;51:116–122
DOI: 10.1159/000137411

Received: January 9, 2008
Accepted: April 2, 2008
Published online: June 9, 2008

Human Bocavirus – A Novel Parvovirus to Infect Humans

Juha Lindner Susanne Modrow

Institute of Medical Microbiology and Hygiene, University of Regensburg, Regensburg, Germany

Key Words
Human bocavirus · Parvoviridae · Respiratory tract infection

Abstract
For almost three decades parvovirus B19 has been described as the only member of the Parvoviridae to infect and cause illness in humans. This statement was correct until 2005 when a group of Swedish scientists identified a previously uncharacterized virus in pools of human nasopharyngeal aspirates obtained from individuals suffering from diseases of the respiratory tract. Comprehensive sequence and phylogenetic analysis allowed the identification of the new virus as a member of the Parvoviridae. Based on its close relation to the minute virus of canines and the bovine parvovirus, it was named human bocavirus (HBoV). Since the identification of HBoV, viral genomes have been frequently detected worldwide in nasopharyngeal swabs, serum and fecal samples almost exclusively derived from young children with various symptoms of the respiratory or the gastrointestinal tract. The detection of HBoV genomes tends to be associated with elevated rates of coinfections with further respiratory viruses, e.g. respiratory syncytial virus or metapneumovirus. First studies on virus-specific immune responses have described the presence of ubiquitous humoral and cellular immune reactions against HBoV in adults and adolescents, indicating a high seroprevalence of this new virus in humans.

Human Bocavirus and the Parvoviridae

The Parvoviridae represent a large family of viruses that is divided into two subfamilies, the Parvovirinae and the Densovirinae. Whereas members of the Densovirinae infect exclusively arthropod and insect hosts, parvoviruses known to cause infection in vertebrates are merged in the Parvovirinae and are further subdivided into four genera: Parvovirus, Erythrovirus, Amdovirus and Bocavirus (table 1). Besides HBoV, two additional parvoviruses, termed parvovirus B19 (B19V) and human parvovirus (PARV) 4, are currently known to infect humans. An additional genotype of PARV4, termed PARV5, has been recently identified [1, 2]. B19V infections frequently result in the childhood rash disease of erythema infectiosum, transient arthritis and anemia due to the narrow tropism of B19V for erythrocyte progenitors of specific differentiation state. In addition, a broad range of additional illnesses have been associated with B19V infection [3, 4]. In contrast, no distinct clinical symptoms have been confirmed for infections of PARV4 and PARV5.

Molecular Characteristics of HBoV

Parvoviruses are characterized by small-sized, icosahedral non-enveloped particles (diameter 20–26 nm), a linear single-stranded DNA genome, and by their depen-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2008 S. Karger AG, Basel
0300–5526/08/0512–0116$24.50/0

Accessible online at:
www.karger.com/int

Susanne Modrow
Institute of Medical Microbiology and Hygiene, University of Regensburg
Franz-Josef-Strauss Allee 11, DE–93053 Regensburg (Germany)
Tel. +49 941 944 6454, Fax +49 941 944 6402
E-Mail susanne.modrow@klinik.uni-regensburg.de

Table 1. Taxonomy of the Parvoviridae

Subfamily	Genus	Human species	Veterinary species
Parvovirinae	Parvovirus		Feline parvovirus Minute virus of canines Porcine parvovirus Minute virus of mice
	Bocavirus	Human bocavirus	Canine minute virus Bovine parvovirus
	Amdovirus		Aleutian mink disease virus
	Erythrovirus	Parvovirus B19 (3 genotypes) PARV4/5	Parvovirus of cynomolgus monkeys Rhesus parvovirus Pig-tailed macaque parvovirus
	Dependovirus	Adeno-associated virus 2/3/5	Adeno-associated virus 1/4 Bovine adeno-associated virus
Densovirinae	Densovirus Iteravirus Brevidensovirus		Culex pipiens densovirus Bombyx mori densovirus Aedes aegyptii densovirus

The taxonomic classification of PARV4 and -5 remains to be confirmed. BPV = Bovine parvovirus.

dence to replicate exclusively in dividing cells. The viral genome of HBoV encompasses 5,217 nucleotides (Stockholm 1 isolate, DQ000495) and shows an organization similar to that of other members of the Parvoviridae with information for nonstructural and structural proteins in the 5′ and 3′ half of the genome, respectively (fig. 1). Several isolates of HBoV with high sequence homology have been described [5]. Herein, the nonstructural genes were shown to represent conserved regions of the viral genome, while variations were preferentially observed in the genes encoding the capsid proteins.

Parvoviruses encode two structural proteins VP1 and VP2, which are identical in sequence and only differ in the amino-terminal extension of VP1, termed VP1-unique region (VP1u). The size of this domain varies and encompasses 129 and 227 amino acids in HBoV and B19V, respectively. In HBoV and many other parvoviruses, e.g. B19V [6] and the minute virus of canines (MVC) [7], the VP1u possesses a phospholipase A_2-like activity (PLA_2) [8]. This viral PLA_2 has been shown to be of crucial importance for parvoviral infectivity as it is thought to mediate the transfer of the viral genome from endocytic compartments to the nucleus of host cells [7]. During productive B19V infection, the enzyme activity is furthermore associated with the production of anti-phospholipid antibodies [9–11].

In addition to the structural components, parvoviruses encode a limited number of regulatory nonstructural

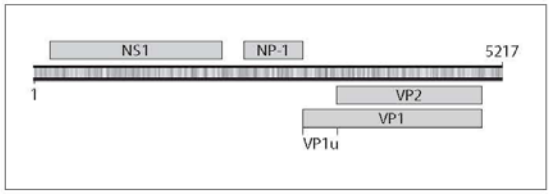

Fig. 1. Genome organization of HBoV ST1 isolate.

proteins. HBoV and animal bocaviruses generally possess the genetic information for two nonstructural proteins, NS1 and NP1 [12, 13], whereas B19V encodes one single major nonstructural protein NS1 [14]. Until today, detailed data on the functional activities associated with the NS1 and NP1 proteins of HBoV are not available. However, the NS1 protein of human B19V has been shown to posses numerous regulatory functions such as transactivation of viral and cellular promoters and induction of apoptosis [15–19]. The NS1 protein of HBoV might exhibit similar functions, as multiple conserved domains can be observed in its amino acid sequence when compared to the NS1 protein of B19V.

A further common feature of parvoviruses is the exceptional stability and the structural simplicity of the small-sized virions (Lat. *parvus* = small) [20, 21]. Recent-

Table 2. Prevalence of HBoV infection and associated clinical symptoms in young children

Study subjects	HBoV DNA, %	Sample type	Clinical symptoms/diagnosis	Country of study
962	0.8	stool	acute gastroenteritis	Korea [33]
1,435	2.1	stool	acute gastroenteritis	Hong Kong [32]
257	2.7	RS	RTI, fever, cough	China [24]
540	3.1	RS	wheezing, bronchitis/asthma, chronic lung disease	Sweden [12]
512	3.9	RS	pneumonia	Thailand [43]
112	4.5	RS	acute RTI	Switzerland [56]
315	4.8	RS	acute RTI	Australia [66]
1,265	5.1	RS	cough, fever, rhinitis, bronchiolitis, pneumonia	Canada [31]
425	5.2	RS	RTI, fever, cough, rhinorrhea, diarrhea	USA [36]
733	5.5	RS, stool	fever, cough, rhinorrhea, dyspnea, diarrhea	France [26]
1,474	5.6	RS	cough, rhinorrhea, fever, diarrhea	USA [39]
318	5.7	RS	bronchitis, bronchiolitis, pneumonia, asthma	Japan [69]
1,200	6.9	RS	upper or lower RTI	Hong Kong [32]
252	7.2	RS	RTI	Thailand [5]
520	7.7	RS	RTI	Spain [34]
261	8	RS	fever, wheezing, otitis media, cough, sore throat	Iran [28]
574	8.2	RS	RTI	UK [44]
527	9.1	stool	gastroenteritis	Spain [34]
835	10.3	RS	acute upper and lower RTI	Germany [38]
341	11	RS	RTI	South Africa [30]
515	11.3	RS	fever, wheezing, bronchiolitis, pneumonia, asthma	Korea [41]
231	11.3	RS	upper RTI	Israel [48]
94	12.8	RS	lower RTI	Germany [27]
917	13.4	RS, stool/urine	lower RTI	Spain [35]
231	13.8	RS	acute wheezing	Korea [40]
50	14	RS	asthma	France [67]
312	18.3	RS	acute RTI	Jordan [68]
259	19	RS, serum	acute wheezing	Sweden [25]

RS = Respiratory samples; RTI = respiratory tract infection.

ly, HBoV particles were detected by electron microscopy in nasopharyngeal aspirates of viremic individuals [22]. Herein and by the recombinant generation of virus-like particles consisting of VP2 capsid proteins [23] HBoV was shown to share typical structural characteristics with other parvoviruses with an approximate capsid diameter of 21–25 nm.

