VACCINATION AGAINST MEASLES

Evaluation of novel approaches

VACCINATIE TEGEN MAZELEN

Evaluatie van nieuwe benaderingen

Proefschrift

Ter verkrijging van de graad van doctor
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op gezag van de Rector Magnificus

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voor Mariëlle en Vera

aan mijn ouders

Contents

ABBREVIATIONS

A (Ala)	Alanine
AIDS	acquired immunodeficiency syndrome
AMS	atypical measles syndrome
APC	antigen presenting cell
ATG	anti thymocyte globulin
BFA	Brefeldin A
B-LCL	B lymphoblastic cell line
Boc	tert-butyloxycarbonyl
BPL	beta propiolactone
C (Cys)	Cysteine
C.	centrifugation method
$CCID_{50}$	cell culture-infectious dose 50%
CD	cluster of differentiation
CDV	canine distemper virus
CEF	chick embryo fibroblasts
CM	culture medium
CMC	critical micelle concentration
CNS	central nervous system
Con A	concanavaline A
Cpm	counts per minute
CTL	cytotoxic T lymphocyte
D (Asp)	Aspartic acid
D.	dialysis method
DC	dendritic cell
DCM	dichloromethane
Dde	1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl
DIPEA	*N,N*-diisopropylethylamine
DLS	dynamic light scattering
DMA	*N,N*-dimethylacetamide
DNA	desoxyribonucleic acid
DPH	diphenylhexatriene
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
EM	electron microscopy
EMVIE	experimentally measles virus-induced encephalitis
ER	endoplasmic reticulum
E-to-T ratio	effector-to-target cell ratio
F	measles virus Fusion protein
FAB MS	fast atom bombardment mass spectrometry
FACS	fluorescence-activated cell scanner
FB	FACS buffer
FBS	fetal bovine serum

FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	fluorescein
Fmoc	9-fluorenylmethoxycarbonyl
H	measles virus Haemagglutinin
hCG	human chorionic gonadotrophin
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HOBt	1-hydroxybenzotriazole
HP	herpes papio virus
HPLC	high pressure liquid chromatography
HRPO	horseradish peroxidase
IFN	interferon
Ig	Immunoglobulin
ii	intra-intestinal
IL	interleukin
im	intramuscular
iscom	immune-stimulating complex
IU	international units
K (Lys)	Lysine
kb	kilo base pairs
L (Leu)	Leucine
L	measles virus Large protein
L16	Leningrad-16
LAV	live attenuated vaccine
LCMV	lymphocytic choriomeningitis virus
LLC	lung lavage cells
M	measles virus Matrix protein
MHC	major histocompatibility complex
MIBE	measles inclusion body encephalitis
MIIC	MHC class II containing compartment
MMR	measles-mumps-rubella
MOI	multiplicity of infection
mono	monocyte
MTT	3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide
Mtt	4-methyltrityl
mφ	macrophage
MV	measles virus
MVA	Modified Vaccinia virus Ankara
N	measles virus Nucleocapsid protein
NK	natural killer
NMM	*N*-methylmorpholine
OD_{450}	optical density at 450 nm
P (Pro)	Proline
P	measles virus Phosphoprotein
Pal	Palmitoyl
PAHO	Pan American Health Organization

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDV	phocid distemper virus
PE	phycoerythrine
PEC	pharyngeal epithelial cells
PFA	paraformaldehyde
PFU	plaque forming units
PHA	phytohaemagglutinin
PIV	parainfluenza virus
Pmc	2,2,5,7,8-pentamethylchromansulfonyl
PMV	porpoise morbillivirus
PPRV	Peste-des-petits-ruminants virus
PTA	phosphotungstic acid
PWM	pokeweed mitogen
PyBOP	benzotriazolyloxy-tris-[*N*-pyrrolidino]phosphonium hexafluorophosphate
R (Arg)	Arginine
RNA	ribonucleic acid
rpm	rounds per minute
RPV	rinderpest virus
RT-PCR	reverse transcriptase-PCR
rVV	recombinant vaccinia virus
S (Ser)	Serine
SCID	severe combined immune deficiency
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFC	spot forming cells
SLAM	signalling lymphocytic activation molecule
SSPE	Subacute sclerosing panencephalitis
*N*Su	N-succinimidyl
TAP	transporter associated with antigen processing
TBI	total-body irradiation
$TCID_{50}$	tissue culture-infectious dose 50%
TFA	trifluoroacetic acid
Th	T helper lymphocyte
TIPS	triisopropylsilane
TMB	tetramethylbenzidine
TMR	tetramethylrhodamine
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
UNICEF	United Nations Children's Fund
V (Val)	Valine
VN	virus neutralization
WBC	white blood cell
WHO	World Health Organization
wt	wild-type
Y (Tyr)	Tyrosine

Chapter 1

INTRODUCTION

Measles and measles virus

Disease

Measles, also called morbilli or rubeola, is a highly contagious disease of humans. After an incubation period of 9-11 days characteristic clinical signs develop like coryza, cervical lymphadenitis, so-called Koplik's spots in the mouth, conjunctivitis, photophobia, myalgia, malaise, sneezing and coughing lasting for about 1 week. Fever precedes the typical rash consisting of generalised maculopapular lesions that first appear on the face and soon after on the trunk and the extremities [1].

Measles is one out of six infectious diseases that cause 90% of infectious disease deaths, worldwide (Fig.1a). It is a major childhood killer in developing countries, accounting for about 900,000 deaths a year (Fig.1b). Data from the pre-vaccination era showed that in Europe by 10 years of age virtually everyone had evidence of past measles virus (MV) infection [2].

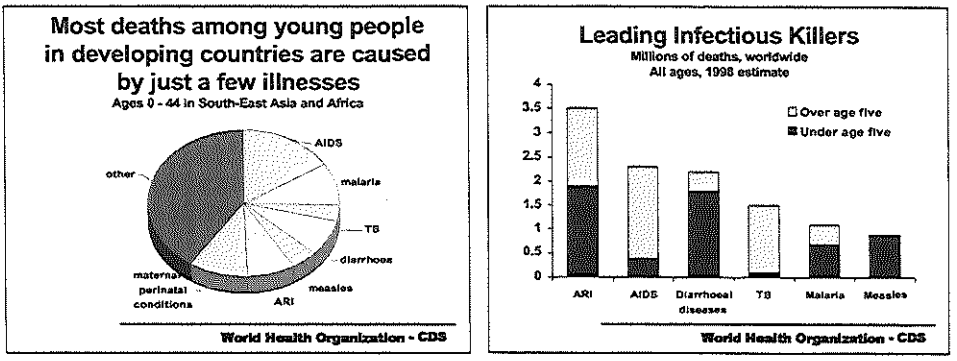

Figure 1. (left) Measles is a very contagious disease, associated wit h high mortallity and morbidity in developing countries, that assembles with just a few other illnesses causing most deaths among young people. (right) Measles predominantly causes high mortallity among infants younger than five years of age. Taken from Statement by Dr. David L. Heymann World Health Organization before the Committee on International Relations U.S. House of Representatives 29 June 2000.

History

In 1757 measles was formally shown to be caused by an "infectious" agent when the Scottish physician Francis Home, attempting immunization, transmitted the disease to naive individuals using blood taken from measles patients during the early stages of the rash [3]. About two centuries later the virus was grown in tissue culture for the first time [4]. Enders and Peebles inoculated primary human kidney cells with blood of David Edmonston, a child with measles [4]. This isolate was passaged numerous times over different cell lines, which resulted in attenuation of the virus. This live attenuated virus facillitated the development of diagnostic tests and the live attenuated vaccines (LAV) [5].

Hereafter, several animal diseases, such as rinderpest, peste des petits ruminants, and canine-phocine-, porpoise- as well as dolphin-distemper, were recognized as diseases caused by more or less closely related morbilliviruses [6,7](Fig. 2).

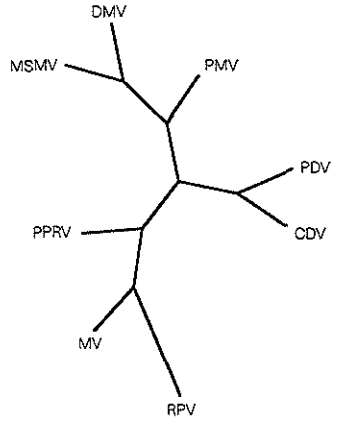

Figure 2. A maximum likelihood tree of N protein fragment sequences (121 nucleotides) of different morbilliviruses. DMV, dolphin morbillivirus; PMV, porpoise morbillivirus; PDV, phocine distemper virus; CDV, canine distemper virus; RPV, rinderpest virus; MV, measles virus; PPRV, peste des petits ruminants virus; MSMV, monk seal morbillivirus. Taken from Osterhaus et al. Nature 1997:388;838-839.

Measles is a relatively new disease of humans and probably evolved from the interspecies transmission of an animal morbillivirus[8]. MV shows a high level of homology with rinderpest virus, a pathogen of cattle (Fig. 2)[9]. It is postulated that rinderpest virus is the so-called "archevirus" of human MV [10]. Rinderpest is likely to have evolved as a "childhood" disease in herds of ungulates large enough to maintain a continuous supply ofsusceptible humans and establish a pattern of endemic infection. Black et al. estimated that a population of at least 300.000 individuals is needed to maintain the circulation of MV[11]. Measles may therefore be considered a disease of civilization[8].

Although MV exists as a single immunotype, sequence analysis of variable regions within the viral genome has identified different genotypes[12]. Although this genetic diversity apparently has little or no consequences for vaccine efficacy[13,14], it can be used for surveillance and epidemiological studies[13,14].

Infection

MV enters the respiratory tract via aerosols and infects the tracheal and bronchial epithelial cells. Here the virus starts to replicate during the first 2 to 4 days and spreads throughout the lymphoid tissues producing lymphoid or reticuloendothelial giant cells. Hereafter, cell associated viraemia is detectable and subsequently, after about ten days, a characteristic maculopapular rash appears. The onset of the rash coincides with the appearance of the immune responses and initiation of virus clearance (Fig. 3). The MV-specific immune responses will be further discussed below.

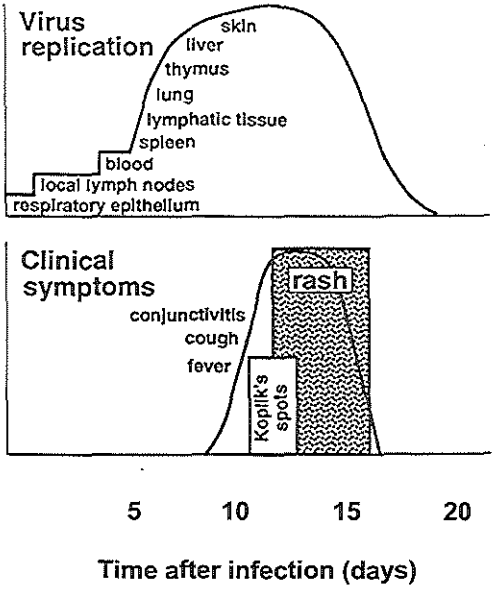

Figure 3. Diagram of the pathogenesis of measles. Virus replication begins in the respiratory epithelium and spreads to monocytes/macrophages, endothelial cells, and epithelial cells in the blood, thymus, spleen, lymph nodes, liver, skin, and lung and to the conjunctivae and the mucosal surfaces of the gastrointestinal, respiratory, and genitourinary tracts. Clearance of virus is approximately coincident with fading of the rash. Taken from Griffin DE, Bellini WJ. Measles virus. In: Fields BN, Knipe DM, Howley PM, eds.Fields Virology. 3 ed. Philadelphia: Lippincott - Raven Publishers, 1996:1267-312.

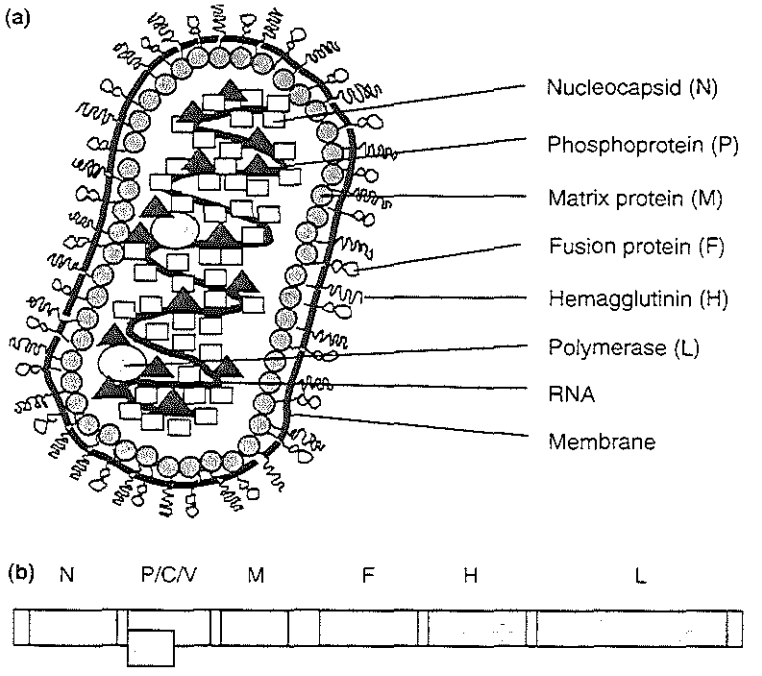

Figure 4. The MV particle and genomic organization. Taken from Ian C. D. Johnston *et al.* Trends in Microbiology 1995:3;361-365.

Virion

MV is an enveloped virus with a non-segmented negative RNA genome. The genome encodes six major structural proteins and three nonstructural proteins (C, R and V)(Fig. 4a). The envelope is composed of the fusion (F) and the hemagglutinin (H) transmembrane glycoproteins and the matrix or membrane (M) protein. The nucleocapsid is composed of the nucleoprotein (N), the phosphoprotein (P), the large protein (L) and the RNA (Fig.4b). Infection of a cell begins with binding of H to the receptor (described below). Subsequently, F, which is closely associated with H on the virus envelope [15], causes the virus envelope to fuse with the plasma membrane of the target cell [16] after which the viral genome is released into the cytoplasm. The process of transcription and replication starts with the synthesis of N followed by P, M, F, H and L. P and L form the viral polymerase complex. Interaction of tubulin, a host cell protein, with the viral polymerase may be required for these processes [17]. M regulates the assembly and budding of virions by interaction with the cytoplasmic tails of F and H, but also inhibits transcription by binding to the nucleocapsid [18]. However, others were not able to demonstrate interaction between N and M [19]. The latter function is often defective in viruses causing persistent infection [18]. The process of MV assembly, association of virus envelope and the ribonucleoparticle, and subsequent budding occurs in detergent-resistant glycosphingolipids and cholesterol-rich cellular membranes, so-called rafts [20]. The function of C, R and V, encoded by the same gene as P, has not been elucidated yet but it has been suggested that these proteins have a share in the regulation of transcription [21]. C- and V-defective viruses have been tipped to be more-attenuated MV vaccine candidates [22]. F, synthesized as an inactive transmembrane polyprotein (F_0), is post-translationally glycosylated, cleaved by furin into its active form (composed of F_1 and F_2) [23-25], tetramerized and transported to the plasma membrane. H is glycosylated and dimerized before it is transported to the plasma membrane where it associates with F [15].

Cell entry

CD46, a complement-regulatory protein, was first identified as being the putative receptor for MV [26]. However, several pieces of information suggested that other proteins are involved in cell entry [27]. Recently, a Japanese group reported that CDw150, the signalling lymphocytic activation molecule (SLAM), is used by wild-type isolates which do not use CD46 [28-30]. SLAM has a tissue distribution that is more consistent with MV pathology than CD46 [28]. It has been suggested that MV utilizes multiple receptor pathways to target different tissues *in vivo* [31,32].

Complications

Complications of measles include bronchopneumonia, otitis media (with or without secondary bacterial infections), gastroenteritis and encephalitis [1]. Subacute sclerosing panencephalitis (SSPE) results from a rarely occurring chronic MV infection in which the virus multiplies in the brain with the expression of a limited repertoire of virus genes, resulting in neurodegenerative disease (for review see)[33]. SSPE is characterised by an underrepresentation of antibodies to the M protein in serum [34] and *ex vivo* experiments with human SSPE brain tissue revealed an interferon-dependent transcriptional attenuation of MV in brain cells [33]. Another rare complication is measles inclusion body encephalitis (MIBE), which is found in malnourished or immunocompromised individuals [35]. However, there is a report on the occurrence of MIBE in an apparently healthy young child after measles-mumps-rubella (MMR) vaccination [36].

Table 1. Overview of selected literature on MV-induced immunosuppression.

Cell type	Consequence of MV infection	Ref
Dendritic cells (DC)	IL-12 production ⇩ (IL-12 elicits production of IFNγ by T- and NK cells)	[a]
	IL-1alpha/beta production ⇩	[b]
	IL-10 production ⇧	[b]
	Type I interferon (IFN-α/β) ⇧ (IFN-α/β may enhance terminal differentiation of DC)	[a][c][d]
	Fas-mediated apoptosis 3-4 days after infection (CD95 and Fas-L ⇧; In syncytia massive virus replication occurs)	[a]
	Syncytia formation in activated DC cultures	[a]
	tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	[e][f]
	CD25, CD69, CD71, CD40, CD80, CD86, and CD83 expression ⇩	[b]
	CD40 signaling dependent maturation ⇩	[b]
	Affect T cell function by MV-F and -H expression	[g]
Monocytes	Apoptosis	[h]
	TRAIL ⇧	[e][f]
T cells	Proliferation after mitogen stimulation ⇩	[i]
	Mixed lymphocyte reaction ⇩	[j]
	Syncytia formation	[a]
	Apoptosis 7 days after infection	[a]
	Skewing of Th cells towards a type-2	[k]
	Cell cycle arrest in G1 phase	[j][l]
B cells	Cell cycle arrest in G1 phase	[m]
	Antibody production ⇩	[m][n][o]
CNS cells	Apoptosis	[p]
plasma	Interferon-gamma ⇧	[d]
	IL-18 production (apoptosis)⇧	[d]
PBMC	IFN-alpha/beta (IFN-α/β) production ⇩ (IFN-α/β are mediators of antiviral immunity)	[q]

[a] Fugier-Vivier I, Servet-Delprat C, Rivailler P, Rissoan M-C, Liu Y-J, Rabourdin-Combe C. Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and T cells. J.Exp.Med. 1997;186:813-23.

[b] Servet-Delprat C, Vidalain PO, Bausinger H, Manie S, Le Deist F, Azocar O, Hanau D, Fischer A, Rabourdin-Combe C. Measles virus induces abnormal differentiation of CD40 ligandactivated human dendritic cells. J Immunol 2000;164:1753-60.

[c] Servet-Delprat C, Vidalain P-O, Azocar O, Le Deist F, Fischer A, Rabourdin-Combe C. Consequences of Fas-mediated human dendritic cell apoptosis induced by Measles virus. J.Virol. 2000;74:438793.

[d] Okada H, Sato TA, Katayama A, Higuchi K, Shichijo K, Tsuchiya T, Takayama N, Takeuchi Y, Abe T, Okabe N, Tashiro M. Comparative analysis of host responses related to immunosuppressioon between measles patients and vaccine recipients with live attenuated measles vaccines. Arch.Virol. 2001;146:859 74.

[e] Vidalain PO, Azocar O, Lamouille B, Astier A, Rabourdin-Combe C, Servet-Delprat C. Measles virus induces functional TRAIL production by human dendritic cells. J Virol. 2000;74:5569.

[f] Hoskin DW. TRAIL: A newly described effector mechanism of cytotoxic lymphocytes. Mod.Asp.Immunobiol. 2000;1:136-9.

[g] Klagge IM, TerMeulen V, Schneider-Schaulies S. Measles virus-induced promotion of dendritic cell maturation by soluble mediators does not overcome the immunesuppressive activity of viral glycoproteins on the cell surface. Eur.J.Immunol. 2000;30:2741-50.

[h] Ito M, Yamamoto T, Watanabe M, Ihara T, Kamiya H, Sakurai M. Detection of measles virusinduced apoptosis of human monocytic cell line (THP-1) by DNA fragmentation ELISA. FEMS Immunol Med.Microbiol. 1996;15:115-22.

[i] McChesney MB, Altman A, Oldstone MBA. Suppression of T lymphocyte function by measles is due to cell cycle arrest in G1. J.Immunol. 1988;140:1269-73.

[j] Grosjean I, Caux C, Bella C, Berger I, Wild F, Banchereau J, Kaiserlian D. Measles virus infects human dendritic cells and blocks their allostimulatory properties for CD4+ T cells. J.Exp.Med. 1997;186:801-12.

[k] Karp CL, Wysocka M, Wahl LM, Ahearn JM, Cuomo PJ, Sherry B, Trinchieri G, Griffin DE. Mechanism of suppression of cell-mediated immunity by measles virus. Science 1996;273:228-31.
[l] Niewiesk S, Ohnimus H, Schnorr JJ, Gotzelmann M, Schneider-Schaulies S, Jassoy C, ter M, V. Measles virus-induced immunosuppression in cotton rats is associated with cell cycle retardation in uninfected lymphocytes. J Gen.Virol. 1999;80:2023-9.
[m] McChesney MB, Kehrl JH, Valsamakis A, Fauci AS, Oldstone MB. Measles virus infection of B lymphocytes permits cellular activation but blocks progression through the cell cycle. J Virol. 1987;61:3441-7.
[n] McChesney MB, Fujinami RS, Lampert PW, Oldstone MB. Viruses disrupt functions of human lymphocytes. II. Measles virus suppresses antibody production by acting on B lymphocytes. J Exp.Med. 1986;163:1331-6.
[o] McChesney MB, Oldstone MB. Virus-induced immunosuppression: infections with measles virus and human immunodeficiency virus. Adv.Immunol. 1989;45:335-80.
[p] McQuaid S, McMahon J, Herron B, Cosby SL. Apoptosis in measles virus-infected human central nervous system tissues. Neuropathol.Appl.Neurobiol. 1997;23:218-24.
[q] Naniche D, Yeh A, Eto DS, Manchester M, Friedman RM, Oldstone MBA. Evasion of host defenses by measles virus: wild-type measles virus infection interferes with induction of alpha/beta interferon production. J.Virol. 2000;74:7478-84.

Immune-suppression

One of the best-documented aspects of MV infection is the acute immune-suppression that can lead to secondary infections by bacteria and fungi or other viruses [37,38]. An overview of selected literature on the mechanisms that are possibly responsible for the MV-induced disruption of immune functions is provided in Table 1. It appeared that the immunosuppression associated with MV infection only occurs if both MV glycoproteins, F and H, are expressed on the membrane of infected cells and that F needs to be proteolytically activated [39,40]. Furthermore, it has been suggested that immunomodulatory peptide somatostatin (SRIF) may be involved through MV-dependent reduction of the capacity of adenylyl cyclase [41]. Adenylyl cyclase, which cyclizes ATP into 3'5' cyclic AMP, is essential for cell signalling pathways. Moabs against CD46 selectively depress IL-12 production [42].

Natural immunity (illustrated by figure 5)

Cell mediated immune (CMI) response to MV

Natural measles is generally believed to result in a lifelong protection against reinfection [1], although secondary immune responses against MV have been observed in adults [43]. Upon infection, the first encounter of MV with the immune system, the so-called innate or non-specific immune reaction, is the capturing (phagocytosis) of virus and viral antigens by monocytes (mono's), macrophages (mφ) and dendritic cells (DC). However, these cells may also become infected [44-46]. After encounter with the virus or its antigens, these cells, especially the DC, maturate into professional antigen presenting cells (APC) and migrate to the T cell areas of lymphoid tissues [47,48]. Meanwhile IL-12, primarily produced by MV-infected mono's, mφ and DC, activates natural killer (NK) cells, which together with the above-mentioned cell types establish a cytokine-mediated defence [49]. Activated NK cells produce IFNγ, which enhances IL-12 production, exerts direct antiviral effects and stimulates T cells [50]. IL-12 production is downregulated through IL-10, a product of Th2 cells (see below) and phagocytic cells, which in turn is induced by IL-12 [51]. MV-infected DC have been described to produce IFNα/β that stimulates IFNγ production but inhibits IL-12 production [52]. IFNα limits MV replication [53], and IFNβ increases the expression of major histocompatibility complex (MHC) class I molecules on infected cells [54,55]. NK cell cytotoxic killing is negatively regulated by MHC class I antigens on infected cells in the presence of IFNγ [56]. In measles patients increased levels of IL-18 have been detected [57]. IL-18, produced by virus-infected mφ, induces IFNγ production, enhances Fas ligand and perforin-mediated T cell and NK cell cytotoxicity, stimulates Th1 development (see below), decreases IL-10 expression in T cells, and modulates Ig secretion from B cells [58]. Thus, in the MV-infected tissues and in the draining lymphoid organs a cytokine milieu develops that regulates the quality of the specific or adaptive immune reaction by influencing APC functions and T cell differentiation. MV-specific T cells migrate and expand in response to MV-derived antigenic peptides exposed on the surface of APC in the context of MHC molecules.

There are two major types of T cells: T helper (Th), marked by the expression of CD4, and cytotoxic T cells (CTL), marked by the expression of CD8 [59]. Th cells become activated upon recognition of a peptide (a strand of 10 to 30 amino acids) bound in the antigen binding cleft of a MHC class II molecule of antigen presenting cells (APC), which mainly present exogenously derived antigen (for review see) [60]. In the lumen of endocytic compartments peptides are generated by enzymatic cleavage of viral proteins [61,62]. In the "MHC class II containing compartment" (MIIC) HLA-DM exchange the invariant chain CLIP fragment for antigenic peptides [63]. Subsequently, the peptide-MHC class II complex is exposed at the surface of the APC. CTL recognise a peptide (a strand of 8 to 11 amino acids) in the context of MHC class I molecules, which mainly sample the interior of the APC (for review see) [64]. From *de novo* synthesized viral proteins antigenic peptides are generated in the cytosol by proteolytic degradation by a multicatalytic protease, the proteasome [65-69]. The resulting protein fragments are translocated into the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing, TAP [70,71]. In the ER, the antigenic peptides bind to and stabilize the complex of MHC class I heavy chains and beta-2-microglobuline (β2M) [72]. The resulting trimolecular complex migrates to the cell surface via the secretory pathway. *De novo* viral protein synthesis is not required to charge MHC class I molecules on DC [73]. DC, but also mφ, have been shown to capture necrotic infected cells leading to so-called cross-

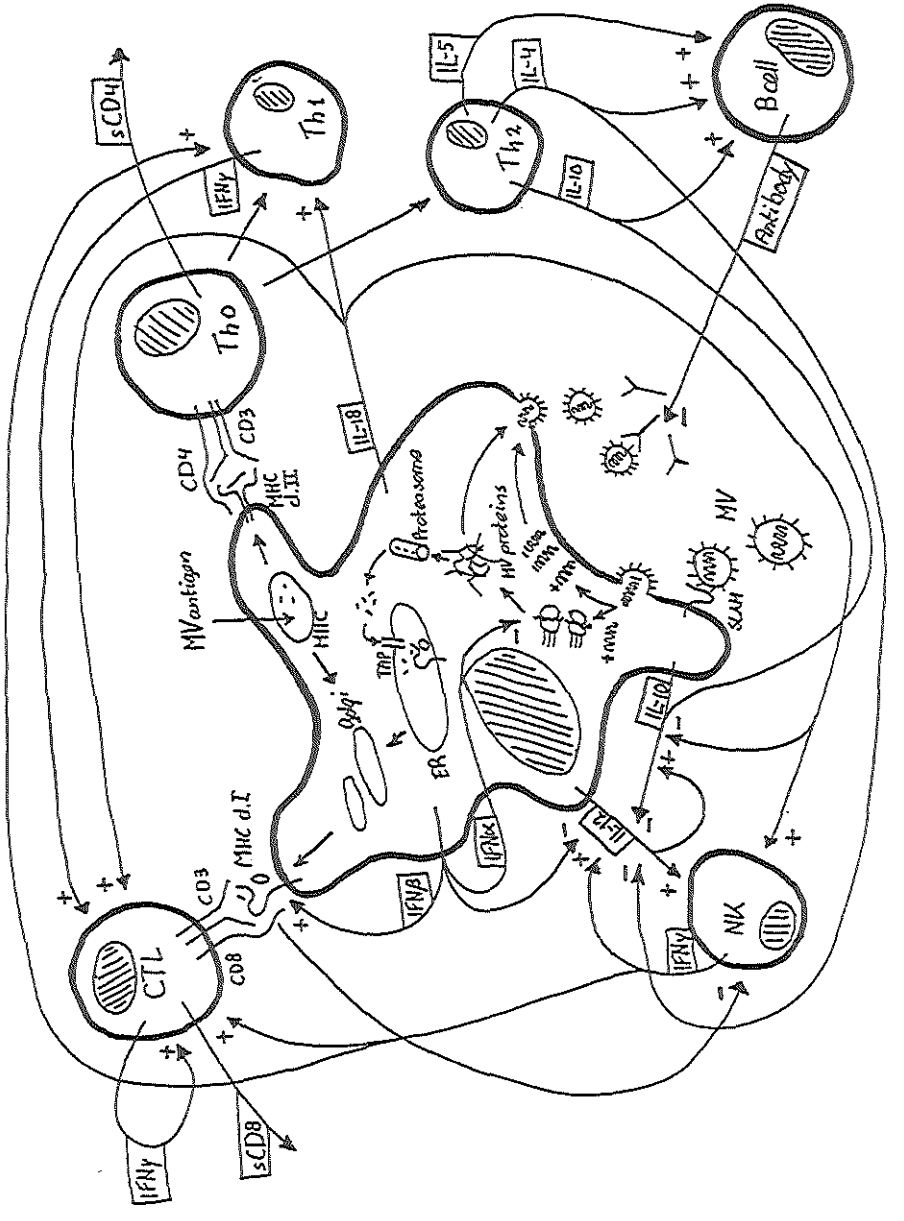

Figure 5. MV replication cycle, antigen processing and presentation, and innate and adaptive immune responses.

presentation of antigen and cross-priming of CTL [74]. Gromme *et al.* showed that recycling MHC class I molecules may acquire peptides in MIIC [75].
MV-specific CTL are detectable in blood from the moment of the rash and in bronchealveolar lavage from the moment of pneumonitis onward [76]. In addition, specific Th cells are activated in response to MV infection, with or without cytolitic capacities [77-82]. CTL are directly involved in the elimination of MV infected cells [83], whereas Th-cells regulate interactions between the humoral and T-lymphocyte-mediated immune responses by means of their differentiated subsets: Th1-cells (producing type-1 cytokines like IFNγ and TNFα) that succor the CMI, and Th2-cells (producing type-2 cytokines like IL-4) that succor the humoral immune response (for review see) [84]. In serum neopterin (a product of stimulated mφ), IFNγ and soluble IL-2 receptor are elevated before the appearance of the rash followed by the emergence of IL-2, soluble CD4, and soluble CD8 at the time of the rash [85]. Soon hereafter, IL-4 can be measured in serum, which remains detectable for weeks [86]. IL-4 negatively regulates NK responses [87]. Thus, an initial type I response gradually changes into a type II response.

Humoral immune response to MV
Upon infection, MV-specific serum antibodies are first detectable when rash appears (for review see) [1]. The isotype of MV-specific antibody is initially immunoglobulin (Ig)M and a few days later other MV-specific Ig classes and subclasses are found. IgA in respiratory secretions can be demonstrated in measles [88], and this class of antibodies may be important for protection against infection [89]. Antibodies to most viral proteins are eventually produced but the most abundantly and rapidly produced antibodies recognize the N protein. F- and H-specific Ig correlate well with neutralization of virus infectivity in tissue culture, i.e. virus neutralizing (VN) antibody [90]. MV-specific neutralizing antibody has been shown to be protective against measles in adoptive transfer experiments [91-93], in immune therapy and by transplacental passive immunization of the foetus [94]. As little as 0.1-0.2 IU/ml MV-specific VN antibody has been shown to be protective [95-97]. Maternal immunity in infants is detectable until an average age of six months (half-life of about 21 days) and is uncommon by 12 months of age [98]. Only in very rare cases the mother is naive for MV or antibody is not transferred through the placenta adequately [99]. Maternal immunity prevents MV infection, but also interferes with replication of the LAV virus, limiting its immunizing and protective effect [100]. Therefore, it can be concluded that MV-specific VN antibody plays a key-role in protection from MV-infection. However, children with agammaglobulinemia do not develop more severe measles after exposure than children without this immunologic handicap [101,102]. Furthermore, children with T cell deficiencies were shown to experience more severe disease, highlighting the importance of specific T-lymphocyte-mediated immune responses [1]. Human immunodeficiency virus (HIV)-infected children may show prolonged MV-shedding as compared to HIV-uninfected children ($\leq$46 days versus $\leq$28 days after the onset of rash) [103]. VN antibody clear cell-free virus, but also MV-infected cells can be cytolytically killed in an antibody-mediated fashion [104]. However, in the presence of VN antibody, MV spreads from cell-to-cell, when both H and F are present in the plasma membrane [105]. This illustrates that VN antibody can effectively protect the host from infection, but apparently, once MV is replicating, CTL are crucial for efficient clearance of the virus.

Vaccines and immune-pathogenesis

Measles, under natural circumstances, does not result in a persistent infection in man, no animal reservoir for MV exists, no vector is involved in its spread, only one serotype of MV has been defined and it is antigenically stable. Therefore, measles is a vaccine preventable disease and moreover, it should be considered theoretically eradicable [106].

Inactivated measles vaccine

In the 1960's children have been vaccinated with tween-ether or formalin-inactivated whole virus vaccines adjuvated with alum.. Although high seroconversion rates were observed (>95%) the virus neutralizing titers were short-lasting which necessitated multiple immunizations [107]. Furthermore, upon natural infection with MV, children vaccinated with the inactivated vaccine appeared to be predisposed for enhanced disease, i.e. atypical measles syndrome (AMS) [108,109]. AMS was characterized by a prolonged high fever; an urticarial, vesicular or petechial rash that began on the extremeties rather than a maculopapular rash beginning on the face and trunk, and severe pneumonitis, often requiring hospitalization [108,110,111]. Abdomal pain, hepatic dysfunction, headache, eosinophilia, pleural effusions, hilar adenopathy and edema were also described [112]. This had led to the abolishing of the use of inactivated vaccines. Curiously, a field trial was performed in the Netherlands from 1973 to 1976 to evaluate a tween-ether inactivated measles vaccine incorporated in the diphtheria-pertussis-tetanus-polio (DPTP) vaccine [113]. It took long before the underlying mechanism of AMS was elucidated, and even today we do not have a full understanding of the underlying immune-pathogenesis. First it was thought that a lack of functional antibody against the fusion protein caused AMS [114], but it appeared that AMS rather resulted from previous priming for a MV-specific but non-protective Th2 response [115].

Live attenuated measles vaccine

In the 1970's vaccination against measles was persued again, but now with the use of LAV vaccines which induced more balanced responses (antibody and cell-mediated immune responses). The use of LAV resulted in an impressive decline of measles cases especially in the developed countries [100]. Furthermore, recent evidence suggested that vaccination against measles and tuberculosis also reduce mortality from many other causes [116]. In most developed countries vaccination led to the almost complete elimination and control of measles [100].

Mechanism of attenuation and safety

Attenuation of MV for vaccination purposes was obtained by further propagation of the Edmonston-Enders MV strain in CEF cells, resulting for instance in the Schwarz vaccine [117]. Another measles vaccine strain derived from the Edmonston-Enders virus, the Edmonston-Zagreb strain, was at the end passaged in human diploid cells. Generally, the EZ vaccine is more immunogenic, showing higher sero-conversion rates as compared to the Schwarz vaccine. It has even been reported that a second dose of EZ vaccine is able to increase antibody levels [118].

Despite many efforts, the underlying mechanism of attenuation has not yet been elucidated. LAV, in which the mechanism of attenuation would be defined at the molecular level, would offer many advantages. Amino acid changes and silent mutations scattered over the MV proteins in vaccine strains have been described, but how they relate to the molecular basis of

attenuation is not known [119]. Sequence comparison between a wildtype MV that was isolated in B95a cells and its Vero cell-adapted form showed a few amino acid changes in the polymerase, and V and C [120]. Nevertheless, these mutations can be very useful as "vaccine strain signature" for comparison with wild-type MV isolates, which may help to recognise vaccine-related illness [119]. Although the first licensed LAV gave undesired side effects in a large proportion of immunized children, the currently used LAV, which was further attenuated, cause a mild clinical reaction in a minority of immunized children [121]. The vaccine should not be given to pregnant women because of a potential risk for teratogenic effects [122]. Furthermore, the occurrence of LAV-associated disease in immunocompromised individuals has been reported [123-125]. A point of concern is the question whether LAV could revert to more virulent strains. It has e.g. been reported that the oral polio vaccine (OPV), also an attenuated virus, had regained the ability to cause an outbreak of disease [126]. However, in more than 30 years of experience with the application of measles LAV, reverted viruses have not been discovered. Another point for attention is that contamination of LAV with other agents in the production process needs to be ruled out. The measles LAV have a history of passages in chicken embryonic fibroblasts (CEF) [117]. Reverse transcriptase activity, associated with the presence of avian retroviruses has been detected in chicken cell-derived measles and mumps vaccines [127]. However, data that support transmission of such viruses to vaccine recipients have not been reported. Recently, evidence was found for the presence of monkey DNA in oral polio vaccine [128]. Together with reports like "monkey virus DNA found in rare human cancers", such concerns cause quite some disturbance [129].
The safety of administering LAV, alone or as MMR, in children with allergy to egg proteins has been addressed [130,131]. A single dose showed only minor reactions at the site of injection, and cases of anaphylaxis after the administration of MMR could not be explained by allergy to eggs [131].

Stability and application of LAV
A disadvantages of the LAV, which probably contributes to primary vaccine failure is that for keeping the vaccine effective, maintenance of the cold chain is essential. This necessitates special logistics. The vaccine is distributed as freeze-dried virus that should be reconstituted just before administration. The currently used LAV is subcutanously administered using needles, which may cause people to refuse vaccination but more importantly, may lead to cross-infections.

Timing of vaccination and vaccination programs
A major stumblingblock of the currently used LAV is vaccine failure due to the presence of MV neutralizing antibody, of maternal origin or from previous vaccination [100]. The moment at which the amount of maternally derived antibodies has dropped to levels that do not interfere with vaccine efficacy is individually determined by the immune status of the mother. This is determined by her infection or vaccination history [132]. In an attempt to overcome vaccine neutralization by pre-existing immunity against MV, LAV has been applied with a dose 100-fold to 1000-fold higher. However, this apperently led to a poorly understood increased mortality in girls in subsequent years as compared to infants vaccinated with standard titer LAV [133-136].
In developed countries epidemiological studies showed that the average age at infection is about 2 years, which makes it possible to vaccinate children with a standard dose at about 14 months of age. In contrast, timing of the optimal moment for vaccination is difficult in

developing countries where measles is endemic [100,137]. Here, vaccination cannot be delayed until all children have lost maternal antibody, because by then many children have already been infected with MV. In developing countries, vaccination at an age of 6-9 months was predicted to maximally reduce mortality and morbidity [138].
High coverage with a single dose of LAV has controlled measles in many countries. However, the wish to eliminate measles makes higher demands. Seroconversion rates upon LAV vaccination are about 95%, even under optimum conditions, which leaves susceptible individuals in the population, and it has been recognised that LAV immunization does not warrant life-long protection [139,140]. Therefore, several countries introduced a two-dose vaccination regimen (for review see) [137]. A 'second' dose of MMR has been shown to prevent the persistence of MV susceptible individuals by reaching people that had not been vaccinated before and people with waning vaccine-induced immunity, who would otherwise sustain transmission of MV [141,142]. Different vaccination regimens, like one dose at one year of age versus a first dose at six months of age followed by a second dose at 15 months of age, have been evaluated for infants born to naturally immune mothers versus vaccinated mothers [143].
It has been suggested that primary vaccine failure (non-protective antibody response after vaccination) may in part be attributed to immunogenetic parameters like HLA genes [144]. In vaccinated populations with waning vaccine-induced immunity occurrence of subclinical measles has been reported [145]. Recently, a comparative analysis of host immune responses related to immunosuppression between measles patients and LAV immunized children revealed that LAV induces lower immune responses to the virus compared to wild-type infection, which may explain waning vaccine-induced immunity [57].

At this moment, none of these drawbacks are a reason to withdraw the LAV from the market, but they need to be taken into consideration in the process of developing new vaccines or vaccination strategies for the eradication of MV.

Surveillance
Effective surveillance of measles and measles vaccine coverage is a prerequisite for the control of measles. Therefore, laboratory methods to confirm measles virus infection that are simple, inexpensive, and practical in field conditions are indispensable. Recently, it has been shown that a small volume of blood spotted on filter paper is stable and is suitable for the laboratory diagnosis of measles using a combination of reverse transcriptase PCR analysis and IgM detection [146].

The future of vaccination against measles
Despite all global efforts to control measles, an estimated 36.5 million cases and almost 1 million deaths caused by measles still occur each year. Therefore, the CDC, the Pan American Health Organization (PAHO), the World Health Organization (WHO), and the United Nations Children's Fund (UNICEF) collectively adopted a strategy that includes "catch-up"-, "keep-up"- and "follow-up" vaccination campaigns [147,148]. Application of this strategy has substantially reduced measles transmission in the Americas. For these approaches the LAV were selected, leaving questions like: "do we need new measles vaccines" or "can the present vaccine be used more efficiently", unanswered [149].
New generation of candidate measles vaccines include: live virus vectors (like pox viruses, adenoviruses), live bacterial vectors (like *Shigella flexneri*, *Salmonella typhimurium*), sub-unit

or synthetic vaccines (like iscoms, biodegradable particles, lipopeptides) and nucleic acid vaccines (plasmid DNA). In this thesis representatives of the different categories were studied and their advantages and disadvantages are discussed in chapter 7 (Summarizing discussion, page).

Animal models

Over the past decades several animal models have been used for studying the pathogenesis of measles as well as for the evaluation of new vaccine candidates and new vaccination strategies. Different rodents including mice [150], rats [151], ferrets [152] and hamsters [153] have been used to study aspects of experimental MV-induced encephalitis (EMVIE) as a model for neurologic disease, and to study MV antigen-induced immune responses using EMVIE as a read out for protection. These animal species are not susceptible to wildtype MV. However, several MV strains have been adapted for use in rodents, although virus replication is in general only detectable after intracerebral inoculation of very young animals. Rodent-adapted MV strains do not produce acute disease similar to that seen in humans, since the infection does not become disseminated. CD46 transgenic mice and rats proved to be more susceptible to EMVIE (for review see) [32], but no virus replication was detectable in the respiratory tract. It has been proposed that this non-permissiveness is due to an intracellular block of MV replication [154], which may also be an obstacle in future SLAM transgenic animals. On the other hand, CD46 transgenic mice defective for the IFNα/β receptor showed lymphatic dissemination [155]. Small animals in which wildtype MV is able to replicate are humanised mice or human-mouse chimeras. Severe combined immune deficiency (SCID) mice, xeno-grafted with human fetal thymus and liver cells, were used to study virulence of wildtype and vaccine MV strains [156,157]. CBA/N mice, grafted with human PBL, were used to study MV vaccine-induced protection in transfer experiments [158]. It needs no explaining that these animals do not develop measles-like disease.

The most successful rodent model for measles research appeared to be the cotton rat (*Sigmodon hispidus*) model [159-163]. Cotton rats can be infected intranasally with LAV and non-culture adapted widtype MV isolates. MV can be re-isolated from lung tissue, pharyngeal epithelial cells (PEC), draining lymph nodes and spleen for at least 7 days after virus inoculation, and using RT-PCR MV RNA has also been demonstrated in other organs. Furthermore, immune suppression of infected cotton rats has been demonstrated by reduced proliferation of spleen cells upon mitogenic stimulation [154]. The interference of pre-existing VN antibody with vaccination in cotton rats was addressed by transfering MV-specific antibodies of human- or cotton rat origin and by vaccination of the offspring from sero-positive dams [162,164,165].