HBoV Epidemiology

Since its discovery, HBoV has been frequently detected worldwide in nasopharyngeal, serum, fecal and urine samples obtained mainly from young children (table 2). In infants with respiratory tract disease, the prevalence of HBoV infection has been described to vary considerably between 2.7–19% [24, 25], with most virus-positive individuals being younger than 2 years of age [25–31]. These variations in HBoV prevalence may possibly be caused by seasonal fluctuations of the infection or by differences in populations that were included into the respective studies. In addition, viral DNA has been detected in 0.8–9.1% of fecal specimens obtained from individuals with acute gastroenteritis [32–34] and in urine samples [35].

HBoV infections have been predominantly detected during the winter season [12, 32, 35–38], yet single studies report increased numbers of HBoV infections in spring or summer [39–41]. HBoV DNA loads have been described to range between <500 to 5.6×10^{10} and $<10^3$ to 5.9×10^5 copies/ml in nasopharyngeal aspirates and serum samples, respectively [24, 25, 27]. The fact that HBoV

may be routinely detected in sera of infected individuals indicates the establishment of a systemic infection, like it is known for most veterinary parvoviruses and B19V [5, 42].

To date, the majority of performed analyses have focused on the detection of HBoV DNA in symptomatic individuals. Therefore, only limited data are available on the prevalence of HBoV in asymptomatic healthy children and adults. Some studies included asymptomatic age-matched children and HBoV genomes were detected in 3/280 (1%) and 4/62 (6.5%) of them [Karalar et al., in preparation; 43]. No viral DNA was observed in 96 healthy control individuals studied by Kesebir et al. [36].

Clinical Manifestations of HBoV Infection

HBoV infections have been frequently detected in young children around the age of 2 years with acute diseases of the upper and lower respiratory tract, e.g. pneumonia, bronchiolitis and wheezing (table 2). Lung infiltrates and abnormal radiologic findings indicating lower respiratory tract infection have been described [36, 39]. Further symptoms including cough, fever, rhinitis and, more rarely, conjunctivitis or rashes were observed in HBoV-infected individuals [39]. In adults, acute HBoV infections seem to be rare and have been currently detected only in single immunocompromised and immunocompetent individuals [44, 45]. As with children, the majority of adults with HBoV infection presented with acute respiratory symptoms.

Using sensitive PCR methods, HBoV genomes have been detected in respiratory aspirates and in the peripheral blood of infected patients. Recently, the presence of elevated viral loads in nasopharyngeal aspirates (>10^4 genome copies/milliliter) has been suggested to correlate with symptomatic HBoV infection, whereas low viral loads (<10^4 genome copies/milliliter) may represent viral persistence [25]. These data are in contrast to those published by Kleines et al. [27], who could not find a relation between the viral load and the severity of HBoV-associated illness, indicating that further work is necessary to study the influence of the viral load on disease manifestation. In addition to respiratory symptoms, HBoV is currently discussed to be associated with gastroenteritic disease [32–34]. Similar features are known from veterinary infections with the closely related bovine parvoviruses (BPV) and MVC, which are known to induce gastric illness in their respective hosts [46, 47].

Table 3. Viral and bacterial coinfections in HBoV-positive individuals

Pathogen	Rate of coinfection, %	References
HRV	9.8–46.7	[25, 32, 35, 40, 43, 56, 66]
HAdV	5.9–69.2	[12, 25, 28, 30, 31, 35, 41, 44, 48, 56, 66, 68]
RSV	1.3–41.7	[12, 25, 27, 28, 30, 35, 38, 40, 41, 44, 43, 48, 56, 66–68]
HPEV	13.30	[66]
HHV-6	6.70	[66]
HEV	2.5–14.3	[25, 32, 66]
IFVA/B	1.8–10.4	[28, 31, 38, 44, 68]
HPIV	1.9–10	[30, 41, 43, 44, 48]
HCoV-NL63	2.6–20	[30, 31, 56]
HMPV	1.8–14.3	[32, 41, 67, 68]
HRoV	2.5–10.4	[32, 33]
EBV	2.50	[32]
HSV	2.50	[32]
Bacterial coinfections	2.5–13.2	[30, 32, 39, 68]

HRV = Human rhinovirus; HAdV = human adenovirus; RSV = respiratory syncytial virus; HPEV = human parechovirus; HHV-6 = human herpesvirus 6; HEV = human enterovirus; IFVA/B = influenza viruses A/B; HPIV = human parainfluenza virus; HCoV = human coronavirus; HMPV = human metapneumovirus; HRoV = human rotavirus; EBV = Epstein-Barr virus; HSV = herpes simplex virus.

HBoV infections tend to be linked with high rates of coinfections with other viral and bacterial respiratory pathogens, e.g. human rhinovirus, adenovirus, respiratory syncytial virus, and *Streptococcus* spp. (table 3). Thereby, coinfecting pathogens have been found in up to 69.2% of HBoV DNA-positive individuals [48]. A prospective study recently performed by our group observed coinfections in 21% of children with HBoV viremia. Together with the fact that the majority of epidemiological studies have been performed retrospectively and long-term follow-up studies with detailed clinical characterization of symptomatic individuals are rare, it is difficult to clearly determine HBoV as the sole infectious agent of human respiratory illnesses.

Infections caused by animal bocaviruses, e.g. MVC and BPV, have been described to cause fetal infections leading to reproductive failure and hydrops fetalis [49–53]. Similar problems are well known to be associated with B19V infections in pregnant women [4]. This raises the question whether also HBoV has the capacity to be

vertically transmitted to the fetus. However, analysis of more than 100 amniotic fluid samples from pregnant women with hydrops fetalis caused by unknown reasons did not reveal detectable amounts of HBoV genomes. Yet, as primary HBoV infections predominantly occur in young children and are rare in adults due to the presence of an ubiquitous virus-specific immunity [23, 54], a potential effect of acute HBoV infections on pregnancy cannot be entirely excluded.

Diagnosis of HBoV Infection

To date, diagnosis of HBoV infection is based on the PCR amplification of viral genome fragments present in human respiratory, serum, stool and urine samples. A great number of different PCR techniques employing varying sets of primers specific for the viral genes NP1 [12, 55], NS1 [44, 56], and VP1/2 [31, 33] have been described. In addition to the detection of viral genomes by PCR, recent reports describe the detection of HBoV-specific antibodies in serum samples using Western blot [57] or immunofluorescence assays [54]. Furthermore, a standardized ELISA for the quantitative determination of HBoV-specific antibodies has been established by our group. No cell culture systems for the in vitro replication of HBoV have been described to date.

In total, the methodological heterogeneity used for diagnosis of HBoV infection raises questions about the specificity and comparability of the described results, highlighting the urgent need for internationally standardized diagnostic guidelines and reference samples for the detection of HBoV genomes and virus-specific immune responses in human samples.

Immune Responses against HBoV

Using an immunofluorescence assay, ubiquitous antibody responses against the viral capsid protein VP1 have been detected in up to 94.7% of adult individuals and children older than 2 years of age, while seronegative individuals were mainly detected among infants around 1 year of age [54]. An ELISA assay based on the use of recombinant HBoV VP2 virus-like particles (VLP) as antigen for the detection of HBoV-specific antibodies in human serum samples was established [Lindner et al., in preparation]. Thereby, IgG_1 subclass antibodies against HBoV VP2-VLP were observed to be ubiquitously detectable in up to 98.3% of samples obtained from healthy adult blood donors. Virus-specific IgG were also detected in infants with active viremia and respiratory illness (41.7%) and in young children without detectable HBoV genomes in blood (51.9%). IgM antibodies were present in 41.7% of sera from HBoV DNA-positive children, whereas HBoV-specific IgM were not observed in samples obtained from children without detectable amounts of HBoV genomes in blood.