From the earliest days of measles vaccine research, primates have been used because of their high susceptibility to MV [166]. Non human primates including marmosets (*Saguinus mystax*) cynomolgus- and rhesus macaques (*Macaca fascicularis* and *Macaca Mulatta*, respectively) and baboons (*Papio hamadryas* and *Papio hybridus*), proved to be most relevant for measles research (for review see) [167,168]. Macaques have been shown to be highly susceptible to MV infection as is illustrated by natural outbreaks and the fact that intratracheal inoculation with 1 $TCID_{50}$ is enough to cause MV viraemia [169]. It has been shown that the pathogenesis

of MV infection and development of specific immunity in macaques is largely similar to that in humans [169-171]. Clinical signs in macaques, albeit with varying severity in different macaque species, include mild fever, leukopenia, mild respiratory signs and rash. Upon intratracheal infection with wildtype MV, infectious MV can be quantitatively demonstrated in peripheral blood mononuclear cells (PBMC), lung lavage cells (LLC) and PEC, showing kinetics of viral loads that resemble MV viraemia in humans. The macaque models have allowed research on vaccine efficacy, in the presence and absence of passively acquired VN antibody [96,172,173]; vaccine safety [174], including comparison of the virulence of different virus strains [169,175]. Today, techniques and reagents to study immunological mechanisms in non-human primates like T-cell proliferation assays, methods to detect specific antibody and reagents to measure cytokine production and cytokine producing cells, are to a large extent available.

Conventional mouse- and rat-strains have been used for studying the antigenicity of MV-derived antigens and candidate vaccines, and the type of immune response induced by these antigens. However, these studies are complicated by the fact that the type of immune reaction (Th1/Th2-like responses) varies among inbred laboratory animals. Transgenic and knock-out mice have been used to study different aspects of MV-specific immune responses. Transgenic mice expressing human MHC and CD8 molecules mounted CTL with similar specificities as compared to humans with natural MV infection [176].

Although the pathogenesis of other morbillivirus infections in several animal species is often quite similar to that of MV infection in humans, models using other animal morbilliviruses, like canine distemper virus (CDV) in ferrets and dogs, are not selected for this thesis.

Aims and outline of the thesis

The development of more efficacious vaccines and vaccination strategies for human and animal virus infections is subject of considerable effort. In the present thesis several approaches to develop a new generation of measles vaccines have been addressed. A major theme was related to studies aiming at the induction of both VN antibodies and HLA class I-restricted cytotoxic T lymphocyte (CTL) responses. The latter are considered to play a major role in the clearance of MV: their role in the elimination of MV infected cells during infection is considered essential. To induce CTL or activate memory CTL, MV antigens have to enter the endogenous Ag processing and presentation pathway in an APC, which generally requires *de novo* Ag synthesis. Certain vaccine formulations, however, may allow non replicating, exogenous Ag to enter this pathway. Developments in organic chemistry, biochemistry and molecular biology in the past decades have boosted efforts to formulate new generations of vaccines, which indeed allow the efficient induction of both VN antibodies and HLA class I-restricted CTL responses.

The investigations described in chapter 2 aim at the elucidation of the mechanism by which lipidated peptides as a vaccine candidate, gain access to the MHC class I processing and presentation pathways. This is important to understand how CTL can be activated by this antigen presentation form. Chapter 2.1 describes the synthesis of (double) fluorescent labelled lipopeptides and chapter 2.2 describes a series of experiments exploring the potential of such lipopeptides, with different spacer sequences between the lipid tail and the CTL epitope, using T cell stimulation assays and confocal microscopy.

In chapter 3 the use of purified Quil A components as candidate adjuvants for whole-inactivated or subunit measles vaccines, is studied. The selection of purified Quil A components in vitro and the immunogenicity in mice are described in chapter 3.1, immunogenicity and protective efficacy in a cotton rat model for measles in chapter 3.2 and the long-term immunity is studied in a macaque model for measles in chapter 3.3.

Chapter 4.1 describes the immunogenicity and protective efficacy of a recombinant MVA-based candidate measles vaccine in the absence and presence of VN antibody in macaques. The safety of the vaccine vector in immune-suppressed macaques is addressed in chapter 4.2.

Application of LAV via the oral route, as alternative to the current parental route, is evaluated in Chapter 5.1.

Chapter 6. describes the immunogenicity and protective efficacy of nucleic acid vaccination against measles in macaques. Different combinations of DNA plasmids encoding F, H or N were transdermally delivered using a gene gun.

Chapter 7. provides a summarizing discussion of the thesis

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Chapter 2.1

In vitro processing and presentation of a lipidated cytotoxic T cell epitope derived from measles virus fusion protein

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ABSTRACT

Lipopeptidic formulations have been described as efficient activators of cytotoxic T lymphocytes (CTL). To better understand the pathway via which lipopeptides reach the MHC class I molecules we studied the intracellular processing and presentation of a measles virus-derived CTL epitope, to which a palmitoyl moiety was added synthetically. The palmitoyl group was conjugated to the N-terminus either directly or via a spacer sequence. The use of single or double fluorescent-labeled lipopeptides allowed the visualization of intracellular processing of these antigens using confocal microscopy. Our data indicate that the spacer composition influences internalization of the conjugate into the cell, proteasomal degradation, translocation into the ER by the Transporter associated with Antigen Processing (TAP), and the intracellular trafficking of lipopeptides.

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) play an important role in the elimination of intracellularly replicating microorganisms [1-3]. The efficacy of vaccines directed against intracellular pathogens therefore strongly depends on the induction of an effective CTL response against pathogen-encoded antigens. In order to activate CTLs, the antigen has to enter the endogenous antigen processing pathway, which generally requires *de novo* synthesis of pathogen-encoded proteins. Although living microorganisms usually activate CTLs efficiently, their pathogenicity frequently precludes their application in vaccines. In addition, the presence of pre-existing antibodies may interfere with the induction of effective T cell immunity. In case of measles virus, neutralization of the vaccine virus by maternal antibodies is a well-established phenomenon [4].

In addition to wild type or attenuated microorganisms, chemically prepared antigens have been used successfully to induce cytotoxic T cell responses. In particular, lipopeptides have been shown to be efficient activators of cytotoxic T lymphocytes [5-7]. The presence of a fatty acid tail, covalently linked to the peptide moiety, mediates translocation of the antigen into the cell [8]. In addition, the lipid tail provides the peptide with intrinsic adjuvant activity [5,9] and allows anchoring of the peptide into larger antigen delivery systems such as liposomes [10,11] and ISCOMS [12,13].

The activation of CTLs requires the presentation of pathogen derived peptides in the context of MHC class I molecules at the surface of the infected cell. The antigenic peptides are generated in the cytosol by proteolytic degradation of viral proteins by a multicatalytic protease, the proteasome [14-16]. The resulting protein fragments are translocated into the lumen of the endoplasmic reticulum (ER) by the Transporter associated with Antigen Processing, TAP [14]. In the ER, the antigenic peptides bind to and stabilize the complex of MHC class I heavy chains and beta-2-microglobuline. The resulting tri-molecular complex migrates to the cell surface via the secretory pathway.

The effect of palmitoylation of peptide epitopes on their immunogenicity has been investigated using functional T cell assays, such as *in vitro* cytotoxicity assays. Specific CTL responses have been demonstrated to be of a magnitude similar to that observed after viral infection [5,6]. The mechanism of membrane passage by lipopeptides has been studied previously using lipidated analogues of a pseudosubstrate sequence derived from protein kinase C [8]. Peptides that are palmitoylated at their N-terminus appear to be translocated into the cytosol efficiently.

It is unclear, however, at what stage lipopeptides enter the endogenous antigen presentation pathway. It has not been established how lipopeptides reach the cytosol, nor whether these preparations are translocated into intracellular organelles, such as the ER, the compartment where newly synthesized MHC class I molecules mostly are loaded with peptides. If the lipopeptides do enter the ER, it remains to be established whether the translocation process depends on transport by TAP. It has been proposed that the peptide encompassing the actual CTL epitope has to be liberated from the lipid tail prior to binding to the MHC class I molecule [9]. It is unknown where such a modification would take place and what enzymes would catalyze this process.

In earlier studies, we have investigated T cell immunity to the measles virus-encoded fusion (F) and hemagglutinin (H) glycoproteins in measles virus patients [17-20]. CTL clones have been isolated from these patients. Using these T cell clones we have identified a CTL epitope, $F_{438-446}$, that is recognized in the context of HLA-B*2705 [21]. In the present study we have prepared a series of N-terminally palmitoylated analogues of $F_{438-446}$ (Table 1). The palmitoyl moiety was either coupled directly to the $F_{438-446}$ peptide or a spacer sequence was included, composed of two or four lysyl

residues or the sequence Cys-Ser-Ser [22]. The measles virus F protein-specific T cell clones were used to evaluate the biological properties of these lipopeptides. We have investigated whether the palmitoylated peptides bind to MHC class I molecules at the cell surface or whether they enter the endogenous antigen processing pathway. For lipopeptides that are translocated into the cytosol we have investigated whether CTL activation is dependent on a functional TAP molecule. The role of the proteasome in lipopeptide processing was studied using a proteasome-specific protease inhibitor and an *in vitro* proteasomal degradation assay. Finally, with (double) fluorescent-labeled lipopeptides the intracellular processing of these antigens was visualized using confocal microscopy.

MATERIALS AND METHODS

Chemicals

Brefeldin A (BFA; Sigma, St. Louis, USA), the aldehyde carbobenzoxy-*L*-Leucyl-*L*-Leucyl-*L*-Leucinal (CbzL3; Peptide Institute Inc., Minoh-shi Osaka, Japan), Pronase (Sigma, St. Louis, USA), DNase II (Boehringer Mannheim, Almere, The Netherlands) and 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma, St. Louis, USA) were used at the concentrations indicated in the figures and the legends.

Peptides

Table 1 shows an overview of the peptides used in the present study. The peptides were synthesized and HPLC-purified as described earlier [23]. Fluorescent-labeled peptides were lyophilized and stored at –20 °C. Before use, the labeled peptides were dissolved in water. The solutions thus obtained were stored at 4 °C for a maximum period of one week. Unlabeled peptides were dissolved in 10% acetic acid at a concentration of 1 mM and stored frozen at –20 °C. Before use, the stock solutions were thawed and aliquots were diluted with water and neutralized with sodium hydroxide.

Table 1. Summary of the synthetic peptides and their abbreviations used in these studies

Arg-Arg-Tyr-Pro-Asp-Ala-Val-Tyr-Leu	$F_{438-446}$
Palmitoyl-Arg-Arg-Tyr-Pro-Asp-Ala-Val-Tyr-Leu	$Pal\text{-}F_{438-446}$
Palmitoyl-Lys-Lys-Arg-Arg-Tyr-Pro-Asp-Ala-Val-Tyr-Leu	$Pal\text{-}K_2\text{-}F_{438-446}$
Palmitoyl-Cys-Ser-Ser-Arg-Arg-Tyr-Pro-Asp-Ala-Val-Tyr-Leu	$Pal\text{-}CSS\text{-}F_{438-446}$
Palmitoyl-Lys-Lys-Lys-Lys-Arg-Arg-Tyr-Pro-Asp-Ala-Val-Tyr-Leu	$Pal\text{-}K_4\text{-}F_{438-446}$
Palmitoyl-Lys-Lys-Arg-Arg-Tyr-Pro-Asp-Ala-Val-Lys^{FL}-Leu	$Pal\text{-}K_2\text{-}F_{438-446}{}^{FL}$
Palmitoyl-Lys^{TMR}-Lys-Lys-Lys-Arg-Arg-Tyr-Pro-Asp-Ala-Val-Lys-Leu	$Pal\text{-}K_4{}^{TMR}\text{-}F_{438-446}$
Palmitoyl-Lys^{TMR}-Lys-Lys-Lys-Arg-Arg-Tyr-Pro-Asp-Ala-Val-Lys^{FL}-Leu	$Pal\text{-}K_4{}^{TMR}\text{-}F_{438-446}{}^{FL}$

Determination of the critical micelle concentration of lipopeptides

Lipopeptides can occur in a solution in monomeric- or multimeric forms, depending on their physical chemical properties and concentration. Due to their amphipathic character, lipopeptides have the potency to form micelles. Lipopeptides forming micelles and the monomeric forms are likely to interact with cells differently. At concentrations below the CMC, the amphipathic molecules will bind to the cell membrane by partitioning into the lipid bilayer [27]. At concentrations above the CMC, the cell membrane will disintegrate to form mixed

micelles with the amphipathic molecules. Thus, the concentration dependent micelle formation will influence delivery of the lipopeptides antigens to the cell and may affect the composition of the cell membrane.

To determine whether the lipopeptides used in this study occur in a monomeric or multimeric form, the critical micelle concentration (CMC), i.e. the lowest concentration at which monomers cluster to form micelles, was measured. The CMC was determined using a hydrophobic fluorescent probe, 1,6-dephenyl-1,3,5-hexatriene (DPH) [15]. In this assay, the concentration of the lipopeptide was increased gradually in the presence of 5 μM DPH in phosphate buffered saline. After 18 h of equilibration in the dark at room temperature, the fluorescence intensity was measured at 25 °C using a luminescence spectrometer type LS-50B (Perkin Elmer, Norwalk, Connecticut, USA) supplied with a FL Winlab software package. A sharp increase of fluorescence was observed when the CMC was reached. The steepness of the curve depends on the amount of DPH-molecules entrapped in the micelles. The non-ionic detergent Triton X-100 (CMC = 0.2-0.9 mM) and the zwitterionic detergent Zwittergent 3-16 (Zwitt.3-16, CMC = 0.01-0.06 mM) were included for comparison. Zwitt.3-16 was selected because its hydrophobic tail resembles palmitic acid.

For Triton X-100 and Zwittergent 3-16 a steep increase of the fluorescence was observed, which reflects the formation of micelles (Fig. 1). The CMC of Pal-$F_{438-446}$ ranges from 20-500 μM. At the concentrations tested, no CMC could be determined for Pal-K_2-$F_{438-446}$ and Pal-K_4-$F_{438-446}$.

From these experiments we can conclude that some of the lipopeptides used in this study are in principle capable of forming micelles, but none of them will do so at the concentrations at which they are used.

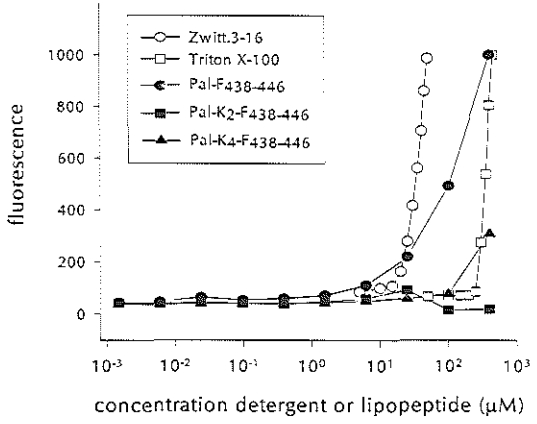

Figure 1. CMC of Zwitt. 3-16 (○); Triton X100 (□); Pal-$F_{438-446}$ (●); Pal-K_2-$F_{438-446}$ (■); and Pal-K_4-$F_{438-446}$ (▲) and shown the fluorescence of DPH in the presence of increasing lipopeptide or detergent concentrations.

Cells

The MV-$F_{438-446}$ specific human CTL clone (WH-F40) and the autologous EBV transformed B cell line (B-LCL WH) have been described elsewhere [21]. The tumor necrosis factor-alpha (TNF-α)-sensitive cancer cell line WEHI 164 [24] was cultured in RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with 10% (v/v) fetal calf serum (FCS), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies)) and 2 mM L-glutamine (Life Technologies), referred to as complete medium. The TAP2 mutant cell line BM36.1 [25] was transfected with HLA-B*2705 (Geneticin selection, 600 μg/ml; Calbiochem-Novabiochem corporation, La Jolla). The resulting cell line is

called BM36.1/B*2705. The TAP2 mutant cells were also transfected with TAP2 (L-histidinol dihydrochloride selection, 750 μg/ml; Sigma, St. Louis, USA). The latter cell line is referred to as BM36.1/B*2705/TAP2. The monocyte-like cell line U937 was cultured in DMEM (Life Technologies) with the supplements indicated for the RPMI 1640 complete medium. The human melanoma cell line Mel-JuSo transfected with HLA*B2705 is referred to as Mel-J*B2705.

Paraformaldehyde fixation of antigen presenting cells

Antigen presenting cells (APCs) were washed twice with PBS and fixed with 1% (w/v) paraformaldehyde (PFA) in PBS for 15 min at room temperature. The cells were then treated twice with 0.2 M glycine in PBS for 15 min at room temperature. After a final wash with PBS, the cells were quantified and immediately used as antigen presenting cells.

Determination of antigen presentation

The autologous B-LCLs WH or the transfectants BM36.1/B*2705 or BM36.1/B*2705/TAP2 cells were used as APCs. Incubations with (lipo)peptides were carried out at 37 °C in serum free medium. The CTL clone WH-F40 was used to monitor the presence of functional HLA-B*2705-peptide complexes at the surface of the APC. The activation of the T cell clones was quantified using a ^{51}Cr-release assay or by measurement of TNF-α production by the CTL clone.

^{51}Cr-release assay

APCs (10^6) were labeled with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham, Buckinghamshire, UK) during a 1 h incubation. The ^{51}Cr-labeled cells were washed three times, counted, and resuspended in the appropriate volume. The ^{51}Cr-labeled target cells (10^4 cells in a final volume of 150 □l) were co-cultivated with the CTL clone at different effector-to-target (E:T) ratio's during 4 h at 37 °C. The amount of ^{51}Cr released into the supernatant was measured using a gamma counter. The specific CTL activation was calculated as (cpm_{sample}- $cpm_{spontaneous\ release}$ / $cpm_{maximal\ release}$ - $cpm_{spontaneous\ release}$) x 100%. The experiments were carried out in triplicate and were repeated at least twice.

TNF-α bioassay

In case of fixed APCs were used the TNF-α production by the CTL clone was measured. The APCs were co-cultivated with the CTL clone at different E:T ratio's in a final volume of 150 μl. After overnight incubation at 37 °C, the supernatants were collected. Aliquots of 100 μl of diluted supernatants were incubated with 5 x 10^4 WEHI 164 cells in medium supplemented with 40 mM lithium chloride and 2 μg/ml actinomycin D [24]. After incubation at 37 °C for 20 h, 50 μl of 2.5 mg/ml 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) in PBS was added. The cells were incubated for another 2 to 4 hrs. The reaction was terminated by addition of 100 μl 20 % SDS in *N,N*-dimethylformamide. Reduced MTT was dissolved during overnight incubation at 37 °C. Using an ELISA microplate reader the absorbance (A) was measured at 550 nm and 690 nm. The specific CTL activation was calculated as 1- (A_{sample} / $A_{TNF\text{-}\alpha\ control}$) x 100%. The experiments were carried out in triplicate and were repeated at least twice.

Removal of surface exposed MHC class I molecules

To remove the MHC class I molecules from the cell surface, the cells were incubated with 4 mg/ml Pronase in the presence of 1 μg/ml DNAse II at 37 °C during 30 min [26]. Membrane stripping was stopped by the addition of two volumes of undiluted FCS, followed by two washes with serum-free medium (PFHM, Gibco Laboratories).

In vitro proteasome assay

U937 cells (10^8) were cultured in the presence of human IFN-γ (10^5 U/l, SanverTECH, Heerhugowaard, Netherlands). The cells were lysed in demineralized water and homogenized with a Potter-Evehem homogenizer. After homogenization, 20 mM Tris-HCl, pH 7.5, with 2 mM ATP, 5 mM $MgCl_2$ and 1 mM DTT (compensation buffer) was added. Post-nuclear supernatants (~1 ml) were separated on a sucrose gradient (10% to 40% sucrose in compensation buffer). The lysate was centrifuged for 21 h at 35,000 rpm in a Beckman SW41Ti rotor. Fractions of ~0.65 ml were collected. From each fraction a sample of 10 µl was collected and assayed for proteasome activity by measuring degradation of the fluorogenic substrate Suc-LLVY-MCA (Sigma) at a concentration of 100 µM at 37 °C for 40 minutes. The reaction buffer contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 5 mM DTT. Fractions that were shown to contain proteasomal activity were pooled and dialyzed overnight against compensation buffer. The dialyzed protein solutions were concentrated to a volume of about 1 ml using a 100k centrifugal concentrator (Filtron, Northborough, MA 01532). The proteolytic activity of the preparations was measured once more.

Usually, about 1000 units (U) of proteasome activity were used for each digestion. An activity of 1 U hydrolyses 1.5 nmol Suc-LLVY-MCA per h. After incubation for 1-4 h, digests were fractionated on a 2.1 mm x 10 cm HPLC C2/C18 column (Pharmacia SMART) using an acetonitrile in water gradient (from 0% to 60% acetonitrile), containing 0.1 % trifluoroacetic acid (TFA), at a flow rate of 0.1 ml/min. The column effluent was monitored using UV-detection at 214 nm. The samples were freeze-dried and stored at -70 °C before analysis.

Peptide fractions were loaded onto a microcapillary reversed phase-HPLC (mLC) column switching system, consisting of an C8 precolumn (Alltima, 7 mm x 0.1 mm i.d., mobile phase 0.1 M HOAc in water, flow 10 ml/min) and an RP18 analytical column (Purospher, 225 mm x 0.1 mm i.d., mobile phase 0-85% acetonitrile gradient, containing 0.1 M HOAc, 5%/min, flow 0.5 µl/min). The eluent flow was directed into the electrospray ionization (ESI) source. The eluent consisted of 1.5 ml/min of water/methanol (3:7 v/v). Mass spectra were recorded every 5 s on a mass spectrometer (Finnigan MAT 95, Bremen, FRG) over a mass range of 400-1500 Da. The digestion products were identified on the basis of their molecular mass.

Confocal microscopy.

Binding and internalization of lipopeptides was studied using Mel-J*B2705 cells. For microscopical studies, trypsinized Mel-J*B2705 cells were allowed to grow on 8-wells Lab-Tek glass chamber slides (Nalge Nunc International, USA) overnight and washed in PFHM. The cells were pretreated with BFA or Cbz-L3 for 2 h at 37°C. The cells were incubated with the different lipopeptides in the continuous presence of BFA or Cbz-L3 for up to 60 min at 4°C or 37°C. The cells were washed with PFHM at room temperature, fixed in 2% paraformalin (pH 7.6), mounted in Vectashield and observed under a NIKON Optiphot 2 microscope. Identification of Golgi apparatus and endoplasmatic reticulum was done with immunolabeling for golgin-97 (CDF4, Mol.Probes) and calnexin (AF8, gift from Dr. M.B. Brenner) on fixed cells after incubation with the lipopeptide [Pal-K_3-$F_{438-446}^{Fl}$]. Both monoclonal antibodies were stained with a Cy5 labelled $F(ab)_2$ DoM-IgG (Jackson ImmunoRes lab, Inc.). The fluorescence was imaged using a BioRad 1024 confocal laser scanning microscope equipped with an Argon/Krypton laser. The 488-nm line was used to excite fluorescein and the 568-nm line was used to image rhodamine. Confocal images were recorded with a ×20 and ×60 Plan Apo objective lenses. Images were processed with Adobe Photoshop 4.0 (Adobe Systems, USA). The fluorescence of fluorescein was pseudocoloured as green and that of rhodamine or Cy5 as red, respectively.

Statistical analysis

The two-tailed Student's t-test was used for a pair wise comparison of means, assuming a Gaussian distribution of each of both variables concerned. As multiple pair wise comparisons were made in this study, the type-1 error rate α was lowered from the usual 0.05 to 0.01. Hence, for each tests a *p*-value of less than 0.01 was considered to denote statistical significance.

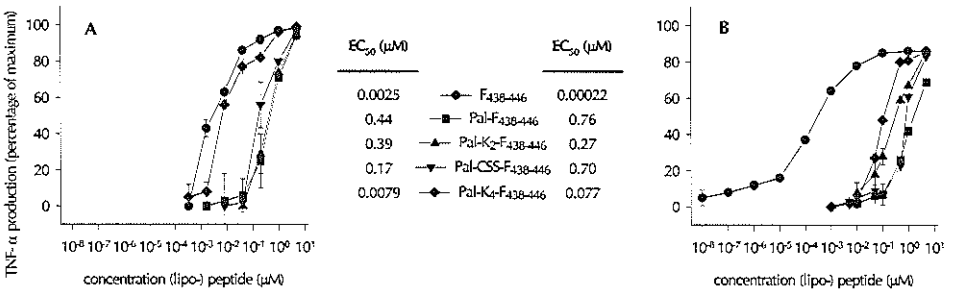

Figure 2. T cells recognize lipopeptides presented by vital and fixed APCs. EBV -transformed B cells (APCs) were washed with PBS and either left vital (A) or fixed with 1% paraformaldehyde solution (B). The cells were incubated with different concentrations of $F_{438-446}$ (●), Pal-$F_{438-446}$ (■), Pal-K_2-$F_{438-446}$ (▲), Pal-CSS-$F_{438-446}$ (▼) and Pal-K_4-$F_{438-446}$ (◆) for 1 hour. Subsequently, the cells were co-cultivated with the MV-$F_{438-446}$ specific $CD8^+$ T cell clone for 4 hours. Supernatants were harvested and the TNF -α concentration was measured in a 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay using TNF-α sensitive WEHI 164 cells. The concentration of peptide at 50% of the maximal response (EC_{50}) was determined by sigmoidal regre ssion calculations on the dose-response curves using SigmaPlot software.

RESULTS

Intracellular processing of lipopeptides: presentation by vital APCs versus PFA-fixed APCs

The antigenicity of the lipopeptides was tested using a T cell clone recognizing the measles virus fusion protein-derived epitope $F_{438-446}$. To investigate whether the lipopeptides require processing by the APCs, CTL activation was measured using vital and pre-fixed target cells. All lipopeptides appear to be capable of activating the CTL clone in a dose dependent manner. In the presence of vital APCs (Fig. 2*A*), a significantly higher response was observed to the parental peptide and Pal-K_4-$F_{438-446}$, compared to Pal-$F_{438-446}$, Pal-K_2-$F_{438-446}$ and Pal-CSS-$F_{438-446}$ (see EC_{50} values).

A completely different pattern is observed when the APCs are fixed prior to incubation with the peptides (Fig. 2*B*). The peptide $F_{438-446}$ induces TNF-α production at very low concentrations when fixed APCs are used. This is probably due to the fact that cytotoxic T cells do not eliminate the fixed APCs, which can therefore continue to stimulate the T cells. Fixed APCs present all lipopeptides tested to the T cell cells, but the responses are much lower than those observed with vital APCs. The response to Pal-K_4-$F_{438-446}$ drops dramatically when this lipopeptide is presented by pre-fixed APCs. These data indicate that the lipopeptides can activate T cells without uptake or prior

modification by the APC, albeit with lower efficiency. Intracellular processing by the APC, however, seems to favor proper loading of the peptides onto MHC class I complexes.

Presentation of lipopeptides is sensitive to brefeldin A treatment

Knowing that the lipopeptides can be processed intracellularly, we investigated whether these antigens are processed via the classical, endogenous MHC class I presentation pathway. Transport of MHC class I-peptide complexes from the ER to the cell surface can be inhibited by treating the cells with brefeldin A (BFA) [28,29]. To prohibit presentation of lipopeptides via MHC class I molecules already present at the surface of the APCs, the cells are first subjected to treatment with Pronase. This enzyme removes the MHC class I molecules from the cell surface [26]. To determine whether MHC class I molecules are being removed effectively, cells pulsed with the peptide $F_{438-446}$ have been treated with different concentrations of Pronase (Fig. 3). Treatment of the cells with 4 mg/ml Pronase during 30 minutes strongly reduces the response of the T cell clone. Thus, if the cells are treated with Pronase *after* the peptide pulse, CTL activation will be entirely dependent on MHC class I molecules that have acquired antigenic peptides intracellularly.

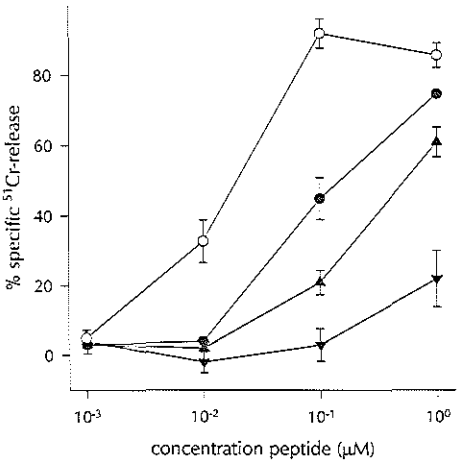

Figure 3. The removal of MHC class I molecules from the cell surface with Pronase inhibits antigen presentation. B-LCLs were incubated with 1 μM of $F_{438-446}$ peptide for 1 hour. The antigen pulse was performed in the continuous absence (open circles) or presence (closed symbols) of 5 μg/ml brefeldin A (BFA). The MHC class I molecules exposed at the cell surface were removed by treatement with 1 (●), 2 (▲) or 4 (▼) mg/ml Pronase. The cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ for 1 hour and co-cultivated with the MV-$F_{438-446}$ specific $CD8^+$ T cell clone for 4 hours. Supernatants were then harvested and ^{51}Cr-release was measured using a standard CTL assay.

When the presentation of Pal-K_4-$F_{438-446}$ is investigated using cells treated with Pronase after the peptide pulse, a strong reduction of CTL activation is observed (Fig. 4*A*). In the absence of BFA, the CTL response is restored within 2 hours of regeneration. A much lower response is observed in the presence of BFA, which indicates that an intact secretory pathway is essential for optimal recognition of this lipopeptide. After four hours, a significant response nevertheless is observed to the internalized Pal-K_4-$F_{438-446}$. This suggests that the dependence on an intact Golgi apparatus is not absolute for this lipopeptide.

Using the same experimental conditions, intracellular processing and presentation of other lipopeptides was investigated (Fig. 5). Also for these lipopeptides a drastic reduction of CTL activation is observed when the MHC complexes are removed from the cell surface (see TAP2+/+ APCs, black bars). The presentation to Pal-K_2-$F_{438-446}$ and Pal-CSS-$F_{438-446}$ is inhibited completely by BFA, which indicates that presentation of these antigens is strongly dependent on an

intact secretory pathway. In cells that have not been treated with BFA, the response to Pal-K2-F438-446 and Pal-CSS-F438-446 is restored 2 hours after removal of the MHC molecules from the cell surface (Fig. 5*A* and 5*B*, TAP+/+ APC's, open bars). Pal-F438-446 is not presented at all after Pronase treatment (Fig. 5C), which suggests that this lipopeptide is only presented by surface-exposed MHC class I molecules.

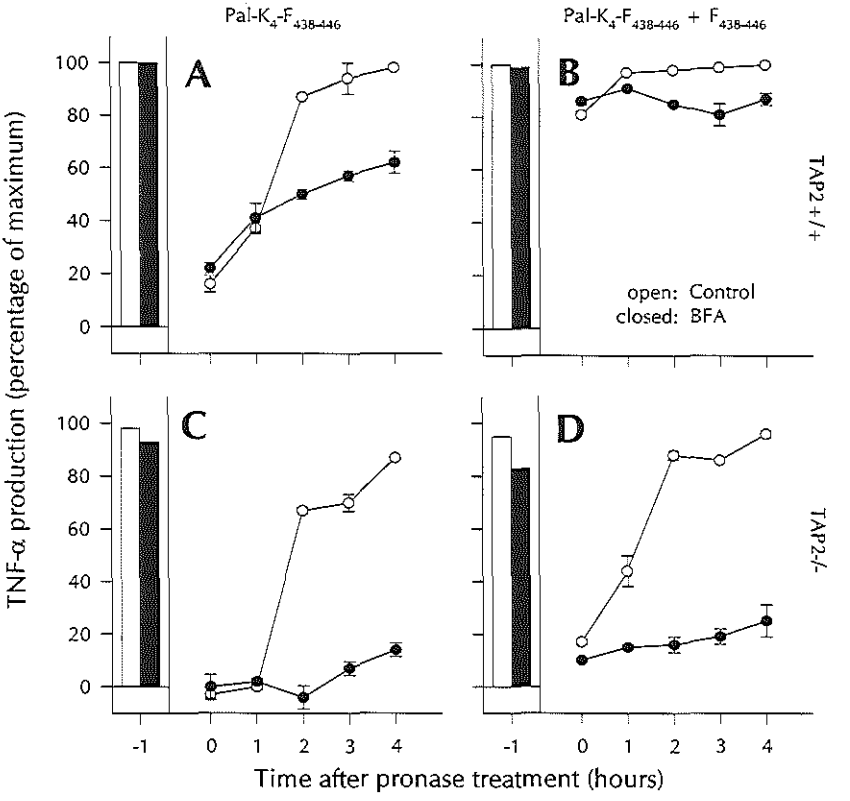

Figure 4. T cell recognition of Pal-K4-F438-446 is brefeldin A sensitive and TAP independent. TAP-2 reconstituted B-LCLs (A and B) or TAP2-/- B-LCLs (C and D) were incubated with 1 μM Pal-K4-F438-446 in the continuous presence of 5 μg/ml BFA for one hour. The surface-exposed MHC class I molecules were removed using 4 mg/ml Pronase. The cells were cultured during 0, 1, 2, 3, or 4 hours in the absence (open circles) or presence (closed circles) of 5 μg/ml BFA. Subsequently, the cells were fixed with 1% PFA and divided into two equal portions, one of which was incubated with 1 μM F438-446 for 1 hour at 37°C (B and D). Both portions were co-cultivated with the MV-F438-446 specific $CD8^+$ T cell clone for 4 hours. Supernatants were harvested and the TNF-α concentration was measured using TNF-α sensitive WEHI 164 cells. The adjacent bars indicate the responses without proteolytic removal of membrane exposed MHC class I molecules. These cells were cultured for 2 hours in the absence (open bar) or presence (closed bar) of 5 μg/ml BFA prior to PFA fixation.

Interestingly, the cell membrane is re-occupied with MHC class I molecules very rapidly. This becomes apparent when a 'split well analysis' of the APCs is performed (Fig. 4*B*). Half of the cells that have been incubated with Pal-K_4-$F_{438-446}$ and treated with Pronase have been fixed with PFA and then incubated with $F_{438-446}$. Whereas the CTL response to Pal-K_4-$F_{438-446}$ reappears very slowly after the Pronase treatment, (Fig. 4*A*), the response to $F_{438-446}$ is high from the beginning (Fig. 4*B*). This is indicative of the existence of a pool of post-ER MHC class I molecules, that can be exposed at the cell membrane relatively fast. Once the Pronase is removed, the expression of $F_{438-446}$-MHC complexes is restored so quickly, that a reduction of the response is not visible within the time frame of the experiment.

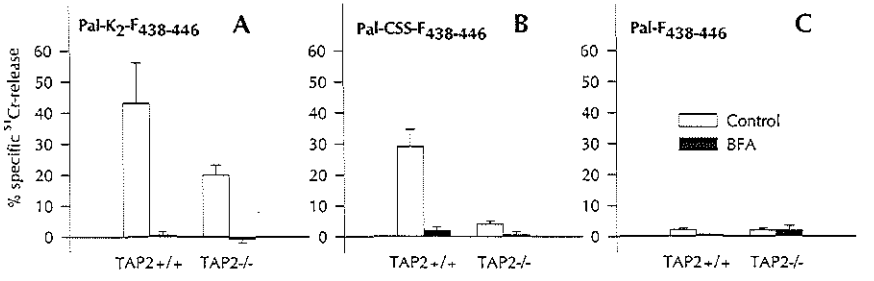

Figure 5. Sensitivity to BFA treatment and requirement of a functional TAP complex varies a mong the different lipopeptides and is influenced by the spacer composition. TAP -2 reconstituted B-LCLs or TAP2-/- B-LCLs were incubated with 1 μM of the indicated lipopeptides. The experiment was performed in the continuous absence (open bars) or presence (closed bars) of 5 μg/ml BFA. After subjecting the cells to digestion with 4 mg/ml Pronase the cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ for 1 hour. After extensive washing, the cells were co-cultivated with the MV-$F_{438-446}$ specific $CD8^+$ T cell clone for 4 hours. Supernatants were then harvested and ^{51}Cr-release was measured.

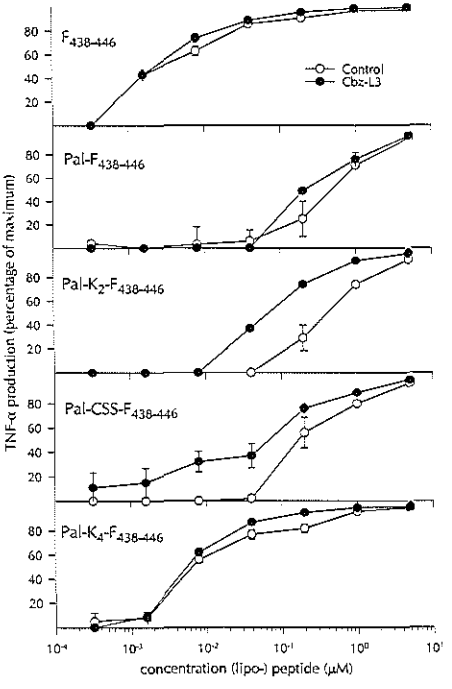

Figure 6. Inhibition of the processing increases presentation of some of the lipopeptides. B-LCLs were pre-treated or mock-treated with 10 μM Cbz-L3 during 1 hour. Subsequently, the cells were incubated with different concentrations of peptides during 2 hrs in the absence (open circles) or presence (closed circles) of 10 μM Cbz-L3. The cells were co-cultivated with the MV-$F_{438-446}$ specific $CD8^+$ T cell clone during 4 hours and the TNF-α concentrations were measured in the supernatants using the TNF-α sensitive WEHI 164 cells.

Translocation of lipopeptides by TAP

To investigate whether the lipopeptides are translocated into the ER by TAP, we have used cells that lack a functional TAP transporter due to deletion of the TAP2 gene [30]. The cells are subjected to Pronase treatment to remove surface-exposed MHC class I complexes. Presentation of Pal-K_4-$F_{438-446}$ appears to be largely independent on an intact TAP transporter (compare Fig. 4*A* and 4*B*, open circles; $p = 0.23$). The presentation of Pal-K_2-$F_{438-446}$ is partially dependent on TAP (Fig. 5, $p = 0.018$), whereas recognition of Pal-CSS-$F_{438-446}$ TAP is completely TAP-dependent (Fig. 5, $p < 0.001$).

In the TAP2-/- cells, presentation of Pal-K_4-$F_{438-446}$, but also $F_{438-446}$, is much more sensitive to BFA treatment than in the TAP2+/+ cells (compare Fig. 4*A* with 4*C*, and 4*B* with 4*D*). This may be due to the ER retention and degradation of newly synthesized MHC class I molecules that occurs in cells lacking a functional TAP transporter [31]. This results in a decreased pool of mature MHC class I molecules in post-ER compartments, which appears to be the population of MHC class I molecules that contributes to presentation of Pal-K_4-$F_{438-446}$ and $F_{438-446}$.

Cbz-L3 influences the processing and presentation of some of the lipopeptides

The observed brefeldin A sensitivity and TAP dependency strongly suggest that some of the lipopeptides can enter the classical MHC class I presentation pathway via translocation into the ER. To investigate whether the proteasome takes part in processing of cytosolic lipopeptides we have used Cbz-leucyl-leucyl-leucinal (Cbz-L3) to inhibit proteolysis by the proteasome. Presentation of Pal-K_2-$F_{438-446}$ and Pal-CSS-$F_{438-446}$ appears to be enhanced in the presence of Cbz-L3 (Fig. 6).

Proteasomal degradation of lipopeptides in vitro

The degradation of Pal-K_2-$F_{438-446}$ and Pal-K_4-$F_{438-446}$ has been examined *in vitro* using isolated proteasomes (Table 2). Both lipopeptides appeare to be processed by proteasomes. Pal-K_2-$F_{438-446}$ is degraded more efficiently than Pal-K_4-$F_{438-446}$. After 4 hours of digestion, the sequence PDAVYL forms the most abundant product. The sequence RYPDAVYL is still capable of sensitizing B-LCLs for cytotoxic killing (data not shown).

Intracellular processing of fluorescent lipopeptides

Using confocal microscopy, intracellular trafficking of lipopeptides carrying a fluorescent label was studied. The intracellular distribution pattern of the monovalently labeled lipopeptides Pal-$F_{438-446}^{FL}$ and Pal-K_4^{TMR}-$F_{438-446}$ is illustrated in Figure 7*A* and 7*D*. Accumulation of the fluorescence signal is time dependent for the first 10 to 15 minutes of incubation at 37°C (data not shown). Thereafter, its intensity and also its distribution pattern remain constant. At 4°C, the fluorescence accumulates more slowly, but finally the intensity is comparable to that seen at 37°C (Fig. 7*B*). As expected, fluorescence is absent in pre-fixed cells (Fig. 7*C*).

Treatment of the cells with BFA 2 hours prior to incubation with the fluorochrome-labelled lipopeptides results in a decreased and altered fluorescence pattern. A vesicular fluorescence pattern is seen in the cytoplasm, without a clear accumulation in the perinuclear region (Fig. 7*E*).

To characterize the perinuclear region that contains the peptides in more detail, the Golgi specific protein golgin-97 and the ER-specific protein calnexin were stained by immunolabeling. The accumulated fluorescence of Pal-K_4-$F_{438-446}^{Fl}$ co-localizes with golgin-97, but not with calnexin (Fig. 8), which indicates targeting of the lipopeptide to the Golgi.

A possible role of the Golgi apparatus in the processing of lipopeptides becomes more evident when a double-labelled-lipopeptide is used, which has fluorescein tagged to the C-terminal

Table 2. Proteasomal degradation of Pal-K_4-$F_{438-446}$ and Pal-K_2-$F_{438-446}$[a].

	Relative amount	
	After 1 h	After 4 h
Pal-K_4-$F_{438-446}$		
Pal-KKKKRRYPDAVYL	100	100
Pal-KKK	5	80
Pal-KKKK	n.d.	8
Pal-KKKKR	n.d.	10
PDAVYL	n.d.	40
YPDA	n.d.	3
RRYPDAVYL	1	8
KRRYPDAVYL	n.d.	16
Pal-K_2-$F_{438-446}$		
Pal-KKRRYPDAVYL	100	47
Pal-KKR	34	46
PDAVYL	36	100
RYPDAVYL	n.d.	22

[a] The lipopeptides were incubated with isolated 26S proteasomes for 1 or 4 h. The digests were separated by HPLC and the fractions were analyzed using mass-spectrometry.