Kantola et al. [57] describe the detection of both IgG- and IgM-antibodies against HBoV VP2 by Western blot analysis in 73% and 49% of samples, respectively, obtained from HBoV-infected children suffering from respiratory diseases. Antibodies against the VP1u region of HBoV were detected rarely: only 7% (IgG) and 2% (IgM) of the patients showed positive results. In contrast to data provided by Endo and colleagues [54] and by our group, the prevalence of HBoV-specific IgG was shown to decline with increasing age. This finding may be based on the maturation of IgG specificity during the time period of up to 6 months following acute infection. This process has been well described for B19V infections, where antibodies against linear epitopes are replaced by those preferentially recognizing conformational structures [58–61]. It may be assumed that similar changes take place during HBoV infections.

In addition to humoral reactions, the presence of HBoV cellular immune responses in healthy individuals supports a high prevalence of HBoV-specific immunity in adults. Thereby, frequent interferon-γ-mediated CD4+ T helper cell reactions were observed against HBoV VLP consisting of VP2 proteins [23]. Similar data have been previously found for B19V-specific cellular immune responses [62–65].

Conclusion

Despite the fact that HBoV was detected only 3 years ago, data on the epidemiology and clinics are accumulating. Based on current data, it seems most likely that HBoV represents the second member of the Parvoviridae pathogenic for humans and may be associated with respiratory and potential gastrointestinal symptoms in mainly children around 2 years of age. The seroprevalence is high in older children and reaches values of more than 95% in adults. Acute HBoV infections are often observed to be accompanied by other infectious agents of both the respiratory and gastrointestinal tract. Therefore, as only a limited number of prospective studies have been published, a final statement on HBoV as the causative agent of infec-

tious disease in children cannot yet be given. However, first data obtained from healthy individuals and children with symptoms of noninfectious disease indicate distinctly lower rates of HBoV viremia as compared to those found in patients with respiratory tract disease. As serological diagnostics of HBoV infection will become more important in the future, standardized viral DNA and antibody specimen should be provided as a basis to establish comparable test systems.

References

1 Fryer JF, Delwart E, Hecht FM, Bernardin F, Jones MS, Shah N, Baylis SA: Frequent detection of the parvoviruses, PARV4 and PARV5, in plasma from blood donors and symptomatic individuals. Transfusion 2007;47:1054–1061.

2 Fryer JF, Kapoor A, Minor PD, Delwart E, Baylis SA: Novel parvovirus and related variant in human plasma. Emerg Infect Dis 2006;12:151–154.

3 Kerr JR, Modrow S: Human and Primate Erythrovirus Infections and Associated Disease. London, Hodder Arnold, 2006, pp 385–416.

4 Young NS, Brown KE: Parvovirus B19. N Engl J Med 2004;350:586–597.

5 Chieochansin T, Chutinimitkul S, Payungporn S, Hiranras T, Samransamruajkit R, Theamboolers A, Poovorawan Y: Complete coding sequences and phylogenetic analysis of Human Bocavirus (HBoV). Virus Res 2007;129:54–57.

6 Dorsch S, Liebisch G, Kaufmann B, Von Landenberg P, Hoffmann JH, Drobnik W, Modrow S: The VP1 unique region of parvovirus B19 and its constituent phospholipase A2-like activity. J Virol 2002;76:2014–2018.

7 Suikkanen S, Antila M, Jaatinen A, Vihinen-Ranta M, Vuento M: Release of canine parvovirus from endocytic vesicles. Virology 2003;316:267–280.

8 Qu XW, Duan ZJ, Qi ZY, Xie ZP, Gao HC, Liu WP, Huang CP, Peng FW, Zheng LS, Hou YD: Human bocavirus infection, People's Republic of China. Emerg Infect Dis 2007;13:165–168.

9 Tzang BS, Tsay GJ, Lee YJ, Li C, Sun YS, Hsu TC: The association of VP1 unique region protein in acute parvovirus B19 infection and anti-phospholipid antibody production. Clin Chim Acta 2007;378:59–65.

10 Von Landenberg P, Lehmann HW, Modrow S: Human parvovirus B19 infection and antiphospholipid antibodies. Autoimmun Rev 2007;6:278–285.

11 Von Landenberg P, Lehmann HW, Knoll A, Dorsch S, Modrow S: Antiphospholipid antibodies in pediatric and adult patients with rheumatic disease are associated with parvovirus B19 infection. Arthritis Rheum 2003;48:1939–1947.

12 Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B: Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci USA 2005;102:12891–12896.

13 Chen KC, Shull BC, Moses EA, Lederman M, Stout ER, Bates RB: Complete nucleotide sequence and genome organization of bovine parvovirus. J Virol 1986;60:1085–1097.

14 Shade RO, Blundell MC, Cotmore SF, Tattersall P, Astell CR: Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. J Virol 1986;58:921–936.

15 Mitchell LA: Parvovirus B19 nonstructural (NS1) protein as a transactivator of interleukin-6 synthesis: common pathway in inflammatory sequelae of human parvovirus infections? J Med Virol 2002;67:267–274.

16 Raab U, Beckenlehner K, Lowin T, Niller HH, Doyle S, Modrow S: NS1 protein of parvovirus B19 interacts directly with DNA sequences of the p6 promoter and with the cellular transcription factors Sp1/Sp3. Virology 2002;293:86–93.

17 Raab U, Bauer B, Gigler A, Beckenlehner K, Wolf H, Modrow S: Cellular transcription factors that interact with p6 promoter elements of parvovirus B19. J Gen Virol 2001;82:1473–1480.

18 Moffatt S, Yaegashi N, Tada K, Tanaka N, Sugamura K: Human parvovirus B19 nonstructural (NS1) protein induces apoptosis in erythroid lineage cells. J Virol 1998;72:3018–3028.

19 Gareus R, Gigler A, Hemauer A, Leruez-Ville M, Morinet F, Wolf H, Modrow S: Characterization of cis-acting and NS1 protein-responsive elements in the p6 promoter of parvovirus B19. J Virol 1998;72:609–616.

20 Kaufmann B, Simpson AA, Rossmann MG: The structure of human parvovirus B19. Proc Natl Acad Sci USA 2004;101:11628–11633.

21 Boschetti N, Wyss K, Mischler A, Hostettler T, Kempf C: Stability of minute virus of mice against temperature and sodium hydroxide. Biologicals 2003;31:181–185.

22 Brieu N, Gay B, Segondy M, Foulongne V: Electron microscopy observation of human bocavirus (HBoV) in nasopharyngeal samples from HBoV-infected children. J Clin Microbiol 2007;45:3419–3420.

23 Lindner J, Zehentmeier S, Franssila R, Schroeder J, Barabas S, Deml L, Modrow S: CD4+ T helper cell responses against human bocavirus VP2 virus-like particles in healthy adults. J Infect Dis, in press.

24 Lin F, Zeng A, Yang N, Lin H, Yang E, Wang S, Pintel D, Qiu J: Quantification of human bocavirus in lower respiratory tract infections in China. Infect Agent Cancer 2007;2:3.

25 Allander T, Jartti T, Gupta S, Niesters HG, Lehtinen P, Osterback R, Vuorinen T, Waris M, Bjerkner A, Tiveljung-Lindell A, van den Hoogen BG, Hyypia T, Ruuskanen O: Human bocavirus and acute wheezing in children. Clin Infect Dis 2007;44:904–910.

26 Catalano-Pons C, Bue M, Laude H, Cattan F, Moulin F, Menager C, Cosnes-Lambe C, Chalumeau M, Giraud C, Meritet JF, Rozenberg F, Lebon P, Gendrel D: Human bocavirus infection in hospitalized children during winter. Pediatr Infect Dis J 2007;26:959–960.

27 Kleines M, Scheithauer S, Rackowitz A, Ritter K, Hausler M: High prevalence of human bocavirus detected in young children with severe acute lower respiratory tract disease by use of a standard PCR protocol and a novel real-time PCR protocol. J Clin Microbiol 2007;45:1032–1034.

28 Naghipour M, Cuevas LE, Bakhshinejad T, Dove W, Hart CA: Human bocavirus in Iranian children with acute respiratory infections. J Med Virol 2007;79:539–543.