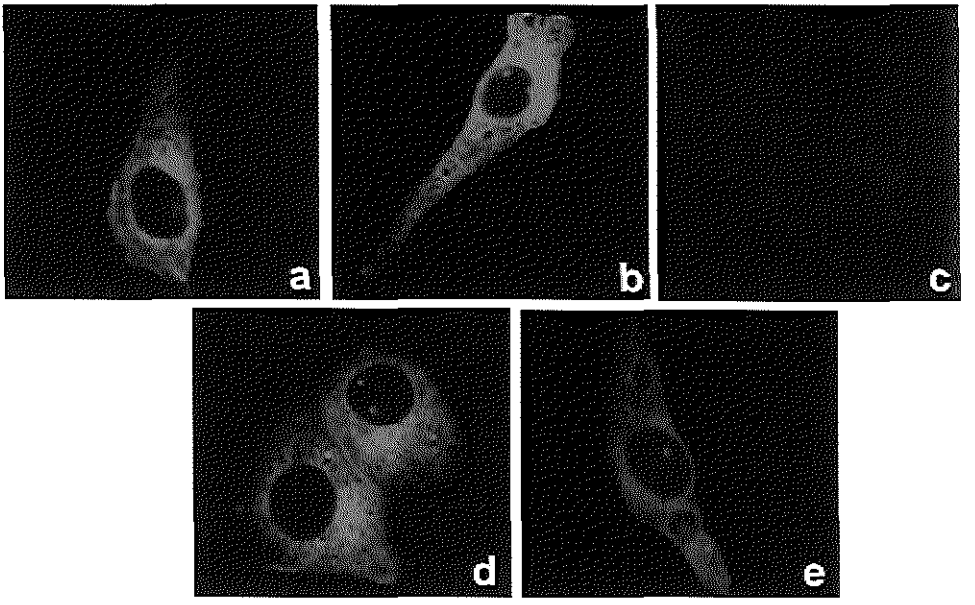

Figure 7. Confocal microscopicy analysis of Mel/J cells incubated with Pal-$F_{438-446}$.^{FL} [panels A, B, C, E and F] or Pal-K_4^{TMR}-$F_{438-446}$ [D] at 37°C [A, C-F] or 4°C [B]. Paraformalin-fixed cells were used in C. BFA-pretreated cells are shown in E. Intracellular membrane-bound fluorescence is seen on vesicle and tubular-like structures and is accumulated into the peri-nuclear region. This distribution pattern is not influenced by the incubation temperature [A, B]. PFA-fixed cells are not able to bind or accumulate the lipopeptides. BFA pretreatment results in a lowered fluorescence intensity and absence of a perinuclear staining [E].

residue of the peptide and rhodamine to the lysine adjacent to the lipid tail (see Table 1). If both fluorochromes are present on an intact lipopeptide molecule, the emission of fluorescein is quenched for about 99% due to intramolecular interaction with rhodamine. Quenching by rhodamine is not possible after enzymatic cleavage of the peptide [23]. These differences in emission characteristics of fluorescein allow a further characterization of the site of processing. In figure 9A and D, the co-localization of fluorescein and rhodamine is shown, whereas the staining of the individual dyes is presented in B and E (fluorescein) and C and F (rhodamine). In the absence of BFA, the cellular distribution patterns of both fluorochromes are similar after 60 min. at 37°C. The fact that the cells predominantly are stained yellow (Fig. 9*A*) indicates that the fluorescein is not quenched by the rhodamine. Thus, the lipopeptide must have been cleaved into fragments, one of which contains the fluorescein and one the rhodamine.
BFA pretreatment of the cells results in a strongly diminished intensity of the fluorescein signal (Fig. 9*E*), whereas the rhodamine signal is about the same as in untreated cells (Fig. 9*F*). The fluorescence for both fluorochromes is restricted to vesicular structures distributed in the cytoplasm (Fig. 9*D*, *E* and *F*). In view of the quenching properties of the fluorophores in the intact lipopeptide we have to conclude that the absence of fluorescein fluorescence in BFA treated cells indicates a lack of cleavage of the lipopeptide, confirming a role for the Golgi apparatus in the processing of these peptides.

DISCUSSION

MHC class I molecules have a preference for binding octameric and nonameric peptides. The physical chemical properties of lipopeptides are quite different from those of the natural peptide ligands of MHC class I molecules and it is assumed that they cannot bind to the MHC class I binding site without prior removal of the lipid tail [32].
In the present study, we have investigated the *in vitro* processing and presentation of lipidated peptides containing a CTL epitope derived from measles virus fusion protein. To establish whether T cell recognition of these lipopeptides requires their modification by APCs, formaldehyde-fixed or vital B cells were incubated with the lipopeptides and co-cultured with cloned T cells recognizing the fusion protein-derived CTL epitope. Surprisingly, formaldehyde-fixed APCs are capable of presenting the lipopeptides to the T cells. Apparently, the presence of a lipid tail and, in case of some of the peptides, a size of 11-13 amino acid residues, does not prohibit binding to MHC class I molecules, nor recognition of the peptides by the T cell receptor. Theoretically, the lipopeptides could be modified by proteolytic enzymes occurring in the serum present during co-cultivation of the APCs with the CTL clone [33]. Although this is not very likely, this possibility cannot be ruled out completely. We favor a different explanation. The CTL clone used in this study recognizes its substrate in the context of HLA-B27. This MHC molecule has the unusual property that it can accommodate peptides of up to 33 amino acids [30,34]. Thus, the lipopeptides may bind to B27 without prior modification, with the fatty acid tail protruding from the peptide binding groove.
Processing by vital APCs significantly increases the presentation efficiency of Pal-K_4-$F_{438-446}$. This lipopeptide has several other interesting properties. Pal-K_4-$F_{438-446}$ can be presented by cells that

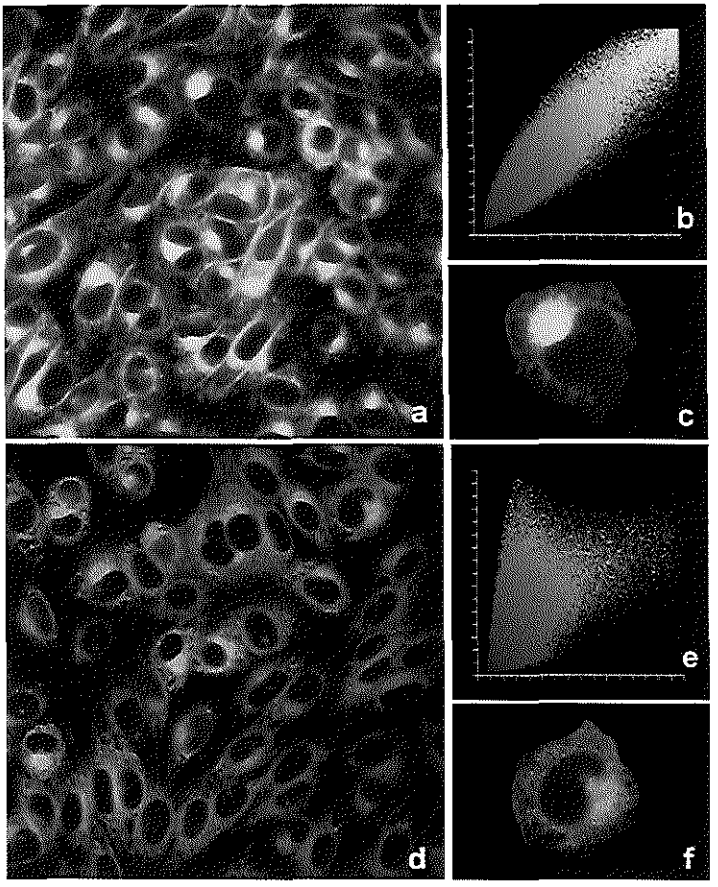

Figure 8. Confocal microscopical pictures of Mel/J cells incubated for 1 hour at 37°C with Pal-K_4-$F_{438-446}^{Fl}$, PFA fixed and immunolabelled for golgin-97 [A-C] and calnexin [D-F].Fluorescein and Cy5 fluorescence are pseudocolored in green and red, respectively, and shown simultaneously. Colocalized pixels are colored in yellow. Fluorescence of fluorescein is accumulated in the perinuclear region and colocalizes with the Golgi-marker golgin-97 [A, C]. There is no colocalization with the ER-marker calnexin [D, F]. Pixel fluorograms of illustrations A and D are shown in B and E respectively. Colocalized pixels (yellow) are located along the diagonal of the cytofluorograms.

lack a functional TAP transporter (Fig. 4), which suggests that the peptide may be translocated into the ER in a TAP-independent fashion. The delivery of the peptide into the ER could occur via a trafficking pathway that facilitates transport of antigen from the extracellular medium to the ER (fluid phase communication') [36]. Alternatively, the lipopeptide may bind to MHC class I molecules in a post-ER compartment. Interestingly, experiments with fluorophoretagged lipopeptides indicate that Pal-K_4-$F_{438-446}$ is preferentially targeted to the Golgi compartment (Fig. 8). A double fluorescent analogue of Pal-K_4-$F_{438-446}$ allowed a more detailed investigation of intracellular trafficking of this lipopeptide. This fluorescent-labeled lipopeptide possesses the novelty of exclusively showing fluorescein fluorescence upon removal of the rhodamine tag[23]. The fact that the fluorescein

and rhodamine fluorescence are both detectable in the Golgi compartment (Fig. 8) indicates that the fluorescein is no longer quenched by the intramolecular rhodamine. This suggests that the lipid moiety is being removed from the peptide in the Golgi compartment. In cells treated with BFA, rhodamine staining is still visible, but the fluorescein signal is not detectable, probably because it is still being quenched by the rhodamine. This supports the idea that the Golgi plays a role in processing of lipopeptides.

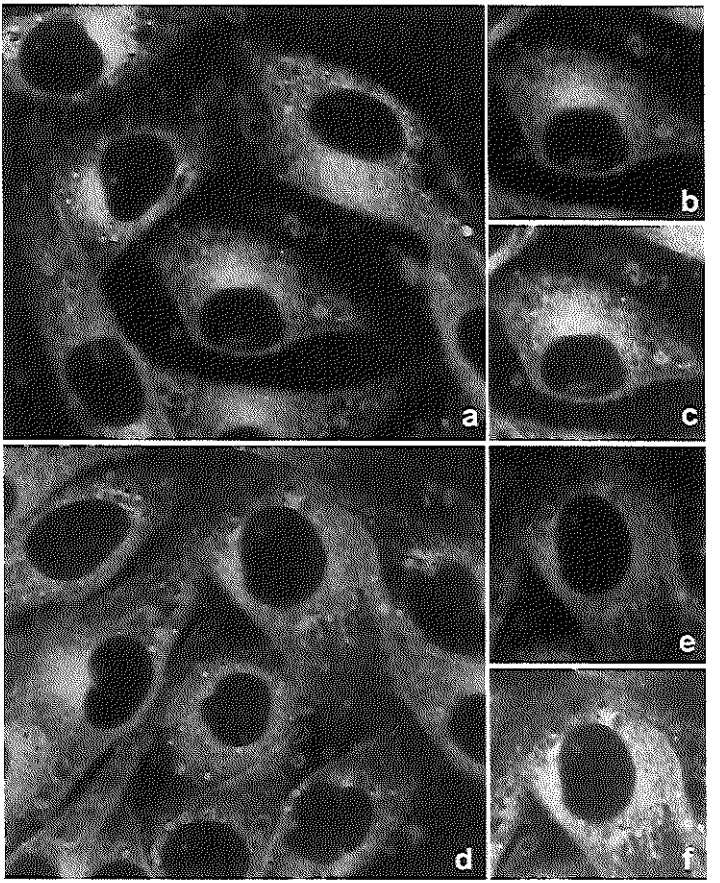

Figure 9. Fluorescent labeling of Mel/J cells incubated with Pal-K_4^{TMR}-$F_{438-446}^{Fl}$. The peptides were incubated with the cells in the absence [A-C] or in the continuous presence of BFA [D-F]. Illustrated is the presence of fluorescein-fluorescence (B and E) and rhodamine (C and F). In A and D, fluoresce in and rhodamine are pseudocolored in green and red, respectively and displayed simultaneously. Colocalized pixels are displayed in yellow. Fluorescence of fluorescein and rhodamine is bound to vesicle and tubular - like structures mainly occurring in the pe rinuclear region. Due to the intramolecular interactions of the labels, fluorescein is quenched and rhodamin staining is more pronounced than fluorescein staining [compare B and C]. After BFA pretreatment, the fluorescence of both fluorochromes is redistri buted to the cytoplasm whereas peri-nuclear accumulation of fluoresence is no longer seen [C, D]. A strong decrease of the intensity of fluorescein is observed [compare B with E].

The observation that Pal-K_4-$F_{438-446}$ can be presented by BFA-treated cells suggests that the recognition of this peptide may not fully depend on the secretory pathway. In addition to the Golgi, other intracellular compartments may be involved in the processing of lipopeptides. In the BFA treated cells, the peptides may be presented via pre-existing MHC molecules, which may re-circulate from the cell surface [37], [38]. In that case, peptide loading could occur in a compartment that is part of the endocytic pathway. Studies with GFP-tagged MHC class I molecules indicate that a fraction of the MHC class I molecules enters the MHC class II loading compartment (MIIC) [39]. MHC class I molecules can acquire peptides in this compartment and can be transported back to the cell membrane. The low pH of the MIIC appears to facilitate the exchange of peptides [39].

T cell recognition of Pal-K_2-$F_{438-446}$ and Pal-CSS-$F_{438-446}$ is completely BFA sensitive. This suggests that these lipopeptides enter the MHC class I pathway in the ER. In accordance with this possibility, the response to Pal-CSS-$F_{438-446}$ is entirely TAP-dependent. The TAP dependency suggests a cytosolic deposition of the peptide. It is unclear, however, how the lipopeptide should enter the cytosol. This could occur via translocation from an intracellular compartment, or the peptide could be translocated into the cytosol via the cell membrane.

The T cells respond poorly to the parental peptide F438-446 and the lipopeptides when these are presented by BFA-treated TAP -/- cells (Fig. 4 and 5). Even the response to Pal-K_4-$F_{438-446}$, which is TAP-independent and relatively insensitive to BFA treatment, is low in the BFA-treated TAP -/- cells. This observation may be related to a decreased availability of MHC molecules in these cells. The antigenic peptide forms an essential component of the MHC class I complex and contributes to its stability. In the absence of a functional TAP transporter, peptides are scarce in the ER and the MHC molecules are retained in this compartment. As part of the cellular quality control process the empty MHC class I molecules are 'dislocated' to the cytosol, where they are degraded by proteasomes [31,40,41]. Thus, less MHC class I molecules will be available for peptide binding in post-ER compartments of TAP -/- cells.

The enhanced T cell response to some of the lipopeptides in the presence of the proteasome inhibitor Cbz-L3 (Fig. 6) indicates that intracellular processing may have an adverse effect on T cell recognition of these antigens. A similar phenomenon has been reported for the presentation of peptides derived from influenza virus proteins [42,43]. Interestingly, the lipopeptides that are most sensitive to proteasomal degradation are also more dependent on TAP for T cell activation (compare Fig. 5 and 6). These data support the hypothesis that these lipopeptides are processed via the classical MHC class I restricted antigen presentation pathway, characterized by proteolysis by the proteasome and translocation into the ER by TAP.

Pal-$F_{438-446}$ fails to activate T cells when it is processed intracellularly by the APCs (Fig. 5). A microscopical evaluation of intracellular trafficking of fluorescent Pal-$F_{438-446}$ indicates that this lipopeptide does enter the cells (Fig. 7). The distribution pattern of fluorescence in cells incubated with Pal-$F_{438-446}$ and Pal-K_4-$F_{438-446}$ is very similar. The lack of T cell recognition of intracellularly processed Pal-$F_{438-446}$ may be due to enzymatic modifications or degradation by intracellular proteases [42]. Using isolated proteasomes, we have shown that Pal-K_2-$F_{438-446}$ and Pal-K_4-$F_{438-446}$ are substrates for proteasomal degradation. Some of the cleavages yield peptides that are no longer capable of activating the T cell clone.

In conclusion, this study shows that the composition of lipopeptides strongly influences intracellular processing of these antigens. The spacer moiety affects intracellular trafficking, proteolytic modifications and T cell activating properties of lipopeptides. Depending on the spacer, a T cell epitope may be loaded onto MHC class I molecules in the ER or in a post-ER compartment. Entry

into the ER may occur in a TAP-dependent or TAP-independent fashion, again depending on the lipopeptide composition.

These results emphasize the importance of a careful evaluation of the spacer composition during the development of lipopeptide immunogens. Additional experiments will be required to investigate how intracellular trafficking of lipopeptide is influenced by the spacer composition. This knowledge will be instrumental in the rational design of lipopeptide vaccines that aim to targeting antigens to specific intracellular compartments to elicit the desired immune response.

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Chapter 2.2

Solid phase synthesis and application of double fluorescent-labeled lipopeptides, containing a CTL-epitope from the measles fusion protein

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ABSTRACT

The mechanism which enables lipopeptides to induce cytotoxicity is not known. By preparing fluorescent-labeled lipopeptides one might unravel the mechanism of their entry into the cell and their intracellular pathway. A method of preparing double fluorescent-labeled peptides by solid phase chemistry is described. As model peptides we have chosen analogs of the sequence RRYPDAVYL, which occurs in the measles fusion protein ($F_{438-446}$) and is an epitope for cytotoxic T-lymphocytes. The peptides Pal-K^{TMR}KKKRRYPDAVK^{FL}L (7) and Pal-K^{FL}KKKRRYPDAVK^{TMR}L (8), in which Pal is palmitoyl and K^{TMR} and K^{FL} are N^{ε}-carboxytetramethylrhodamine- and N^{ε}-carboxyfluorescein-labeled lysyl residues, respectively, were prepared and obtained in approximately 30% yield after purification by high performance liquid chromatography.

The fluorescence of fluorescein and tetramethylrhodamine in lipopeptide Pal-K^{TMR}KKKRRYPDAVK^{FL}L (7) was quenched to a degree of 98-99% due to intramolecular interaction of the labels. On incubation with trypsin (i.e. cleavage at the KKKRR-site) the fluorescence of both labels was restored.

The intracellular routing of lipopeptide Pal- K^{TMR}KKKRRYPDAVK^{FL}L was studied with human melanoma cell line, Mel/J, which was transfected with human leukocyte antigen B*2705. It appeared that the double fluorescent-labeled lipopeptide was able to induce antigen-specific cytotoxicity. Furthermore, preliminary confocal microscopical studies indicated that this lipopeptide is observed intracellularly.

INTRODUCTION

The possibility of inducing specialized cytotoxic T lymphocytes (CTL) by immunization with synthetic lipopeptides is well established [1-8]. However, studies about the cellular processing of these compounds are scarce [9-11] and further investigations of cell binding and entry – and the intracellular trafficking or accumulation – of lipopeptides are required for rational vaccine development. A useful method of studying these aspects may be the application of fluorescent-labeled lipopeptides [11]. With appropriate fluorochrome labeling, the lipid and peptide moieties might be localized specifically by confocal microscopy by performing triple or quadruple (immuno)staining with specific markers for the different cellular organelles.

We have been interested in the immunogenicity and the cellular processing of lipopeptides containing analogs of the sequence RRYPDAVYL, which is a human leukocyte antigen (HLA) B*2705-restricted CTL-epitope of the measles virus fusion protein [12]. Our aim was to introduce two different fluorescence labels into the spacer-containing sequence Pal-KKKKRRYPDAVYL, one at the side-chain of Lys-1, *i.e.* near the lipid tail, and the second one in the epitope. By systematically substituting each amino acid residue in the sequence RRYPDAVYL by a β-naphtylalanine residue, it was found that only the C-terminal Tyr residue is not essential for maintaining the *in vitro* biological activity. In addition, the peptide RRYPDAVC^{FL}L, which was labeled in solution by reaction of the side chain of Cys with 5-(iodoacetamido)fluorescein, proved to be active in the routine Cr^{51} release CTL assay (unpublished results).

A procedure of labeling a peptide with two fluorochromes has already been described [13]. In this case, the peptide GSGSRLPGPSDTC, a sequence derived from human chorionic gonadotrophin (hCG), was labeled in solution with tetramethylrhodamine-5-maleimide at the Cys residue and, thereafter, with 5-carboxyfluorescein succinimidyl ester at the N-terminus. However, unlike labeling of peptide RRYPDAVCL, the modification of lipopeptides in solution proved to be troublesome in our hands. Furthermore, selectivity with respect to the labeling of lysine residues was required. For these reasons, it was decided to prepare the fluorescent-labeled lipopeptides by solid phase chemistry [14,15]. In this paper, we describe the solid phase synthesis and purification of the lipopeptides Pal-K^{TMR}KKKRRYPDAVK^{FL}L (*7*, Fig. 1) and Pal- K^{FL}KKKRRYPDAVK^{TMR}L (8), in which K^{TMR} and K^{FL} are N^{ε}-carboxytetramethylrhodamine- and N^{ε}-carboxyfluorescein-labeled lysyl residues, respectively. In addition, fluorescence spectra of lipopeptide *7* are presented. Finally, the entry of the peptide into the HLA-B*2705-transfected melanoma cell line Mel/J and the intracellular distribution pattern is shown by confocal microscopy.

MATERIALS AND METHODS

5-Carboxyfluorescein and 5-carboxytetramethylrhodamine, as well as the *N*-succinimidyl esters of these compounds were purchased from Molecular Probes Europe BV, Leiden, The Netherlands). Fmoc-amino acids and Fmoc-Leu-Wang polymer (0.84 mmol/g) were obtained from Novabiochem (Läufelfingen, Switzerland) and *N*-succinimidyl palmitoylate from Sigma (St. Louis, MO, USA).

Peptides were assembled on 10 μmol scale by using an automated multiple peptide synthesizer, equipped with a 48-column reaction block (AMS 422, ABIMED Analysen-Technik Gmbh, Langenfeld, Germany). Double couplings were performed with Fmoc-amino acid (50

μmol), benzotriazolyloxy-tris-[*N*-pyrrolidino]phosphonium hexafluorophosphate (PyBOP, 50 μmol), and *N*-methylmorpholine (NMM, 100 μmol) and the Fmoc group was cleaved with piperidine/N,N-dimethylacetamide (DMA), 2/8 (v/v), as described earlier [16,17]. The following side-chain protecting groups (Fig. 1) were used: 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) for Lys-1; *tert*-butyloxycarbonyl (Boc) for Lys-2, -3, and -4; 2,2,5,7,8-pentamethylchromansulfonyl (Pmc) for Arg; tert-butyl (tBu) ether for Tyr and ester for Asp; 4-methyltrityl (Mtt) for Lys-12.