29 Volz S, Schildgen O, Klinkenberg D, Ditt V, Muller A, Tillmann RL, Kupfer B, Bode U, Lentze MJ, Simon A: Prospective study of human bocavirus (HBoV) infection in a pediatric university hospital in Germany 2005/2006. J Clin Virol 2007;40:229–235.

30 Smuts H, Hardie D: Human bocavirus in hospitalized children, South Africa. Emerg Infect Dis 2006;12:1457–1458.

31 Bastien N, Chui N, Robinson JL, Lee BE, Dust K, Hart L, Li Y: Detection of human bocavirus in Canadian children in a 1-year study. J Clin Microbiol 2007;45:610–613.

32 Lau SK, Yip CC, Que TL, Lee RA, Au-Yeung RK, Zhou B, So LY, Lau YL, Chan KH, Woo PC, Yuen KY: Clinical and molecular epidemiology of human bocavirus in respiratory and fecal samples from children in Hong Kong. J Infect Dis 2007;196:986–993.

33 Lee JI, Chung JY, Han TH, Song MO, Hwang ES: Detection of human bocavirus in children hospitalized because of acute gastroenteritis. J Infect Dis 2007;196:994–997.

34 Vicente D, Cilla G, Montes M, Perez-Yarza EG, Perez-Trallero E: Human bocavirus, a respiratory and enteric virus. Emerg Infect Dis 1007;13:636–637.

35 Pozo F, Garcia-Garcia ML, Calvo C, Cuesta I, Perez-Brena P, Casas I: High incidence of human bocavirus infection in children in Spain. J Clin Virol 2007;40:224–228.

36 Kesebir D, Vazquez M, Weibel C, Shapiro ED, Ferguson D, Landry ML, Kahn JS: Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis 2006;194:1276–1282.

37 Manning A, Willey SJ, Bell JE, Simmonds P: Comparison of tissue distribution, persistence, and molecular epidemiology of parvovirus B19 and novel human parvoviruses PARV4 and human bocavirus. J Infect Dis 2007;195:1345–1352.

38 Weissbrich B, Neske F, Schubert J, Tollmann F, Blath K, Blessing K, Kreth HW: Frequent detection of bocavirus DNA in German children with respiratory tract infections. BMC Infect Dis 2006;6:109.

39 Arnold JC, Singh KK, Spector SA, Sawyer MH: Human bocavirus: prevalence and clinical spectrum at a children's hospital. Clin Infect Dis 2006;43:283–288.

40 Chung JY, Han TH, Kim SW, Kim CK, Hwang ES: Detection of viruses identified recently in children with acute wheezing. J Med Virol 2007;79:1238–1243.

41 Choi EH, Lee HJ, Kim SJ, Eun BW, Kim NH, Lee JA, Lee JH, Song EK, Kim SH, Park JY, Sung JY: The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000–2005. Clin Infect Dis 2006;43:585–592.

42 Wilhelm S, Zimmermann P, Selbitz HJ, Truyen U: Real-time PCR protocol for the detection of porcine parvovirus in field samples. J Virol Methods 2006;134:257–260.

43 Fry AM, Lu X, Chittaganpitch M, Peret T, Fischer J, Dowell SF, Anderson LJ, Erdman D, Olsen SJ: Human bocavirus: a novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. J Infect Dis 2007;195:1038–1045.

44 Manning A, Russell V, Eastick K, Leadbetter GH, Hallam N, Templeton K, Simmonds P: Epidemiological profile and clinical associations of human bocavirus and other human parvoviruses. J Infect Dis 2006;194:1283–1290.

45 Kupfer B, Vehreschild J, Cornely O, Kaiser R, Plum G, Viazov S, Franzen C, Tillmann RL, Simon A, Muller A, Schildgen O: Severe pneumonia and human bocavirus in adult. Emerg Infect Dis 2006;12:1614–1616.

46 Binn LN, Lazar EC, Eddy GA, Kajima M: Recovery and characterization of a minute virus of canines. Infect Immun 1970;1:503–508.

47 Freeman KP, Castro AE, Kautz CE: Unusual characteristics of a parvovirus isolated from a clinically ill steer. Vet Microbiol 1986;11:61–68.

48 Hindiyeh M, Keller N, Mandelboim M, Ram D, Rubinov J, Regev L, Levy V, Orzitzer S, Shaharabani H, Azar R, Mendelson E, Grossman Z: High rate of human bocavirus and adenovirus co-infection in hospitalized Israeli children. J Clin Microbiol 2008;46:334–337.

49 Jarplid B, Johansson H, Carmichael LE: A fatal case of pup infection with minute virus of canines (MVC). J Vet Diagn Invest 1996;8:484–487.

50 Harrison LR, Styer EL, Pursell AR, Carmichael LE, Nietfeld JC: Fatal disease in nursing puppies associated with minute virus of canines. J Vet Diagn Invest 1992;4:19–22.

51 Carmichael LE, Schlafer DH, Hashimoto A: Pathogenicity of minute virus of canines (MVC) for the canine fetus. Cornell Vet 1991;81:151–171.

52 Anderson MJ, Khousam MN, Maxwell DJ, Gould SJ, Happerfield LC, Smith WJ: Human parvovirus B19 and hydrops fetalis. Lancet 1988;1:535.

53 Storz J, Young S, Carroll EJ, Bates RC, Bowen RA, Keney DA: Parvovirus infection of the bovine fetus: distribution of infection, antibody response, and age-related susceptibility. Am J Vet Res 1978;39:1099–1102.

54 Endo R, Ishiguro N, Kikuta H, Teramoto S, Shirkoohi R, Ma X, Ebihara T, Ishiko H, Ariga T: Seroepidemiology of human bocavirus in hokkaido prefecture, Japan. J Clin Microbiol 2007;45:3218–3223.

55 Simon A, Groneck P, Kupfer B, Kaiser R, Plum G, Tillmann RL, Muller A, Schildgen O: Detection of bocavirus DNA in nasopharyngeal aspirates of a child with bronchiolitis. J Infect 2007;54:e125–e127.

56 Regamey N, Frey U, Deffernez C, Latzin P, Kaiser L: Isolation of human bocavirus from Swiss infants with respiratory infections. Pediatr Infect Dis J 2007;26:177–179.

57 Kantola K, Hedman L, Allander T, Jartti T, Lehtinen P, Ruuskanen O, Hedman K, Soderlund-Venermo M: Serdiagnosis of human bocavirus infections. J Infect Dis 2008;46:540–546.

58 Corcoran A, Mahon BP, Doyle S: B cell memory is directed toward conformational epitopes of parvovirus B19 capsid proteins and the unique region of VP1. J Infect Dis 2004;189:1873–1880.

59 Manaresi E, Gallinella G, Zerbini M, Venturoli S, Gentilomi G, Musiani M: IgG immune response to B19 parvovirus VP1 and VP2 linear epitopes by immunoblot assay. J Med Virol 1999;57:174–178.

60 Soderlund M, Brown CS, Spaan WJ, Hedman L, Hedman K: Epitope type-specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J Infect Dis 1995;172:1431–1436.

61 Corcoran A, Mahon BP, Doyle S: B cell memory is directed toward conformational epitopes of parvovirus B19 capsid proteins and the unique region of VP1. J Infect Dis 2004;189:1873–1880.

62 Lindner J, Barabas S, Saar K, Altmann D, Pfister A, Fleck M, Deml L, Modrow S: CD4+ T-cell responses against the VP1-unique region in individuals with recent and persistent parvovirus B19 infection. J Vet Med B Infect Dis Vet Public Health 2005;52:356–361.

63 Franssila R, Auramo J, Modrow S, Mobs M, Oker-Blom C, Kapyla P, Soderlund-Venermo M, Hedman K: T helper cell-mediated interferon-gamma expression after human parvovirus B19 infection: persisting VP2-specific and transient VP1u-specific activity. Clin Exp Immunol 2005;142:53–61.

64 Franssila R, Hedman K: T-helper cell-mediated interferon-gamma, interleukin-10 and proliferation responses to a candidate recombinant vaccine for human parvovirus B19. Vaccine 2004;22:3809–3815.

65 Franssila R, Hokynar K, Hedman K: T helper cell-mediated in vitro responses of recently and remotely infected subjects to a candidate recombinant vaccine for human parvovirus b19. J Infect Dis 2001;183:805–809.

66 Arden KE, McErlean P, Nissen MD, Sloots TP, Mackay IM: Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J Med Virol 2006;78:1232–1240.

67 Gendrel D, Guedj R, Pons-Catalano C, Emerian A, Raymond J, Rozenberg F, Lebon P: Human bocavirus in children with acute asthma. Clin Infect Dis 2007;45:404–405.

68 Kaplan NM, Dove W, Abu-Zeid AF, Shamoon HE, Abd-Eldayem SA, Hart CA: Human bocavirus infection among children, Jordan. Emerg Infect Dis 2006;12:1418–1420.

69 Ma X, Endo R, Ishiguro N, Ebihara T, Ishiko H, Ariga T, Kikuta H: Detection of human bocavirus in Japanese children with lower respiratory tract infections. J Clin Microbiol 2006;44:1132–1134.

5.6. Publikation VIII: Clinical and Epidemiological Aspects of Human Bocavirus Infection

Eingereichtes Manuskript.

Juha Lindner[1], Lüdya Karalar[1], Sven Schimanski[1], Heiko Pfister[2], Wilhelm Struff[3], and Susanne Modrow[1*]

[1]Institute of Medical Microbiology and Hygiene, University of Regensburg, Germany.
[2]Mikrogen GmbH, Floriansbogen 2-4, 82061 Neuried, Germany
[3]DRK Blutspendedienst West, Zentrum für Transfusionsmedizin Münster, Sperlichstrasse 15, 48151 Münster, Germany

Keywords: Human Bocavirus, Respiratory Disease, Gastroenteritis

*Corresponding author: Susanne Modrow, Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz-Josef-Strauss Allee 11, 93053 Regensburg, Germany. Phone: (+49)-941-9446454, Fax: (+49)-941-9446402, e-mail: susanne.modrow@klinik.uni-regensburg.de.

Abstract

Human Bocavirus was recently described as a novel member of the *Parvoviridae* to infect humans. Based on accumulating clinical and epidemiological data the virus is currently being associated with respiratory infections in young children and infants and is furthermore discussed as causative agent of gastrointestinal illness.

Introduction

Acute respiratory tract infections (ARTIs) caused by viruses represent a major cause of hospitalization and morbidity in young children and infants worldwide. Pathogens associated with this clinical condition include the respiratory syncytial virus (RSV), human adenovirus, and the recently described human metapneumovirus and coronaviruses NL63 and HKU1 [1]. Although a constantly growing number of pathogens is being associated with ARTIs, a high percentage of infections still remain uncharacterized and their causative agents unknown.

In 2005 Allander *et. al* described a previously uncharacterized virus in pools of human nasopharyngeal aspirates obtained from children suffering from diseases of the respiratory tract [2]. Comprehensive sequence and phylogenetic analyses revealed a close relation of the new virus with the bovine parvovirus (BPV) and the canine minute virus (CnMV), both members of the *Bocavirus* genus of the *Parvoviridae* family. It was therefore provisionally named human bocavirus (HBoV).

Parvoviruses represent a large family of small, non-enveloped viruses characterized by linear single-stranded DNA-genomes and an exceptional structural simplicity. Besides HBoV two additional parvoviruses, parvovirus B19 (B19V) and PARV4 including its second genotype termed PARV5, are currently known or discussed to infect humans [3-5]. For almost three decades B19V has represented the only member of the virus family to cause illness in humans, e.g. the self-limiting childhood disease *Erythema infectiosum* [6]. Additionally, B19V-infections during pregnancy are known to frequently result in intrauterine infections of the fetus, occasionally leading to miscarriage or *Hydrops fetalis* [7]. While the clinical relevance of PARV4 remains unclear up to date, evidence for an influence of HBoV-infections in the manifestation of respiratory and gastric symptoms is accumulating.

Material and Methods

Detection of HBoV-specific antibodies by ELISA

For detection of HBoV VP2-specific IgG and IgM, 100 ng of purified HBoV VP2 virus-like particles (VP2-VLP) were generated as previously described [8] and coated onto Nunc-Immuno™ MediSorp™ plates (Nunc GmbH, Wiesbaden, Germany) in coating buffer (0.2 M Na_2CO_3, 0.2 M $NaHCO_3$, pH 9.5) overnight at 4°C. The plates were subsequently washed six times with washing buffer (PBS, 0.05% Tween 20) and blocked with dilution buffer (PBS, 2% Tween 20, 3% FCS) for 1 h at 37°C. After incubation with respective serum samples for 2 h at 37°C, the plates were washed and rabbit anti-human IgG- or IgM-specific HRP-coupled secondary antibodies were added for 1 h at 37°C (1:6000 and 1:1000 in dilution buffer, respectively; both Dako Deutschland GmbH, Hamburg, Germany). Development was performed using the BD OptEIA™ Substrate (BD Biosciences, Heidelberg, Germany) according to the manufacturer´s instructions.

As an international IgG standard for HBoV is not yet available, serially diluted sera of a healthy adult male (age: 28 years) and of a boy (age: 22 months) both exhibiting strong HBoV-specific IgG- and IgM-responses, respectively, were introduced for internal reference and used for the calculation of HBoV-specific antibody-titers in all performed experiments. Sera with background optical densities were considered negative and used for the determination of respective IgG/IgM cut-off values, which were additionally confirmed by Western blot analysis.

Reviewed Literature

All reports listed in the PubMed-database of the National Library of Medicine (Rockville Pike, MD, USA) until May 2008 have been considered and evaluated in this review.

Diagnosis of HBoV infections

Up to date, no cell culture systems for the *in vitro* replication of HBoV have been described. Therefore, diagnosis of HBoV infection has so far mainly been based on the detection of viral genomes present in human respiratory, serum, stool, and urine samples using different PCR techniques employing numerous sets of primers specific for the viral genes NP1 [2, 9-12], NS1 [12-15] and VP1/2 [11, 12, 16-18].

Recent reports describe the detection of HBoV-specific antibodies directed against the viral capsid proteins VP1 or/and VP2 in serum samples using ELISA [19, 20], Western blot [21], and immunofluorescence assays [22]. Up to date, no cross-reactions of HBoV- and B19V-

specific humoral and/or cellular immune responses have been described. Using HBoV VP2-VLP we established an ELISA for the detection of HBoV-specific IgM- and IgG-antibodies. This test system was used for analyzing the seroprevalenc of HBoV as described below.

HBoV Epidemiology

Prevalence of HBoV-DNA

HBoV-DNA has been frequently detected worldwide in respiratory [2, 9-11, 13-16, 18, 23-66], serum [30, 43], fecal [17, 30, 33, 35, 38, 67, 68], and urine samples [38] obtained from infants mainly around 2 years of age. The prevalence of HBoV-DNA has been described to vary considerably between 2.7%-19% in children suffering from ARTIs and 0.8%-9.1% in patients with gastroenteritis [17, 25, 35, 43]. However, since the majority of currently published studies have been performed retrospectively, these variations in viral prevalence may be explained by differences in the study populations and patient characteristics. In infected infants, viral loads have been described to range between <500 to 10^{10} and $<10^3$ to $5.9x10^5$ genome copies in nasopharyngeal aspirates and fecal samples, respectively [25, 37, 43, 52]. In serum, viral loads of up to $1.2x10^6$ have been reported (Karalar *et al.*, submitted).

Only limited data is available on the prevalence of HBoV viremia in asymptomatic individuals, since most of the studies have focused on children with distinct clinical symptoms of infectious diseases. In a first study a total of 96 healthy controls were included for diagnostic analysis of HBoV, yet no viral DNA was observed in respiratory samples from these individuals [16]. Furthermore, we were unable to detect HBoV-DNA in sera collected from 298 healthy adult blood donors. However, a recent publication describes the detection of viral genomes in 5% of respiratory samples obtained from asymptomatic children [24].

While most studies have detected the virus during the winter season [2, 28, 33, 38, 46, 69], single reports describe increased numbers of viral infections in spring or summer [9, 31, 40]. No information is currently available on the routes of viral transmission. However, since HBoV can be frequently detected in respiratory and fecal samples, a transmission of the virus via aerosols or direct contact has to be presumed. Thereby, the contagiousness of virus-containing body secretions might be potentiated by the exceptional stability of parvoviral virions and might facilitate increased frequencies of nosocomial infections.