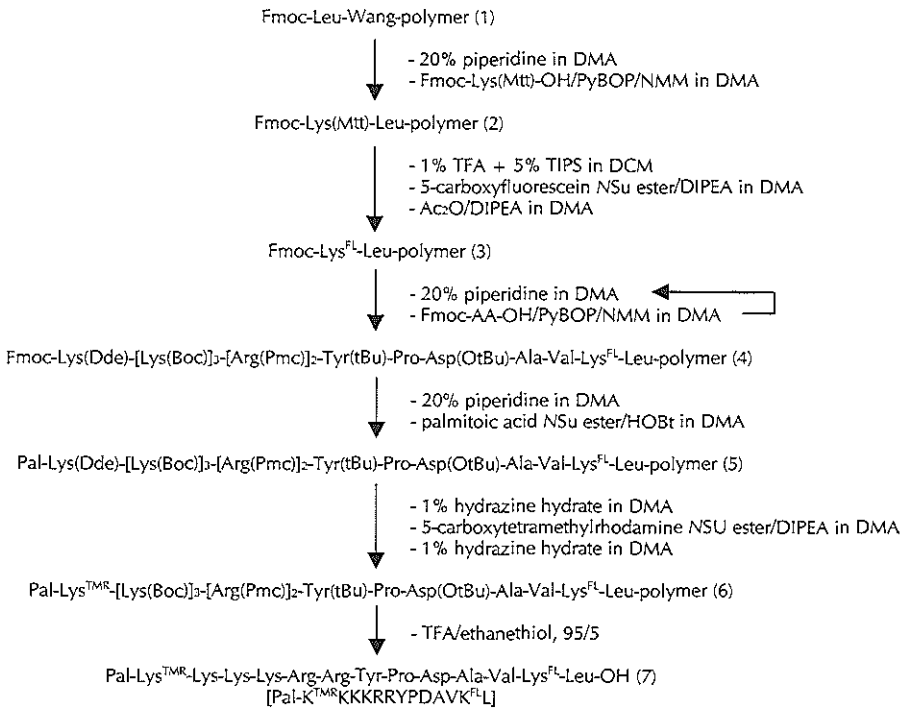

Figure 1. Outline of the synthesis of the 5-carboxytetramethylrhodamine (TMR)- and 5-carboxyfluorescein (FL)-labeled lipopeptide Pal-K[TMR]KKKRRYPDAVK[FL]L (7).

Pal-K[TMR]KKKRRYPDAVK[FL]L (7, Fig. 1), first synthesis

Fmoc-Lys(Mtt)-Leu-Wang resin 2 (10 μmol) was prepared according to the standard protocol. The automatic program of the synthesizer was then interrupted and the synthesis was continued manually. The protected dipeptide-resin obtained was washed with dichloromethane (DCM) (3 × 3 mL), incubated with 1% trifluoroacetic acid (TFA) and 5% triisopropylsilane (TIPS) in DCM (3 × 300 μL, each time for 15 min), and washed again with DCM (3 × 3 mL) and *N,N*-dimethylacetamide (DMA, 3 × 3 mL), successively. 5-Carboxyfluorescein *N*-succinimidyl ester (10 mg, 21 μmol) was dissolved in 300 μL DMA, containing 17.4 μL (100 μmol) *N,N*-diisopropylethylamine (DIPEA), and the red solution obtained was added to the peptide-polymer. After reaction for 3 h, the product was washed with DMA (6 × 3 mL). The orange fluorescent peptide-resin was then incubated with 170 μL acetic anhydride/DIPEA/DMA,

1/2/14 (v/v/v) for 30 min to give a light yellow product (3) which was no longer fluorescent. Finally, the peptide-resin was washed with DMA (3 × 3 mL).

The synthesis was continued automatically. After coupling of the N-terminal Fmoc-Lys(Dde)-residue and Fmoc-cleavage from 4, the synthesis was completed manually. The α-amino group was acylated by double coupling with a solution of *N*-succinimidyl palmitoylate (20 mg, 57 μmol) and 1-hydroxybenzotriazole hydrate (8.5 mg, 55 μmol) in 170 μL DMA for 90 min. Thereafter, the peptide-resin (5) was washed with DMA, DCM, methanol, and DMA, successively (3 × 3 mL of each solvent).

In the final step, Dde was removed from 5 with 1% hydrazine hydrate in DMA (0.5 mL, 6 × 5 min), followed by washing with DMA (6 × 3 mL). The second label was now introduced by reaction with a solution of 5-carboxytetramethylrhodamine *N*-succinimidyl ester (10 mg, 19 μmol) in 300 μL DMA, containing 17.4 μL (100 μmol) DIPEA. After reaction for 5 h, the red peptide-polymer (6) was washed with DMA (6 × 3 mL), incubated with 1% hydrazine hydrate in DMA (0.5 mL, 3 × 5 min), and washed with DMA, DCM, and diethyl ether, successively (3 × 3 mL of each solvent).

Complete deprotection of the peptide was performed with TFA/ethanethiol, 95/5 (v/v) for 3 h, as described earlier (16, 17). HPLC analysis (see below) of the crude product showed the presence of two main products with absorbency at 546 nm (Fig. 2A). These products were separated by semi-preparative HPLC (see below) and analyzed by fast atom bombardment mass spectrometry as described before (16) and thus identified as Pal-K^{TMR}KKKRRYPDAVKL (18 min retention time on HPLC, FAB MS: M^+ (mono-isotopic mass) found/calcd: 2280.4/2280.6) and peptide 7 (19 min retention time, M^+ (av. mass) found/calcd: 2640.7/2640.2), respectively.

Pal-K^{TMR}KKKRRYPDAVK^{FL}L (7), second synthesis

The second synthesis was performed as described above with the following exceptions: (a) before Mtt-cleavage, dipeptide-resin 2 was additionally washed with acetic acid/DCM, 1/9 (v/v) (3 × 3 mL) and (b) the cleavage was performed with 9 aliquots of 300 μL of 1% TFA and 5% TIPS in DCM during 45 min (i.e. 9 × 5 min instead of 3 × 15 min). Fig. 2B shows the analytical HPLC profile of the crude product. The target peptide was purified by semi-preparative HPLC and obtained in approximately 30 % yield, as determined by colorimetric assay. In this assay, the absorbance at 546 nm of equimolar mixtures of 5-carboxyfluorescein and 5-carboxytetramethylrhodamine was used for calibration.

Pal-K^{FL}KKKRRYPDAVK^{TMR}L (8)

The protocol for the second synthesis of peptide 7 was also used to prepare Pal-K^{FL}KKKRRYPDAVK^{TMR}L, with the obvious exception that the fluorescein- and rhodamine-labels were coupled to Lys-1 and Lys-12, respectively. Fig. 2C shows the HPLC profile of the double labeled product which was purified by semi-preparative HPLC and also obtained in approximately 30 % yield. FAB MS: M^+ (av. mass) found/calcd: 2640.7/2640.2), respectively.

Preparative HPLC

Preparative HPLC was performed with a Waters LC Module 1, equipped with a 996 photo diode array detector and controlled by Millennium Chromatography Manager Software (Waters), on a Vydac 208 TP1010 C8 reversed-phase column (10 × 250 mm). Solvent A: 0.1 % TFA in H_2O; solvent B: 0.08% TFA in CH_3CN/H_2O, 2/1 (v/v). A linear gradient from 0 to 100% B in 30 min, a flow rate of 4.5 ml/min and detection at 546 nm was used.

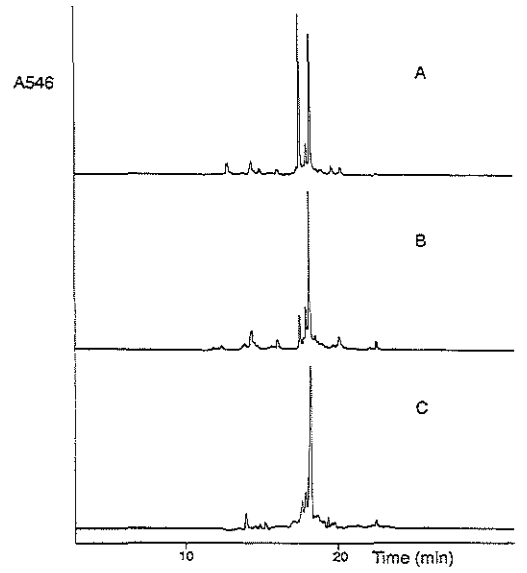

Figure 2. HPLC profiles of the crude Pal-K^{TMR}KKKRRYPDAVK^{FL}L (A, first synthesis; B, second synthesis) and Pal-K^{FL}KKKRRYPDAVK^{TMR}L (C) with detection at 546 nm. In chromatogram A, the products with retention times of 18 and 19 min are the mono-labeled Pal-K^{TMR}KKKRRYPDAVKL and the target peptide Pal-K^{TMR}KKKRRYPDAVK^{FL}L, respectively, as found by off-line FAB MS analysis.

Analytical HPLC

Analytical HPLC was performed with a Waters 2690 Separations moduleon a Vydac 208TP54 C8 reversed-phase column (4.6 × 250mm). The solvents, gradient, and detection were the same as used for semi-preparative HPLC, whereas a flow rate of 1.0 ml/min was used.

Trypsin incubation of lipopeptide 7

A sample of 1 ml of 10^{-6} M peptide 7 in phosphate buffered saline, pH 7.2, was incubated with 100 µl of a solution of trypsin and buffer salts, which was prepared from a ready to use tablet (Sigma, T-7168), at room temperature.

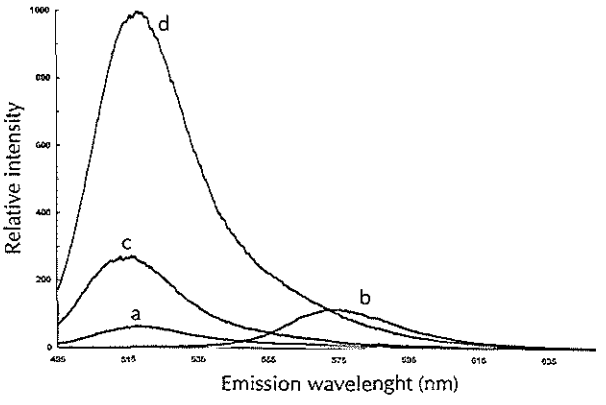

Figure 3. Technical fluorescence emission spectra (excitation at 488 nm) of (a) 10^{-6} M lipopeptide 7, (b) 10^{-6} M 5-carboxytetramethylrhodamine, (c) 40 × 10^{-9} M 5-carboxyfluorescein, and (d) 0.9 × 10^{-6} M lipopeptide 7 after incubation with trypsin for 2 h at ambient temperature.

Fluorescence emission spectra

Fluorescence emission spectra were recorded from 495-650 nm (excitation at 488 nm) and from 575-750 nm (excitation at 568 nm) at ambient temperature with an LS-50B luminescence spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). The excitation and emission bandwidths were set at 2.5 nm. Samples had a maximum absorbency below 0.1 within the range of 450-600 nm.

Confocal microscopy

The human melanoma cell line Mel/J-R6 cells were cultured in RPMI medium supplemented with 10% fetal calf serum (Gibco Laboratories, Paisley, Scotland). For microscopic studies enzymatically dispersed cells were allowed to grow on 8-wells Lab-Tek glass chamber slides (Nalge Nunc International, USA) overnight, washed in serum-free medium (PFHM, Gibco Laboratories), incubated with lipopeptide 7 in an optimal dose for 60 minutes at 37°C together with TO-PRO-3 (1μmol, Molecular Probes Europe BV, Leiden, The Netherlands), washed at room temperature in PFHM, and subsequently fixed in 2% paraformalin (pH 7.6), mounted in Vectashield and directly observed under a NIKON Optiphot 2 microscope. Fluorescence was imaged with a BioRad 1024 confocal laser scanning microscope equipped with an Argon/Krypton laser. The 488-nm line was used to excite fluorescein, the 568-nm line was used to image the rhodamine and the 647-nm line for TO-PRO-3 excitation. Confocal images [with a section thickness of about 0.5 μm] of sequential planes through the cell were recorded with a × 60, Plan Apo objective lens. Images were processed with Adobe Photoshop 4.0 (Adobe Systems, USA). Fluorescence of fluoresceine, tetramethylrhodamine en TO-PRO-3 were pseudocolored as green, red and blue, respectively.

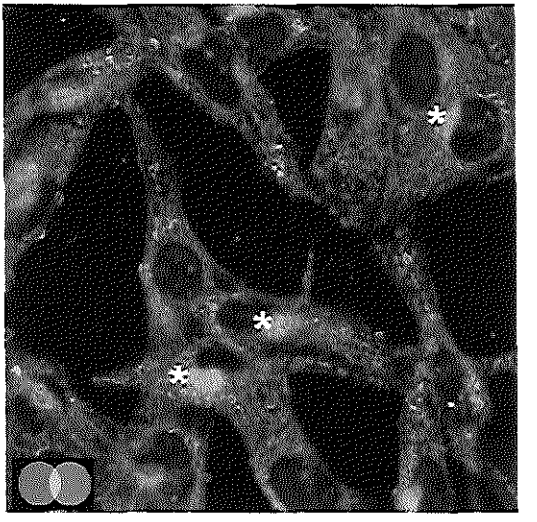

Figure 4. A confocal microscopical picture of Mel/J cells incubated with lipopeptide 7 for 60 minutes at 37°C, washed and directly observed under the microscope. Staining is seen as intracellular membrane-bound fluorescence. Colocalisation of both fluorochrome-labels is colored yellow and is found on vesicle and tubular-like structures, resembling the membranes of the rough endoplasmatic reticulum (RER). Green fluorescence from the fluorescein-label is more pronounced seen in the perinuclear area of some cells (indicated with an asterix), suggesting that in these cells the Golgi area is optically sectioned. Nuclei are counterstained with TO-PRO-3 and pseudocolored in blue.

RESULTS AND DISCUSSION

Peptide synthesis and purification

Starting from Fmoc-Leu-Wang resin (1, Fig. 1), the resin-bound and protected dipeptide Fmoc-Lys(Mtt)-Leu (2) was prepared. In this stage, either the Fmoc or the Mtt [18,19] group could be removed selectively. Since it was expected that the introduction of the label into a longer peptide fragment would be difficult due to steric hindrance [15], the Mtt group was removed from the resin-bound dipeptide with 1% TFA and 5% TIPS in DCM [14]. The label was then introduced by reaction with a twofold excess of 5-carboxyfluorescein *N*-succinimidyl ester [14] in the presence of DIPEA to give a clearly fluorescent peptide-resin. In the next step, capping of remaining ε-amino groups with acetic anhydride, the fluorescence vanished, probably due to acetylation of the fluorescein hydroxyl groups. This acetylation proved to be reversible since the fluorescence reappeared in the next piperidine step, when the Fmoc group was cleaved from the labeled dipeptide resin (3). [When plain 5-carboxyfluorescein was treated with acetic anhydride in the presence of DIPEA, fluorescence also vanished, but could also be restored by piperidine treatment.] During the following Fmoc-aminoacylations and Fmoc-cleavages, the fluorescence was repetitively vanished and revived, respectively, although this process became slower while the peptide chain was growing.

The N-terminal lysyl residue was introduced by coupling of Fmoc-Lys(Dde)-OH [20] instead of Fmoc-Lys(Mtt)-OH, since we were uncertain about the stability of the fluorescein label in the presence of triisopropylsilane which was added during Mtt-cleavage. As Fmoc and Dde are not orthogonal protecting groups, it was necessary to remove the Fmoc group first. The possible use of Dde-Lys(Fmoc)-OH was omitted because an N^{α}-Dde is sensitive towards migration to the N^{ε}-position, although this side reaction can be suppressed by using 1,8-diazabicyclo[5.4.0]undec-7-ene for Fmoc-cleavage [21]. Thus, the Fmoc group was removed from the Fmoc-Lys(Dde)-peptide-resin (4) and acylation was performed by reaction with *N*-succinimidyl palmitoylate and *N*-hydroxybenzotriazole. After cleavage of the Dde group from 5 with 1% hydrazine hydrate, the second label was then introduced by reaction with 5-carboxytetramethylrhodamine *N*-succinimidyl ester in the presence of DIPEA to give a red colored and fluorescent peptide-resin (6). To ensure removal of traces of the rhodamine derivative (or amino acids) which might have coupled to the hydroxyl groups of the fluorescein label, the peptide resin was again treated with 1% hydrazine.

Finally, complete deprotection and cleavage of the peptide from the resin was achieved with 95% TFA. Analysis of the crude product by HPLC with detection at 546 nm, *i.e.* at the wavelength of the maximum absorbency of 5-carboxytetramethylrhodamine, showed the presence of two major products (Fig. 2A). These products were purified by HPLC and the product which eluted from the HPLC column after about 18 min proved to be the carboxytetramethylrhodhamine-labeled descarboxyfluorescein lipopeptide Pal-K^{TMR}KKKRRYPDAVKL, as determined by FAB MS analysis, whereas the product which eluted after about 19 min was the target lipopeptide Pal-K^{TMR}KKKRRYPDAVK^{FL}L (7). The presence of a large amount of mono-labeled peptide in the crude product could hardly be explained by incomplete reaction of the resin-bound Fmoc-Lys-Leu with 5-carboxyfluorescein *N*-succinimidyl ester. In that case, the N^{ε}-acetyl Lys-12 derivative of the lipopeptide would have been expected as a side product due to the capping with acetic anhydride after the first labeling

reaction. Incomplete cleavage of the Mtt from the dipeptide seemed to be a more likely explanation. This could have been caused by the use of a rather small molar excess (about 12-fold) of TFA.

In view of the results obtained, a second synthesis with two modifications was performed. The protected dipeptide resin (2) was washed with 10% acetic acid in dichloromethane before Mtt-cleavage and this cleavage was performed with a larger volume of 1% TFA solution. The analytical HPLC profile (Fig. 2B) shows that the more thorough Mtt-cleavage had improved the quality of the crude product. After semi-preparative HPLC, Pal-K^{TMR}KKKRRYPDAVK^{FL}L (7) was obtained in a yield of about 30%. Lipopeptide 7 showed good biological activity in an *in vitro* CTL assay which is specific for the epitope RRYPDAVYL from the measles fusion protein (data not shown).

Finally, it was decided to prepare an isomeric peptide by interchanging the position of the two fluorescence labels. Differences in the biological properties of Pal-K^{TMR}KKKRRYPDAVK^{FL}L and Pal-K^{FL}KKKRRYPDAVK^{TMR}L (8) were not expected. From a chemical point of view, it seemed advantageously to circumvent the observed repetitive acylation and deacylation of the fluorescein moiety by introducing this label at the final stage of the synthesis. This was not the case, however, since peptide 8 was also obtained in about 30% yield after purification by semi-preparative HPLC.

Fluorescence spectra

Fig. 3 shows the fluorescence emission spectra of lipopeptide 7 (spectrum a), an equimolar solution of 5-carboxytetramethylrhodamine (spectrum b), and a 25-fold more diluted solution of 5-carboxyfluorescein (spectrum c) after excitation at 488 nm. From these three spectra it is estimated that the fluorescence of both labels in lipopeptide 7 was quenched to a degree of about 99%. After excitation at 568 nm, the rhodamine fluorescence was also almost completely quenched (98-99%, data not shown). On the other hand, the fluorescence emission spectrum of a mixture of the two labels corresponded to a simple summation of the spectra of the separate labels (data not shown). These observations are in accordance with the findings of Wei *et al.*, who reported fluorescence quenching of fluorescein and rhodamine of ~ 98% and ~ 90%, respectively, for a fluorescein- and tetramethylrhodamine-labeled peptide derived from hCG [13]. These authors ascribed the fluorescence quenching to dimerization of the two dyes, which presumably occurs when the two labels are within close proximity to each other. When the labeled hCG peptide was forced to adopt a different conformation by binding to an antibody, the rhodamine fluorescence increased up to 7.8-fold, whereas the fluorescein fluorescence was still quenched as a result of fluorescence resonance energy transfer from fluorescein to rhodamine. When lipopeptide 7 was incubated with trypsin, the fluorescence of fluorescein (Fig. 3, spectrum d) as well as tetramethylrhodamine (data not shown) rapidly increased as a result of enzymatic digestion of the peptide. This shows that a covalent linkage between the two labels (by means of the peptide) is a prerequisite for efficient quenching.

Confocal microscopy

Binding and internalization of lipopeptide 7 was studied using human melanoma cell line Mel/J transfected with HLA-B*2705. Incubation of the cells with this lipopeptide revealed a time and temperature dependent fluorescence. Figure 4 shows the fluorescence of cells after an

incubation of one hour at 37°C. Most of the staining is seen as intracellular membrane-bound fluorescence. Colocalisation of both fluorochrome-labels is colored yellow and is found on vesicle and tubular-like structures. These structures resemble the membranes of the rough endoplasmatic reticulum (RER). In some cells a pronounced green fluorescence from the fluorescein-label is seen in the perinuclear area (Figure 4, asterix), suggesting that in these cells the Golgi area is optically sectioned. In view of the quenching properties of intact lipopeptide we have to conclude that the observed fluorescence in the Golgi area and the colocalisation of both fluorochromes into RER-membranes may indicate either unfolding of the peptide by binding to intracellular membranes (analogous to the double labeled hCG) [13] or processing, *i.e.* enzymatic cleavage, of the peptide into smaller HLA-B*2705-binding peptides (class 1 major histocompatibility complex-, e.g. HLA-B*2705-binding peptides usually have a length of 8-10 amino acid residues). Definite proof should be obtained in future parallel studies of lipopeptide 7 and the corresponding mono-labeled lipopeptides.

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CHAPTER 3.1

In vivo antibody response and in vitro CTL activation induced by selected measles vaccine candidates, prepared with purified Quil A components

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ABSTRACT

Semipurified Quil A and purified Quil A were used to prepare well-characterized subunit vaccine candidates against measles. Variation in the relative amounts of the measles virus (MV) fusion (F) protein, Quil A-components and lipids did not influence induction of antibody responses in mice, but had a pronounced effect on the capacity to induce cytotoxic T cell (CTL) activity of a $CD8^+$ MV F-protein specific human T cell clone *in vitro*. A characteristic MV iscom preparation based on the combined use of HPLC-purified Quil A-components QA-3 and QA-22 (QA-3/22) efficiently induced CTL activity *in vitro*. Comparable results were obtained by mixing β-propiolactone inactivated MV with iscom-matrix QA-3/22 or free QA-22. On the basis of the data presented it was concluded that these three preparations are interesting MV vaccine candidates for further evaluation in pre-clinical experiments in a primate model.