Prevalence of HBoV-specific antibodies and cellular immune reactions

Up to date, only a limited number of studies have been focused on the analysis of HBoV-specific adaptive immune responses in healthy individuals and infants suffering from ARTIs, mainly due to the initial lack of recombinant viral antigens and standardized diagnostic methodologies.

In the first report published on HBoV seroprevalence Endo and co-workers describe ubiquitous IgG-responses against the viral capsid protein VP1 in up to 83.3% of children aged ≥ 2 years with respiratory infections [22]. The overall seroprevalence of HBoV-specific IgG in the Japanese population aged between 0 months to 41 years was 71.1%, while seronegative patients were observed most frequently in infants with 6 – 12 months of age.

In a subsequent study the prevalence of HBoV-specific antibodies in Finnish infants suffering from ARTIs was assessed using Western blot [21]. In children determined positive for HBoV DNA, IgG- and IgM-antibodies against the viral VP2 protein were observed in 73% and 49% of analyzed samples, respectively. The mean age of these children was 2.1 years. The overall prevalence of HBoV-specific IgG and IgM in children without detectable viral genomes in nasopharyngeal samples was 35% and 13%, respectively. Antibodies against the aminoterminal domain of the viral VP1 protein, termed VP1-unique region, were detected rarely: only 7% (IgG) and 2% (IgM) of the patients showed positive results. In contrast to the data provided by Endo and colleagues and by our group (see below), the prevalence of HBoV-specific IgG was shown to decline from 52% in 1-2 year old infants to 29% in children aged over 5 years in the Finnish study [21]. Furthermore, maternal VP2-specific IgG were not observed in children <6 months of age despite a seemingly high seroprevalence of HBoV in adults. This finding may be due to the maturation of IgG-specificity in the time period of up to six months following an acute infection, during which antibodies against linear epitopes get replaced by those preferentially recognizing conformational antigen structures. This process has been well documented for B19V-specific humoral immune responses [70], and therefore it may be assumed that similar changes in IgG affinity take place during HBoV infections.

More recently, we and others have established ELISA assays based on the use of recombinant HBoV VP2-VLP for the detection of HBoV-specific antibodies in human serum samples [19, 20]. Herein, our group observed the prevalence of IgG_1 subclass antibodies against HBoV VP2-VLP to rise from 24% in children with 7 to 9 months of age to up to 98.3% adult blood donors (mean age: 42 years).

In addition to humoral immune reactions the presence of HBoV-specific T-cells in healthy adults supports a high prevalence of HBoV-specific immunity in adults. Thereby, frequent

interferon-gamma (IFN-γ) mediated $CD4^+$ T helper cell reactions were observed against HBoV capsid proteins [8]. Similar data have been previously described for B19V-specific cellular immune responses [71-74].

Clinical associations

HBoV infections are frequently linked to high rates of co-infections with viral and bacterial pathogens of the respiratory and/or gastrointestinal system. Together with the fact that most of the studies have been performed retrospectively and long-term follow-up studies with detailed clinical characterization of symptomatic individuals are rare, it is currently difficult to clearly determine HBoV as sole infectious agent of human illnesses.

HBoV and respiratory disease

Up to date, HBoV infections have been detected in young children around the age of 2 years with acute diseases of the upper and lower respiratory tract [2, 9-11, 13, 14, 16, 18, 25-29, 31-60, 62-66], frequently in combination with interstitial lung infiltrates and abnormal radiologic findings [2, 28, 37, 45, 55]. In HBoV-positive individuals, we detected both virus-specific IgG and IgM in 42% of studied sera, whereas no IgM were observed in samples obtained from children without detectable amounts of HBoV genomes in blood.

Symptoms and disease manifestations observed in HBoV infected children include pneumonia, bronchiolitis, wheezing, respiratory distress, hypoxia, fever, rhinitis, laryngeal croup and, more rarely, conjunctivitis or rashes. In adults, acute HBoV infections leading to ARTIs seem to be rare and have been currently detected mainly in immunocompromised [13, 49, 51, 75] and only in single immunocompetent individuals [13, 16].

Recently, the presence of elevated viral loads in nasopharyngeal aspirates ($>10^4$ genome copies/ml) has been suggested to correlate with the severity of respiratory symptoms during HBoV infection, whereas low viral loads ($<10^4$ genome copies/ml) may represent viral persistence [43]. These data are in contrast to those published by Kleines and colleagues, who could not find a relation between the viral load and the severity of HBoV associated illness [37], indicating that further work is necessary to study the influence of the viral load on respiratory disease manifestation. However, infections with the related parvovirus B19 often result in a prolonged replication of the virus in infected individuals [5] and therefore mechanisms of persistence may also apply for HBoV.

Individuals found positive for HBoV-DNA in nasopharyngeal aspirates are frequently found to be co-infected with a multitude of additional viral and/or bacterial respiratory

pathogens. Thereby, high rates of co-infections reaching up to 91% have been observed [26]. Commonly detected additional pathogens include the respiratory syncytial virus (RSV), human adenovirus, rhinovirus, and *Streptococcus* sp.. Despite these high rates of co-infection, HBoV viremia has been frequently described to be significantly more prevalent in infants suffering from ARTIs than in age-matched asymptomatic control groups [24, 26, 28, 43, 51], and therefore a role of HBoV in the development of human respiratory diseases is to be presumed. This finding is supported by our data, which show a significantly higher prevalence of HBoV-infections in young children with lower respiratory tract infections (14.6%, 7/48) as compared to a control group of age-matched individuals hospitalized due to non-infectious conditions such as bone fractures or planned surgeries (5.0%, 3/60).

HBoV and gastrointestinal disease

In addition to respiratory symptoms, HBoV is currently discussed to be associated with gastroenteritic symptoms. Similar features are known from veterinary infections with the closely related BPV and CnMV, which are known to induce gastric illness in their respective hosts [76, 77].

First reports have described the prevalence of HBoV genomes to range between 0.8%-9.1% in fecal samples obtained from children suffering from acute gastroenteritis, often in combination with ARTIs [17, 28, 31, 33, 35, 53, 63, 67]. In a recent prospective study we detected HBoV-DNA in 7.8% (5/64) fecal samples obtained from young children exhibiting gastrointestinal symptoms, e.g. diarrhea, nausea and vomiting. An additional child tested positive for HBoV was diagnosed with inflammatory bowel disease.

As co-infections with additional intestinal pathogens, e.g. human rota- and noroviruses, enteropathogenic strains of *Escherichia coli* or *Salmonella* sp., have been frequently observed in up to 77.6% of HBoV positive individuals, an association of HBoV with gastroenteritis remains unclear [68]. Since in many cases HBoV-DNA has been detected concurrently in both stool samples and nasopharyngeal aspirates obtained from young children with ARTIs [11], the presence of HBoV in fecal samples might represent natural viral shedding during an acute HBoV infection and not play an active role in the pathogenesis of gastric disease.

Conclusions

Although HBoV was detected only three years ago, both epidemiological and clinical data establishing the virus as the second member of the *Parvoviridae* pathogenic to humans are

accumulating. Based on current reports it seems most likely that HBoV may be associated with respiratory infections in young children and infants, while a further connection between HBoV and gastrointestinal symptoms has been suggested. However, as acute HBoV-infections are often accompanied by infections with additional pathogens of both the respiratory and gastrointestinal tract, a final establishment of HBoV as the causative agent of infectious disease in humans needs to be confirmed by additional prospective studies. The methodological heterogeneity used for the diagnosis of HBoV infection raises questions about the specificity and comparability of many published studies, highlighting the urgent need of internationally standardized diagnostic guidelines and reference samples for the detection of HBoV genomes and virus-specific immune responses in human samples. As serological diagnostics of HBoV infection will become more important in the future, standardized viral DNA and antibody specimen should be provided as a basis to establish comparable test systems.

Nevertheless, first data obtained from healthy control individuals and children with symptoms of non-infectious disease indicate distinctly lower rates of HBoV infections in comparison to patients suffering from ARTIs. Whether HBoV might require the presence of helper-viruses to establish human illness or may even act as the provider of such co-factors for other respiratory viruses, remains to be assessed in further studies.