INTRODUCTION

Measles is still a major cause of serious disease and mortality in developing countries, annually causing the deaths of more than 1 million children [1]. The limited ability of the currently used live attenuated measles vaccines to induce protective immunity in the presence of maternally derived measles virus (MV) neutralizing antibodies is considered a major stumbling-block in the control of measles in developing countries [2-6]. Therefore, the development of a vaccine that induces protective immunity in the presence of maternally derived MV specific antibodies, would be a major step forward for the eradication of measles, as is currently envisaged by the World Health Organization (WHO).

The specific immune response after MV infection against most of the MV proteins has been the subject of many studies in humans, non-human primates, and rodent models [7]. Still the individual contribution to protective immunity of the MV-specific humoral and cell mediated immune responses, against each of these proteins is largely elusive. However, the combined induction of long lasting virus neutralizing (VN) antibodies and $CD4^+$ as well as $CD8^+$ memory T cells warrants solid protection against measles [8].

For the successful control and eventual eradication of measles, the exploitation of new generations of vaccines and vaccination strategies may contribute, especially in very young infants with maternal derived MV-specific VN antibodies. Among the currently considered candidate vaccines, recombinant pox-viruses [9,10], recombinant plasmid DNA [11-13] and immune-stimulating complex (iscom) based vaccines [9] have attracted major attention. The iscom formulation proved to be of particular interest since it has been shown to induce both strong VN antibody and cell-mediated immune responses. In the presence of pre-existing VN antibodies, adequate protection could be induced with a MV candidate iscom vaccine in a macaque model [9]. The latter studies have all been carried out with iscoms prepared with semipurified Quil A using 'classical' centrifugation methods [14]. However, iscom based measles vaccines may only be considered for use in infants, if well-defined non-toxic purified Quil A components would be used. Crude Quil A, as was initially used for iscom preparation has been extensively analyzed by semi-preparative reversed phase high-pressure liquid chromatography (HPLC). Some purified fractions have been characterized in terms of sugar composition, hemolytic activity, and adjuvant activity [15-17].

Here we present a study in which we have analyzed five purified Quil A fractions (QA-3; QA-17; QA-20; QA-22 and QA-23) for their capacity to - either alone or in combination - allow the formation of iscoms using various preparation methods. The resulting iscom preparations were characterized with regard to the MV F- and hemagglutinin (H) protein, Quil A components and lipid content, and antigenic properties. In addition, we compared the immunogenic properties (antibody and T cell responses) of these preparations *in vitro* and *in vivo*.

MATERIALS AND METHODS

Virus

Vero cells grown in a 40 litre bioreactor on microcarriers (Cytodex 1, Pharmacia, Uppsala, Sweden) were infected with plaque-purified MV (Edmonston strain) at a m.o.i. of 0.2. After 64 hours, culture supernatant was harvested and concentrated using a 100Kd 0.92 m^2 cross-flow filter (Centracette, Filtron Technology Corporation, Massachusetts, USA). Further purification and concentration of virus were achieved by ultra-centrifugation (SW28 rotor 50' 18.000 rpm) through a 20 % (w/v) sucrose cushion. The resulting purified MV pellet was resuspended in PBS (2 mg/ml protein), contained $2x10^9$ $TCID_{50}$ MV per ml.

BPL inactivation

MV was chemically inactivated, at a dilution of 0.2 mg protein per ml PBS, by an 18 hours treatment with 0.025% (v/v) β-propiolactone (BPL, Aldrich Chemie, Steinheim, Germany) at 4°C. Subsequently, BPL was hydrolysed by incubation at 37°C for 2 hours. The BPL-inactivated MV was tested in Vero cell cultures for the absence of residual infectious MV.

HPLC-purified Quil A components

Quil A (Spikoside™) was obtained from Iscotec AB (Luleá, Sweden). Sub-fractions were isolated by preparative reversed phase HPLC on a Prep Nova-Pak HR C18 column (40 x 200 mm) (Waters, Milford, MA) [15]. Separation was achieved by acetonitrile-water gradient elution. A total number of 23 peaks and peak clusters were identified and were designated QA 1-23. The five major peaks (QA-3; QA-17; QA-20; QA-22 and QA-23) were collected and stored lyophilized until use.

Preparation of MV-iscom and iscom-matrix

MV was either solubilized in 2% Triton X-100 ("centrifugation method" - see below) or in 4% Octyl-glucoside ("dialysis method" - see below) and subsequently ultra-centrifuged through a 20% sucrose cushion [14]. The solubilized fraction was then mixed with semipurified Quil A or HPLC-purified Quil A components, and lipids (cholesterol: 5-cholesten-3β-ol and PC: L-α-phosphatidylcholine, Sigma Chemical Co., St. Louis, USA) in a ratio protein:Quil A:lipid of 1:5:1. Detergent was removed either by ultra-centrifugation over a continuous 10-60% sucrose gradient ("centrifugation method", refered to as C.) or by extensive dialysis against PBS ("dialysis method", refered to as D.). Non-complexed proteins, Quil A and/or lipids were removed by ultra-centrifugation through a sucrose gradient or cushion. For iscom-matrix preparation the same procedure was used with the omission of solubilized MV.

Characterization of MV-iscoms, iscom-matrix and BPL-inactivated MV

Electron microscopy (EM).

Iscom suspensions were adsorbed to glow-discharged carbon-stabilized formvar-coated grids, negatively stained using 2.0% ammonium molybdate (pH 5.1) or 2.0% potassium phosphotungstate (pH 6.0) and analyzed in a Philips TEM400 electron microscope at an operating voltage of 80 kV. Images were digitally stored and analyzed using analySIS (Soft-imaging software, Germany). Images were printed after grey value modification. Using negative contrast EM the capacity to form characteristic iscoms in the presence (MV-iscoms) or absence (iscom-matrix) of solubilized MV with semipurified Quil A and purified components of Quil A

was analyzed. A preparation was regarded as a characteristic iscom formulation if it showed spherical structures of 40 to 100 nm with ring-like subunits. Furthermore, the preparation had to comply with criteria of reproducibility, stability and uniformity.
Hydrodynamic particle sizes were determined by dynamic light scattering (DLS) using a System 4600 size analyzer (Malvern Instruments Ltd., Worcestershire, UK).

Total protein quantification
The protein quantification assay according to Bradford [18] was used to measure the total protein content. Samples were diluted in PBS 0.01% Tween-20. A BSA standard curve was used for calibration purposes.

SDS-PAGE
Protein compositions were analyzed by 10 % SDS-PAGE. After staining with Coomassie Brilliant Blue the gel was scanned using Bio Image® Intelligent Quantifier™ (Ann Arbor, Michigan, USA). Estimations of the protein compositions were in part based upon calculating the volume of the band in the gel by mearuring the area under the curve between the upper and lower edge of the band (densitometric profile), multiplied by the total lane width.

Antigen detection
The relative amounts of the F- and the H-antigens were determined using an inhibition ELISA. ELISA plates (high binding EIA/RIA, Costar, Cambridge, USA) were coated with BPL-inactivated MV, washed with 0.025% Tween-80 in H_2O and blocked with PTB (PBS, 0.1% Tween-20, 1% BSA). Samples were solubilized in 2% Triton X-100 for 30 minutes and serial dilutions were pre-incubated with a fixed concentration of monoclonal antibodies specific for the F- or H-protein (F7-21, C26-15 respectively). Mixtures were incubated on the coated plates and mouse immunoglobulins were detected using rat anti-mouse IgG HRP-conjugate (Jackson Immuno Research, Pennsylvania, USA), followed by incubation with TMB, and the absorbance was measured at $OD450_{nm}$. Relative amounts were determined as the dilution corresponding to 50% of the OD max. Characterized purified MV (2 mg/ml protein with a F-to-H ratio of 0.3) was used as standard.

Estimation of F- and H-protein contents
By combining the data of the total protein quantification, the SDS-PAGE and the antigen detection (see above) the amounts and ratios of F- and H-proteins were estimated.

Quil A quantification
Quil A and purified Quil A-components were quantified by chromatography on a Hypersil ODS 5µ analytical column (150 x 4.6 mm) (Shandon, Runcorn, UK). The acetonitrile concentration was increased from 32% to 40% in ammonium acetate buffer (pH 6.0) during the analysis. Peaks were detected at 208 nm. Quantification was achieved from the peak height. To quantify semipurified Quil A, the height of the 3 major peaks was used. Standard curves were run for calibration purposes.

Lipid quantification

Cholesterol was quantified by gas-chromatography (GC) / mass-spectometry (MS). To 5 µl sample 25 µl of 20 µg/ml cholesterol-d_7 (ICN Biochemicals Inc.) in aceton was added as an internal standard followed by vigorous mixing. The sample was dried and subsequently dissolved in 200 µl derivatization reagent (2 : 2 : 0.2 ethylacetate : acetic acid anhydride : triethylamine) and vigorously mixed. After heating at 60°C for 1h, the mixture was cooled on ice to room temperature and 200 µl H_2O and 300 µl ethylacetate were added successively. The sample was vigorously mixed for 1 minute, and subsequently the upper phase was collected. A volume of 1 ml was injected into a Hewlett Packard 5890A series II GC-instrument that was equipped with a 50 m CP Sil 5CB column of 0.25 mm i.d. and 0.12 µm thickness. A temperature program of 70°C for 1 min and then a 20°C/min rise to 250°C was used for sample loading. The column effluent was admitted to a Finnigan SSQ 710 mass spectrometer operating in negative ion mode. Ions at *m/z* 368.4 corresponding to cholesterol and *m/z* 375.4 corresponding to cholesterol-d_7 were monitored and the area ratio 375.4/368.4 was calculated. The cholesterol concentration was estimated from a standard curve.

Immunisation

Groups of five Balb/c mice were immunised intramuscularly with vaccine preparations at the doses indicated. Four weeks later a booster injection was given with the same dosis as the primary vaccination. Blood samples were collected before immunization and subsequently at weekly intervals.

Quantification of VN serum antibodies

Serial dilutions of heat-inactivated (30 minutes at 56°C) individual mouse sera were incubated with 50 $CCID_{50}$/ml plaque-purified MV (Edmonston strain) for 1 hour at 37°C and subsequently mixed with Vero cells ($2x10^4$ cells/well) and cultured in 96-well flat-bottomed plates (Greiner Labor Technik, Nürtingen, Germany) for seven days at 37°C as previously described [19]. Infected cultures were identified by microscopic monitoring for cytopathic changes. VN antibody titres (^{10}log) were expressed as the reciprocals of the highest serum dilution still giving 100% reduction of cytopathic changes.

Quantification of MV-specific serum antibodies

The MV-specific serum antibody responses were measured by MV-ELISA as previously described [19]. In short, ELISA plates (high binding EIA/RIA, Costar, Cambridge, USA) were coated with BPL-inactivated MV, washed with 0.025% Tween-80 in water and blocked with PTB. Individual samples were incubated as serial dilutions in PTB at 37°C for 1 hour. After washing mouse immunoglobulins were detected using HRP-conjugates of goat-antibodies (Southern Biotechnology Associates, Birmingham, USA) to mouse total-IgG, IgG1, IgG2a, IgG2b, followed by incubation with TMB and the absorbance was measured at $OD450_{nm}$. Serum titers were calculated as the dilution corresponding with 50% of the $OD450_{nm}$ max.

Quantification of MV F-specific serum antibodies

MV F-specific antibody responses were measured in a FACS-measured immunofluorescence assay using a MV F-protein transfected human melanoma cell-line as target cells (FACS-F assay) [20]. Briefly, Mel-JuSo/wildtype or Mel-JuSo/MV-F cells were incubated with serum (pooled per group; n = 5) dilutions in PBS supplemented with 2% FBS (FB) at 4°C for 1 hour. Cells were washed with FB, split into four aliquots and incubated with goat-anti-mouse Ig (Becton

Dickinson, Kranklin Lakes, USA), IgG1, IgG2a, and IgG2b (Nordic Immunology, Tilburg, Netherlands) FITC-conjugates in FB at 4°C for 1 hour. Fluorescence signals were measured using a FACScan (Becton Dickinson). Fluorescence intensity was quantified by determining the peak channel of the fluorescence histograms and corrected for Mel-JuSo/wildtype and normal mouse serum reactions. The experiments were repeated at least twice. The results of a representative experiment are shown.

CTL activation in vitro

Since the MV-iscom preparations proved to contain different protein : Quil A : cholesterol ratios, we adjusted their F-protein concentrations to a pre-defined level of 100 ng F-protein per ml. The CTL assay was essentially performed as described earlier [21]. Briefly, an autologous EBV transformed human B-cell line (B-LCL WH) was used as target cell for cytotoxic killing by a $CD8^+$ T cell clone (WH-F40) which responds to the natural occurring CTL epitope $F_{438-446}$ in the context of HLA-B27 [22]. This CTL clone was expanded and maintained according to previously described methods [22,23]. B-LCL WH were incubated twice within 48 hours at 37°C with vaccine candidates in RPMI-1640 supplemented with 2% FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) at $1x10^6$ cells/well of a 24-well plate (Greiner Labor Technik, Nürtingen, Germany) at the indicated concentrations. Treated B-LCL WH were then harvested and labelled with 100 µCi $Na^{51}CrO_4$ (Amersham, Buckinghamshire, UK). After incubation for one hour at 37°C, cells were washed 3 times with 2% FBS in RPMI and co-cultivated with WH-F40 in 96-well U-bottomed plates (Costar) at different effector-to-target ratios (E-to-T ratio). After incubation for 4 hours at 37°C, supernatants were collected and counted in a gamma counter. The percentage specific ^{51}Cr-release was expressed as $(cpm_{sample} - cpm_{spon}) / (cpm_{max} - cpm_{spon}) \times 100\%$ and corrected for the response to MV-BPL (in accordance with the MV-F concentration), which was never higher than 5%. Furthermore, iscom-matrix or free QA-22 alone were not able to sensitize autologous B-LCL target cells for cytotoxic killing at all. The experiments were carried out in triplicate and were repeated at least twice. In the experiments E-to-T ratios ranging from 1 to 12 were tested. Because at an E-to-T ratio 6 the responses appeared to have reached plateau levels the data presented in the respective figures were obtained using an E-to-T ratio of 6.

Statistical analysis

Results are presented as mean and standard deviations. The two-tailed Student's t-test is used for pairwise comparison of means, assuming a Gaussian distribution of each of both variables concerned. As in this study multiple pairwise comparisons were made the type-1 error rate α is lowered from the usual 0.05 to 0.01. Hence, for each tests a p-value smaller than 0.01 was considered to denote statistical significance. Also for each t-test the p-value is presented as calculated, unless the p-value is smaller than 0.001, in which case $p < 0.001$ is noted.

RESULTS

Iscom formation with MV components

Using semi-purified Quil A and purified MV, both the use of the "dialysis method" (D.) and the "centrifugation method" (C.) yielded characteristic iscom-like structures which could not be distinguished morphologically. The MV-iscoms D./QA-3/22 (ratio 1:4) are shown in Figure 1 as an example. The mean particle size found for the characteristic iscom-like structures was

47±5.8 nm (ranging from 39 to 56 nm) when measured by EM and DLS, which is within the range previously published for these structures [14]. We subsequently studied whether the five most abundant Quil A components were complexed into MV-iscoms, using the "dialysis method". Using component QA-3 characteristic iscom-like structures were obtained. The individual components QA-17, QA-20, QA-22 and QA-23 yielded iscom-like structures of more heterogeneous nature, often larger than 1μm in size (not shown). Next we included QA-22 in the preparations since *in vitro* studies showed that its presence contributed significantly to a functional activation of a CTL clone (see below). Irrespective of the presence of solubilized MV, mixtures of equal amounts of QA-3 and QA-22 (QA-3/22) also resulted in homogeneous-sized population of characteristic iscom-like structures (not shown).

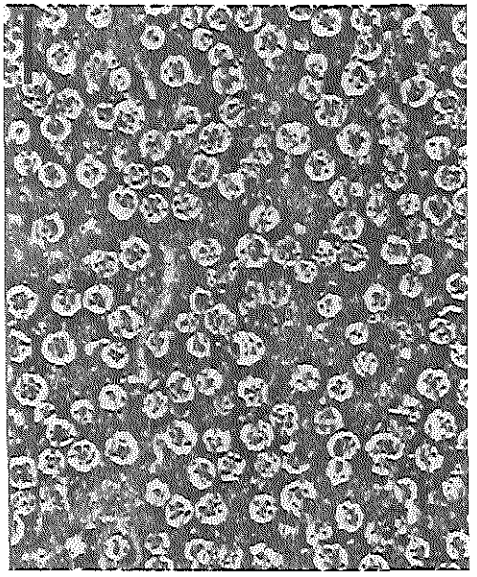

Figure 1. Transmission electron microscopy of MV-iscoms D./QA-3/22 (ratio 1:4) negative-stained with 2.0% phosphotungstic acid (PTA). Bar represents 100 nm.

Composition of the iscom containing vaccines

The total protein-to-Quil A ratios in the MV-iscom preparations varied from 0.17 to 0.34 (Table 1). Analysis of the relative quantities of F- and H-protein that had been incorporated were derived from SDS-PAGE scanning (Figure 2) and protein-specific ELISA. A marked difference between the MV-iscoms prepared by the "centrifugation method" and those prepared by the "dialysis method" was observed. The MV-iscoms prepared with the "dialysis method" had F- to-H-protein ratios that were similar to those in the MV concentrate used for preparation (0.3±0.07). In contrast, MV-iscom prepared with the "centrifugation method" contained a significantly higher F-to-H ratio (43.0±1.5), suggesting that this method resulted in a selective incorporation of the F-protein or an ineffective incorporation of the H-protein. BPL inactivation and the use of Triton X-100 or octylglucoside solubilization did not influence the protein distribution (Table 1).

Table 1. Characteristics of different preparations

Preparation	F-to-H ratio[a]	QA-3-to-QA-22 ratio[b]	Protein[c]: Quil A[b]: Cholesterol[d]
MV-concentrate	0.3 ± 0.07	-	-
BPL-inactivated MV	0.3 ± 0.1	-	-
Triton X-100 solubilisate	0.3 ± 0.05	-	-
octylglucoside solubilisate	0.3 ± 0	-	-
MV-iscom C./semi-purified	43 ± 1.5	ND[f]	1.0 : 4.0 : 0.4
MV-iscom D./semi-purified	0.4 ± 0.08	ND	1.0 : 3.0 : 0.8
MV-iscom D./QA-3/22 (ratio 1:1)	0.5 ± 0	0.64	1.0 : 2.9 : 0.7
MV-iscom D./QA-3/22 (ratio 1:2)	0.6 ± 0	0.35	1.0 : 3.4 : 0.7
MV-iscom D./QA-3/22 (ratio 1:4)	0.6 ± 0.16	0.19	1.0 : 5.9 : 0.9
D./QA-3/22 (ratio 1:4) iscom-matrix	-[e]	0.19	0:3.2 : 1.0

[a] Relative amounts of the F- and H-protein were calculated from both an estimation by SDS-PAGE followed by Coomassie Briljant Blue-staining, and by using an inhibition ELISA based on protein-specific monoclonal antibodies.

[b] Quil A and purified Quil A-components (QA-3 and QA-22) were estimated chromatographically.

[c] The protein quantification assay according to Bradford was used to estimate the total protein content.

[d] Cholesterol content was estimated by gas-chromatography/mass-spectrometry.

[e] Not applicable; C.: "centrifugation method"; D.: "dialysis method".

[f] ND: not determined.

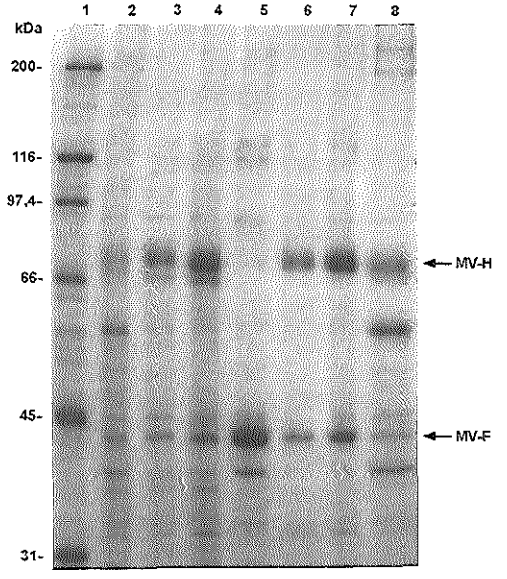

Figure 2. Analysis of viral polypeptides at various stages of purification and iscom formation by 10% SDS-PAGE under reducing conditions followed by Coomassie Brilliant Blue staining. Lane 1: molecular weight markers; lane 2: MV-concentrate; lane 3: supernatant after solubilization of MV with 2% Triton x-100; lane 4: supernatant after solubilization of MV with 4% Octyl-glucoside; lane 5: C./semipurified; lane 6: D./semipurified; lane 7: D./QA-3/22 (ratio 1:4); lane 8: BPL-inactivated MV. The masses in kDa of markers are shown on the left and the hemagglutinin (MV-H; 79 kDa) and the fusion (MV-F; 41 kDa) protein are designated on the right.

The QA-3/22 ratios as measured in the different preparations appeared to be largely similar to the relative amounts of QA-3 and QA-22 used to prepare the respective preparations (Table 1). The MV-iscom preparations showed an average distribution of protein : Quil A : cholesterol of 1.0±0.0 : 3.8±1.2 : 0.7±0.2 (Table 1).