References

1. Kesson AM. Respiratory virus infections. Paediatr Respir Rev 2007; 8:240-8.
2. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, and Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A 2005; 102:12891-6.
3. Fryer JF, Delwart E, Hecht FM et al. Frequent detection of the parvoviruses, PARV4 and PARV5, in plasma from blood donors and symptomatic individuals. Transfusion 2007; 47:1054-61.
4. Fryer JF, Kapoor A, Minor PD, Delwart E, and Baylis SA. Novel parvovirus and related variant in human plasma. Emerg Infect Dis 2006; 12:151-4.
5. Young NS and Brown KE. Parvovirus B19. N Engl J Med 2004; 350:586-97.
6. Anderson MJ, Lewis E, Kidd IM, Hall SM, and Cohen BJ. An outbreak of erythema infectiosum associated with human parvovirus infection. J Hyg (Lond) 1984; 93:85-93.
7. Woernle CH, Anderson LJ, Tattersall P, and Davison JM. Human parvovirus B19 infection during pregnancy. J Infect Dis 1987; 156:17-20.
8. Lindner J, Zehentmeier S, Franssila R et al. CD4+ T Helper Cell Responses Against Human Bocavirus VP2 Virus-like Particles in Healthy Adults. J Infect Dis 2008.

9. Choi EH, Lee HJ, Kim SJ et al. The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000-2005. Clin Infect Dis 2006; 43:585-92.

10. Simon A, Groneck P, Kupfer B et al. Detection of bocavirus DNA in nasopharyngeal aspirates of a child with bronchiolitis. J Infect 2007; 54:e125-e127.

11. Neske F, Blessing K, Tollmann F et al. Real-time PCR for diagnosis of human bocavirus infections and phylogenetic analysis. J Clin Microbiol 2007; 45:2116-22.

12. Choi JH, Chung YS, Kim KS et al. Development of real-time PCR assays for detection and quantification of human bocavirus. J Clin Virol 2008.

13. Manning A, Russell V, Eastick K et al. Epidemiological profile and clinical associations of human bocavirus and other human parvoviruses. J Infect Dis 2006; 194:1283-90.

14. Regamey N, Frey U, Deffernez C, Latzin P, and Kaiser L. Isolation of human bocavirus from Swiss infants with respiratory infections. Pediatr Infect Dis J 2007; 26:177-9.

15. Lu X, Chittaganpitch M, Olsen SJ et al. Real-time PCR assays for detection of bocavirus in human specimens. J Clin Microbiol 2006; 44:3231-5.

16. Bastien N, Brandt K, Dust K, Ward D, and Li Y. Human Bocavirus infection, Canada. Emerg Infect Dis 2006; 12:848-50.

17. Lee JI, Chung JY, Han TH, Song MO, and Hwang ES. Detection of human bocavirus in children hospitalized because of acute gastroenteritis. J Infect Dis 2007; 196:994-7.

18. Bastien N, Chui N, Robinson JL et al. Detection of human bocavirus in Canadian children in a 1-year study. J Clin Microbiol 2007; 45:610-3.

19. Kahn JS, Kesebir D, Cotmore SF et al. Seroepidemiology of Human Bocavirus Defined Using Recombinant Virus-Like Particles. J Infect Dis 2008.

20. Lin F, Guan W, Cheng F, Yang N, Pintel D, and Qiu J. ELISAs using human bocavirus VP2 virus-like particles for detection of antibodies against HBoV. J Virol Methods 2008; 149:110-7.

21. Kantola K, Hedman L, Allander T et al. Serodiagnosis of human bocavirus infection. Clin Infect Dis 2008; 46:540-6.

22. Endo R, Ishiguro N, Kikuta H et al. Seroepidemiology of human bocavirus in Hokkaido prefecture, Japan. J Clin Microbiol 2007; 45:3218-23.

23. Chieochansin T, Chutinimitkul S, Payungporn S et al. Complete coding sequences and phylogenetic analysis of Human Bocavirus (HBoV). Virus Res 2007; 129:54-7.

24. Garcia-Garcia ML, Calvo C, Pozo F et al. Human bocavirus detection in nasopharyngeal aspirates of children without clinical symptoms of respiratory infection. Pediatr Infect Dis J 2008; 27:358-60.

25. Lin F, Zeng A, Yang N et al. Quantification of human bocavirus in lower respiratory tract infections in China. Infect Agent Cancer 2007; 2:3.

26. Fry AM, Lu X, Chittaganpitch M et al. Human bocavirus: a novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. J Infect Dis 2007; 195:1038-45.

27. Arden KE, McErlean P, Nissen MD, Sloots TP, and Mackay IM. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J Med Virol 2006; 78:1232-40.

28. Kesebir D, Vazquez M, Weibel C et al. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis 2006; 194:1276-82.

29. Catalano-Pons C, Bue M, Laude H et al. Human bocavirus infection in hospitalized children during winter. Pediatr Infect Dis J 2007; 26:959-60.

30. Catalano-Pons C, Giraud C, Rozenberg F, Meritet JF, Lebon P, and Gendrel D. Detection of human bocavirus in children with Kawasaki disease. Clin Microbiol Infect 2007; 13:1220-2.

31. Arnold JC, Singh KK, Spector SA, and Sawyer MH. Human bocavirus: prevalence and clinical spectrum at a children's hospital. Clin Infect Dis 2006; 43:283-8.

32. Ma X, Endo R, Ishiguro N et al. Detection of human bocavirus in Japanese children with lower respiratory tract infections. J Clin Microbiol 2006; 44:1132-4.

33. Lau SK, Yip CC, Que TL et al. Clinical and molecular epidemiology of human bocavirus in respiratory and fecal samples from children in Hong Kong. J Infect Dis 2007; 196:986-93.

34. Chieochansin T, Samransamruajkit R, Chutinimitkul S et al. Human bocavirus (HBoV) in Thailand: clinical manifestations in a hospitalized pediatric patient and molecular virus characterization. J Infect 2008; 56:137-42.

35. Vicente D, Cilla G, Montes M, Perez-Yarza EG, and Perez-Trallero E. Human bocavirus, a respiratory and enteric virus. Emerg Infect Dis 2007; 13:636-7.

36. Naghipour M, Cuevas LE, Bakhshinejad T, Dove W, and Hart CA. Human bocavirus in Iranian children with acute respiratory infections. J Med Virol 2007; 79:539-43.

37. Kleines M, Scheithauer S, Rackowitz A, Ritter K, and Hausler M. High prevalence of human bocavirus detected in young children with severe acute lower respiratory tract disease by use of a standard PCR protocol and a novel real-time PCR protocol. J Clin Microbiol 2007; 45:1032-4.

38. Pozo F, Garcia-Garcia ML, Calvo C, Cuesta I, Perez-Brena P, and Casas I. High incidence of human bocavirus infection in children in Spain. J Clin Virol 2007; 40:224-8.

39. Chung JY, Han TH, Kim SW, Kim CK, and Hwang ES. Detection of viruses identified recently in children with acute wheezing. J Med Virol 2007; 79:1238-43.

40. Chung JY, Han TH, Kim CK, and Kim SW. Bocavirus infection in hospitalized children, South Korea. Emerg Infect Dis 2006; 12:1254-6.

41. Gendrel D, Guedj R, Pons-Catalano C et al. Human bocavirus in children with acute asthma. Clin Infect Dis 2007; 45:404-5.

42. Kaplan NM, Dove W, Abu-Zeid AF, Shamoon HE, Abd-Eldayem SA, and Hart CA. Human bocavirus infection among children, Jordan. Emerg Infect Dis 2006; 12:1418-20.

43. Allander T, Jartti T, Gupta S et al. Human bocavirus and acute wheezing in children. Clin Infect Dis 2007; 44:904-10.

44. Sloots TP, McErlean P, Speicher DJ, Arden KE, Nissen MD, and Mackay IM. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol 2006; 35:99-102.

45. Foulongne V, Olejnik Y, Perez V, Elaerts S, Rodiere M, and Segondy M. Human bocavirus in French children. Emerg Infect Dis 2006; 12:1251-3.

46. Weissbrich B, Neske F, Schubert J et al. Frequent detection of bocavirus DNA in German children with respiratory tract infections. BMC Infect Dis 2006; 6:109.

47. Smuts H and Hardie D. Human bocavirus in hospitalized children, South Africa. Emerg Infect Dis 2006; 12:1457-8.

48. Khetsuriani N, Kazerouni NN, Erdman DD et al. Prevalence of viral respiratory tract infections in children with asthma. J Allergy Clin Immunol 2007; 119:314-21.

49. Kupfer B, Vehreschild J, Cornely O et al. Severe pneumonia and human bocavirus in adult. Emerg Infect Dis 2006; 12:1614-6.

50. Schenk T, Huck B, Forster J, Berner R, Neumann-Haefelin D, and Falcone V. Human bocavirus DNA detected by quantitative real-time PCR in two children hospitalized for lower respiratory tract infection. Eur J Clin Microbiol Infect Dis 2007; 26:147-9.

51. Maggi F, Andreoli E, Pifferi M, Meschi S, Rocchi J, and Bendinelli M. Human bocavirus in Italian patients with respiratory diseases. J Clin Virol 2007; 38:321-5.

52. Qu XW, Duan ZJ, Qi ZY et al. Human bocavirus infection, People's Republic of China. Emerg Infect Dis 2007; 13:165-8.

53. Monteny M, Niesters HG, Moll HA, and Berger MY. Human bocavirus in febrile children, The Netherlands. Emerg Infect Dis 2007; 13:180-2.

54. Terrosi C, Fabbiani M, Cellesi C, and Cusi MG. Human bocavirus detection in an atopic child affected by pneumonia associated with wheezing. J Clin Virol 2007; 40:43-5.

55. Volz S, Schildgen O, Klinkenberg D et al. Prospective study of Human Bocavirus (HBoV) infection in a pediatric university hospital in Germany 2005/2006. J Clin Virol 2007; 40:229-35.

56. Hindiyeh MY, Keller N, Mandelboim M et al. High rate of human bocavirus and adenovirus coinfection in hospitalized Israeli children. J Clin Microbiol 2008; 46:334-7.

57. Villa L, Melon S, Suarez S et al. Detection of human bocavirus in Asturias, Northern Spain. Eur J Clin Microbiol Infect Dis 2008; 27:237-9.

58. Christensen A, Nordbo SA, Krokstad S, Rognlien AG, and Dollner H. Human bocavirus commonly involved in multiple viral airway infections. J Clin Virol 2008; 41:34-7.

59. Gerna G, Piralla A, Campanini G, Marchi A, Stronati M, and Rovida F. The human bocavirus role in acute respiratory tract infections of pediatric patients as defined by viral load quantification. New Microbiol 2007; 30:383-92.

60. Redshaw N, Wood C, Rich F, Grimwood K, and Kirman JR. Human bocavirus in infants, New Zealand. Emerg Infect Dis 2007; 13:1797-9.

61. Schenk T, Strahm B, Kontny U, Hufnagel M, Neumann-Haefelin D, and Falcone V. Disseminated bocavirus infection after stem cell transplant. Emerg Infect Dis 2007; 13:1425-7.

62. Longtin J, Bastien M, Gilca R et al. Human bocavirus infections in hospitalized children and adults. Emerg Infect Dis 2008; 14:217-21.

63. Esposito S, Bosis S, Niesters HG et al. Impact of human bocavirus on children and their families. J Clin Microbiol 2008; 46:1337-42.

64. Canducci F, Debiaggi M, Sampaolo M et al. Two-year prospective study of single infections and co-infections by respiratory syncytial virus and viruses identified recently in infants with acute respiratory disease. J Med Virol 2008; 80:716-23.

65. Smuts H, Workman L, and Zar HJ. Role of human metapneumovirus, human coronavirus NL63 and human bocavirus in infants and young children with acute wheezing. J Med Virol 2008; 80:906-12.

66. Rihkanen H, Ronkko E, Nieminen T et al. Respiratory viruses in laryngeal croup of young children. J Pediatr 2008; 152:661-5.

67. Albuquerque MC, Rocha LN, Benati FJ et al. Human bocavirus infection in children with gastroenteritis, Brazil. Emerg Infect Dis 2007; 13:1756-8.

68. Yu JM, Li DD, Xu ZQ et al. Human bocavirus infection in children hospitalized with acute gastroenteritis in China. J Clin Virol 2008.

69. Manning A, Willey SJ, Bell JE, and Simmonds P. Comparison of tissue distribution, persistence, and molecular epidemiology of parvovirus B19 and novel human parvoviruses PARV4 and human bocavirus. J Infect Dis 2007; 195:1345-52.

70. Soderlund M, Brown CS, Spaan WJ, Hedman L, and Hedman K. Epitope type-specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J Infect Dis 1995; 172:1431-6.

71. Franssila R, Auramo J, Modrow S et al. T helper cell-mediated interferon-gamma expression after human parvovirus B19 infection: persisting VP2-specific and transient VP1u-specific activity. Clin Exp Immunol 2005; 142:53-61.

72. Franssila R and Hedman K. T-helper cell-mediated interferon-gamma, interleukin-10 and proliferation responses to a candidate recombinant vaccine for human parvovirus B19. Vaccine 2004; 22:3809-15.

73. Franssila R, Hokynar K, and Hedman K. T helper cell-mediated in vitro responses of recently and remotely infected subjects to a candidate recombinant vaccine for human parvovirus b19. J Infect Dis 2001; 183:805-9.

74. Lindner J, Barabas S, Saar K et al. CD4(+) T-cell responses against the VP1-unique region in individuals with recent and persistent parvovirus B19 infection. J Vet Med B Infect Dis Vet Public Health 2005; 52:356-61.

75. Garbino J, Inoubli S, Mossdorf E et al. Respiratory viruses in HIV-infected patients with suspected respiratory opportunistic infection. AIDS 2008; 22:701-5.

76. Freeman KP, Castro AE, and Kautz CE. Unusual characteristics of a parvovirus isolated from a clinically ill steer. Vet Microbiol 1986; 11:61-8.

77. Binn LN, Lazar EC, Eddy GA, and Kajima M. Recovery and Characterization of a Minute Virus of Canines. Infect Immun 1970; 1:503-8.

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitates gekennzeichnet.

Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar finanzielle Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, den 30.07.2008

_____

(Juha Lindner)

Danksagung

Ich danke Herrn Prof. Dr. Hans Wolf für die Möglichkeit, meine Doktorarbeit am Institut für Medizinische Mikrobiologie & Hygiene durchführen zu können.

Frau Prof. Dr. Susanne Modrow danke ich für die Bereitstellung des interessanten Themas, für die Übernahme des Erstgutachtens, die Vertretung der vorliegenden Arbeit vor der Biologischen Fakultät der Universität Regensburg und für Ihre unermüdliche Hilfs- und Diskussionsbereitschaft. Besonders möchte ich mich auch für die zahlreichen Kongressbesuche bedanken, die Sie mir ermöglicht hat!

Annette Rohrhofer, Barbara Hottenträger, Ingeborg Held, Irene Nebeja und allen weiteren Mitarbeitern der virologischen Diagnostikabteilung danke ich für ihre Hilfestellungen bei Problemen und Arbeiten aller Art!

Dr. Heiko Pfister, Dr. Wilhelm Struff, Prof. Dr. Hugo Segerer, Dr. Michel Noutsias und Dr. Friedhelm Küthe danke ich für die gute Zusammenarbeit.

Dr. Annelie Plentz, Dr. Sven Schimanski, Dr. Jürgen Wenzel und Dr. Hans-Helmut Niller danke ich für ihre stete Bereitschaft den an dieser Studie beteiligten Spendern Blut abzunehmen.

Dr. Sascha Barabas und PD Dr. Ludwig Deml danke ich für die jahrelange gute Zusammenarbeit. Julia Mischner, Richard Klar, Laura Gerber, Adriana Migliorini, Dominik Altmann, Dipl. Biol. Sandra Zehentmeier, Dipl. Biol. Susanne Schiffner und allen weiteren nicht namentlich genannten Praktikanten/-innen danke ich für eine schöne Zeit im Labor! Ihr seid mir zu sehr guten Freunden geworden.

Vielen Dank auch an Josh, Simon, Wolfgang und alle weiteren Freunde und Bekannte, dank derer mein Studium mir immer Spaß gemacht hat!

Besonderer Dank gilt meinen Eltern, Pypi, Opa und Paija und meiner restlichen Familie! Zuletzt möchte ich ganz besonders meiner lieben Valeria danken! Ohne Euch und Eure Unterstützung hätte dies alles nie geklappt! Danke.