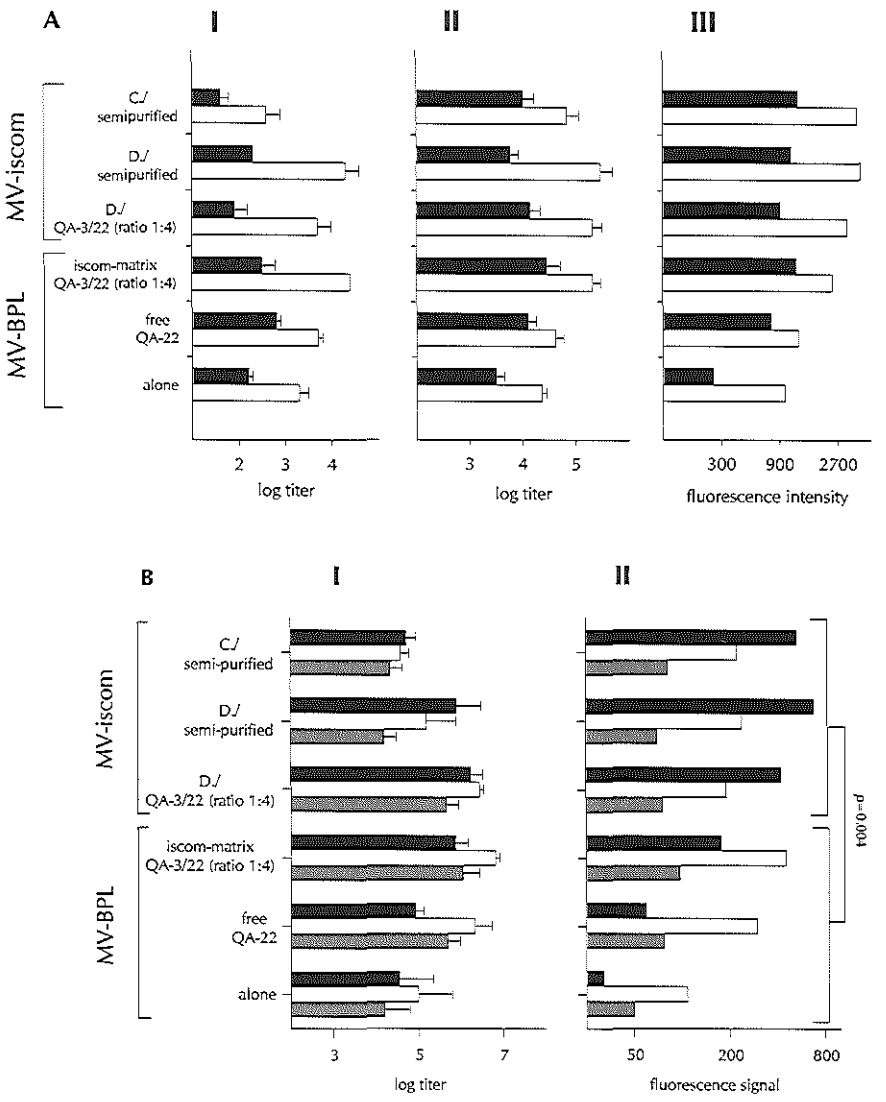

Figure 3. (A) Groups of five Balb/c mice were immunized on days 0 and 28 with different MV-vaccine candidates at a dose of 0.25 μg F-protein. (I) Development of MV-specific VN antibodies in serum collected on days 28 (black bars) and 56 (open bars). Titers represent the highest serum dilution still giving 100% reduction of cytopathic changes in Vero cells. (II) Development of MV-specific IgG antibodies in serum collected on days 28 (black bars) and 56 (open bars) as measured by the IgG MV-ELISA. (III) Development of MV-F protein-specific antibodies in serum collected on days 28 (black bars) and 56 (open bars) as measured by the FACS-F assay. The results are presented as the mean values with error bars representing the standard deviation, except for the FACS-F assay where the results of one representative experiment are shown.
(B) Development of MV-specific IgG1 (black bars), IgG2a (open bars), and IgG2b (grey bars) antibodies in mouse sera as measured by the MV-ELISA (I) or by the FACS-F assay (II) collected on day 42 or 28, respectively. The results are presented as the mean values with error bars representing the standard deviation, except for the FACS-F assay where the results of one representative experiment are shown.

Induction of humoral immune responses in mice

The capacity of MV-iscom preparations and of MV-BPL to which iscom-matrix QA-3/22 or QA-22 had been added, to induce serum antibodies in mice was studied by measuring antibody responses and IgG-isotype distributions upon one and two immunizations in the VN assay, MV-ELISA and FACS-F assay.

Twenty-eight days after immunization of mice with the Quil A based MV-vaccine candidates gave rise to VN serum antibodies in all cases with titers ranging from 1.6±0.2 to 2.8±0.1 (Figure 3a.I). Mice immunised with MV-BPL, either adjuvated with QA-3/22 (ratio 1:4) iscom-matrix or free QA-22 induced the highest levels of VN serum antibodies after one immunization. Immunization with MV-iscom prepared by the "centrifugation method" consistently showed lower levels of VN serum antibodies, which is probably due the low amount of the H-protein in the preparation. In all cases VN antibody responses were boosted significantly by the second vaccination at day 28.

MV-specific IgG ELISA titers were similar for the different preparations, ranging from about $3.1x10^3$ to $28x10^3$ after one immunization and from $23x10^3$ to $300x10^3$ after two immunizations, respectively. MV-BPL alone appeared to be the least immunogenic both after the first and second immunization (Figure 3a.II).

F-protein specific serum IgG levels measured in the FACS-F assay were more or less comparable after one and two immunizations for all preparations containing Quil A components, ranging from 756 to 1271 and 1275 to 4204, respectively. MV-BPL alone, a preparation without Quil A, appeared to be least immunogenic both after the first and the second immunization, resulting in IgG levels which were 3 to 4-times lower.

On basis of the MV-ELISA it was not possible to attribute a certain IgG isotype pattern to the different vaccine formulations (Figure 3b I). However, the induced F-specific IgG responses of different isotypes as measured in the FACS-F assay revealed that the MV-iscom preparations, irrespective of preparation method or Quil A components added, appeared to behave largely similar (Figure 3b II). The MV-iscom preparations induced a more predominant IgG1 F-protein specific response whereas the MV-BPL vaccines, with or without adjuvant, showed a more predominant IgG2a F protein specific response ($p=0.004$ with respect to the IgG1-to-IgG2a ratio).

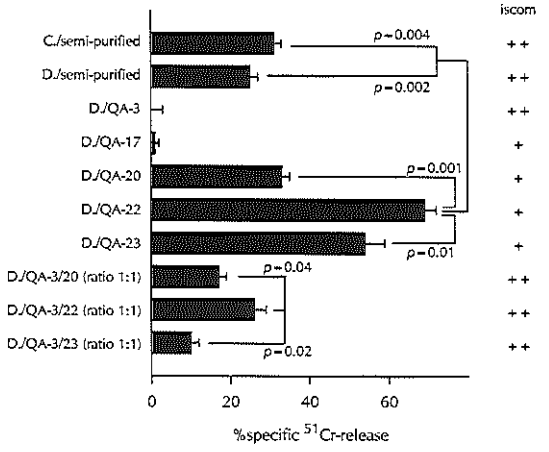

Figure 4. Antigen specific cytotoxic killing of B-LCL treated with different MV-iscom preparations, produced according to the "centrifugation method" (C.) or "dialysis method" (D.) using semi-purified Quil A or (com-binations of) purified Quil-A components (QA-3, QA-17, QA-20, QA-22, QA-23). F-protein concentrations were adjusted to a pre-defined level of 0.1 mg/ml. The results are presented as the average percentage specific ^{51}Cr-release with error bars representing the standard deviation. The quality of the different iscom preparations as determined by EM is indicated arbitrarily at the right: characteristic ultrastructural iscom-like structures (++) or iscom-like structures of a more heterogeneous nature (+).

In vitro activation of a F-protein specific CTL clone

The MV-iscom prepared with semi-purified Quil A either using the "centrifugation method" or the "dialysis method" elicited lytic activity of the CTL clone with lysis percentages of 31±2 and 25±2 respectively (Figure 4). The MV-iscom preparations prepared with single purified Quil A components, QA-3 or QA-17 using the "dialysis method", both failed to elicit lytic activity of the CTL clone with lysis percentages of <3%. The MV-iscom preparations prepared with QA-20, QA-22 or QA-23, using the "dialysis method" all elicited lytic activity of the CTL clone lysis percentages of 33±2, 69±3 and 54±5, respectively. The lysis elicited with QA-22 containing preparation was significantly higher than that with the preparations prepared with the semi-purified Quil A ("centrifugation method" and "dialysis method"), and QA-20 ($p=0.004$; 0.002 and 0.001, respectively). MV-iscoms prepared with purified Quil A components QA-22 and QA-23 elicited the highest CTL lysis. However, both failed to show the characteristic and well-defined iscom-like structures in negative contrast EM. Since it is not clear at present what the contribution of the iscom structure is for the induction of protective immunity, MV-iscom preparations were made with equimolar mixtures of QA-3 on the one hand and QA-20, QA-22 or QA-23 on the other hand using the "dialysis method". QA-3 was chosen since the MV-iscom preparation based on QA-3 showed characteristic iscom-like structures in negative contrast EM but failed to induce CTL lysis. Each of these MV-iscom preparations were indeed able to elicit lytic activity of the CTL clone with lysis percentages of 17±2, 26±3 and 10±2, respectiVely, but significantly lower than the MV-iscoms prepared with QA-20, QA-22 and QA-23 alone ($p=0.003$, 0.004 and 0.002, respectively (not indicated in Figure 4)). Each preparation did show the characteristic iscom-like structures in negative contrast EM (Figure 1). Since the MV-iscom prepared with equimolar quantities of QA-22 and QA-3 elicited the highest percentage of specific lysis of the three MV-iscom preparations prepared with mixtures ($p=0.04$ and 0.02, respectively). Next, MV-iscoms prepared from QA-3/22 mixtures with decreasing relative amounts of QA-3 (ratios 1:1; 1:2 and 1:4) were tested for their capacity to elicit lytic activity of the CTL clone (Figure 5).

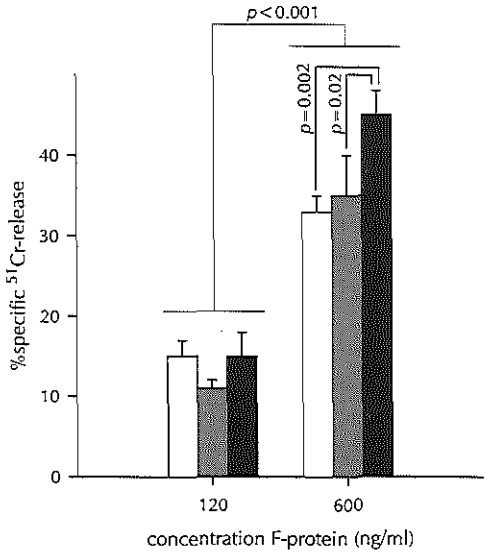

Figure 5. Antigen specific cytotoxic killing of B-LCL treated with two different concentrations of MV-iscom DJ/QA-3/22 preparations with different ratios between QA-3 and QA-22 (open bars: QA-3/22 (ratio 1:1); grey bars: QA-3/22 (ratio 1:2); black bars: QA-3/22 (ratio 1:4). The results are presented as the average percentage specific ^{51}Cr-release with error bars representing the standard deviation.

The resulting preparations were tested in two concentrations, standardized on the basis of the F-protein concentration (120 and 600 ng/ml F-protein). The percentage of cytolytic activity elicited with the highest doses were significantly higher for each of the resulting three MV-iscom preparations tested, ranging from about 11 to 15% lysis at the lowest and from about 33 to 45 % at the highest dose of F-protein ($p<0.001$). At the highest dose of F-protein the addition of QA-3 apparently had a negative effect on eliciting lytic activity, since higher percentages were obtained with the MV-iscoms prepared with the lowest QA-3/22 ratio ($p=0.002$ and 0.02, respectively). This effect was not seen at the lowest amount of F-protein but the specific release was borderline.

Dose-response curves of MV-iscom prepared with semi-purified Quil A and with QA-3/22 are shown in Figure 6. The MV-iscoms tested *in vivo* have been prepared using the "centrifugation method" or the "dialysis method" with semi-purified Quil A. Since the best CTL responses were obtained with MV-iscoms prepared with purified Quil A components in the "dialysis method" these preparations were selected for further testing in a dose response analysis (Figure 6). Using concentrations of the F-protein ranging from 0.03 to 9.0 μg/ml to compare with the semi-purified Quil A-besed preparations, a clear dose-response was obtained with all three preparations. With the MV-iscom based on QA-3/22 (ratio 1:4) the optimal response was obtained for 2 μg/ml F-protein, resulting in 65±12% lysis. Similarly, with the MV-iscom prepared with the "centrifugation method" using semi-purified Quil A, the optimal response was obtained for 0.1 μg/ml F-protein, resulting in 28±4% lysis. With the MV-iscom prepared with the "dialysis method", using semi-purified Quil A, a plateau was reached for 3 μg F-protein per ml or higher, also resulting in about 29% lysis.

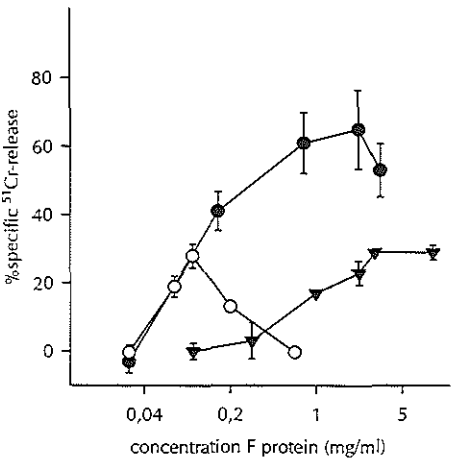

Figure 6. Dose response curves of antigen specific cytotoxic killing of B-LCL treated with different concentrations of MV-iscom preperations (indicated as concentration F-protein): MV-iscom C./semi-purified (○), MV-iscom D./semi-purified (▼), MV-iscom D./QA-3/22 (ratio 1:4) (●). The results are presented as the average percentage specific ^{51}Cr-release with error bars representing the standard d eviation.

To investigate whether cytolytic activity can also be elicited with preparations, in which the F-protein was not incorporated in the iscom-like structure prior to use, MV-BPL was mixed with QA-22 or with QA-3/22 (ratio 1:4) iscom-matrix. In analyzing the capacity of these preparations to elicit cytolytic activity of the F-protein specific CTL clone, MV-BPL was mixed either with a standard amount of Quil A-components (2 and 4 μg/ml) and a varying amount of F-protein (Figure 7a) or with a standard amount of MV-BPL (1 μg/ml F-protein) and a varying amount of Quil A-components (Figure 7b). The data show a clear dose response in the former experiment,

resulting in a maximal lysis of about 80% when 6.4 μg/ml F-protein was used in the presence of 4 μg/ml QA-3/22 (ratio 1:4) iscom-matrix. Also a dose response was observed, reaching a plateau value of about 57% when more than 1.2 μg/ml F-protein was used in the presence of 2 μg/ml free QA-22. In the latter experiment a maximum CTL lysis was observed at 4 μg/ml QA-3/22 (ratio 1:4) iscom-matrix and at 2 μg/ml free QA-22 of about 65 and 54%, respectively. In combining the data of these two experiments, an optimal F-protein-to-Quil A-components ratio could be identified for both preparation methods resulting in optimal ratios of about 0.6 (Figure 7c).

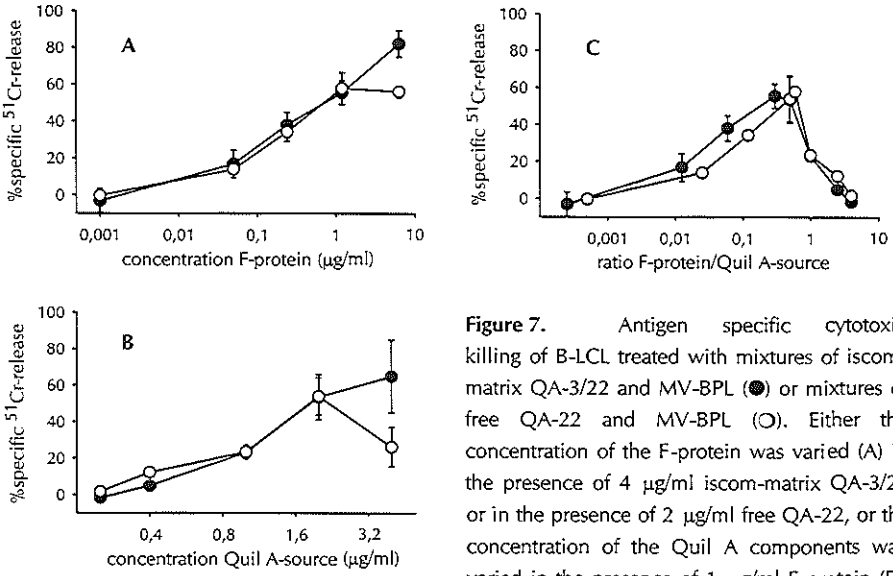

Figure 7. Antigen specific cytotoxic killing of B-LCL treated with mixtures of iscom-matrix QA-3/22 and MV-BPL (●) or mixtures of free QA-22 and MV-BPL (○). Either the concentration of the F-protein was varied (A) in the presence of 4 μg/ml iscom-matrix QA-3/22 or in the presence of 2 μg/ml free QA-22, or the concentration of the Quil A components was varied in the presence of 1 μg/ml F protein (B). In panel C the CTL-mediated killing is plotted versus the ratio F-protein/Quil A-components. The results are presented as the average percentage specific ^{51}Cr-release with error bars representing the standard deviation.

DISCUSSION

In the present paper we have shown that selected Quil A based measles vaccine candidates induced stronger and qualitatively different MV-specific antibody responses in mice than BPL-inactivated MV without Quil A-components. Furthermore, we showed that only preparations containing Quil A components induced *in vitro* cytolytic activity of a F-protein specific CTL clone.

A series of HPLC-purified Quil A components was used to study their individual contribution to the formation of the characteristic iscom-like structures and their potential to contribute to the induction of antibody responses in mice and *in vitro* CTL activation. These data were compared to those obtained with MV-iscoms, prepared with semi-purified Quil A using the "centifugation method" and the "dialysis method". Although the use of each of the purified Quil A components resulted in the formation of iscom-like structures, only QA-3 containing

preparations, including semipurified Quil A, allowed the formation of the characteristic iscom-like structures. The "dialysis method" allowed the incorporation of the F- and H-protein in ratios resembling those found in the intact virus, whereas the "centrifugation method" resulted in the selective incorporation of the F-protein. Therefore further experiments were carried out with MV-iscoms prepared with the dialysis method. A difference observed in the *in vivo* antibody responses was that MV-iscoms seemed to induce a more predominant F-protein specific IgG1 response than the preparations in which the antigen was not deliberately incorporated in the iscom structure (Figure 3b), suggesting that the iscom preparations may induce a more Th2-like response [24]. In the light of recent speculations about a possible role of Th2 type cells in the immunopathogenesis of the atypical measles syndrome observed in infants previously vaccinated with inactivated whole MV preparations [25], this may be a point of concern, that would need further investigation. In contrast the use of MV-BPL preparations mixed with the purified QA-22 or QA-3/22 iscom-matrix, did not show this production of a predominant IgG1 response.

The MV vaccine candidates were tested for their ability to present the F-protein through the MHC class I pathway, the prerequisite for activation of F-specific $CD8^+$ CTL, using a well characterized human T cell clone and autologous B-LCL as antigen presenting cells. There proved to be a marked difference in the ability of the individual purified Quil A components, to contribute to the *in vitro* sensitization of B-LCL for cytotoxic killing, QA-22 being the most and QA-3 being the least potent in this respect. QA-3 was apparently needed for the formation of characteristic iscom-like structures, and at present it is unknown to what extent this structure would contribute to the induction of protective immunity. This prompted us to further evaluate the potential of QA-22 and a mixture of QA-3 and QA-22, either after incorporation into iscoms or as free components, to elicit CTL activation *in vitro*. It was shown that increasing amounts of QA-3 had an inhibitory effect on the induction of CTL activity, when mixtures of QA-3 and QA-22 were used to prepare iscoms. A MV-iscom prepared with QA-3/22 (ratio 1:4) was selected to compare its potential to induce CTL activity with two classically prepared MV-iscoms, using the "centrifugation-" and the "dialysis methods". Higher levels of cytolysis (up to 65%) were obtained with the MV-iscoms prepared with the QA-3/22 mixture over a broad concentration range. It may be speculated that the MV-iscom prepared with the "centrifugation method" had an inhibitory effect at the higher concentrations tested.

Finally the question was addressed whether CTL activation could also be elicited when MV antigen was not deliberately incorporated in the iscom structure. This would be interesting for practical reasons, since it would allow the simple mixing of purified Quil A components or Quil A iscom-matrix with the antigen, rather than the more laborious construction of iscoms. Also in the light of the observed antibody responses in the mice (see above), this would be an attractive alternative. Therefore a series of CTL assays was performed in which the antigen used consisted of MV-BPL mixed with free QA-22 or QA-3/22 (ratio 1:4) iscom-matrix. In these experiments either a standard quantity of the Quil A-components with varying quantities of F-protein was used or *vice versa*. The data showed a clear dose-response curve both for the F-protein and for the Quil A-components, and an optimum F-protein-to-Quil A-components ratio of 0.6. This is largely comparable with the average F-protein-to-Quil A-components ratio of 0.3±0.1 (Table 1) in the MV-iscom preparations tested. Collectively these data show that for the induction of CTL activity *in vitro*, incorporation of the antigen during iscom preparation is not necessary. We speculate that QA-22 exerts a hydrophobic interaction with the MV membrane and thereby enhances presentation by the MHC class I pathway. Although at first sight this seems to conflict with our previous data [21], it can probably be explained by the differences in

the physico-chemical characteristics of the antigen preparations used in both studies. In the present study purified whole virus was used, whereas in the previous study affinity purified F-protein was used which spontaneously forms stable micelles.

In HPLC analysis, we have compared the purified Quil A components used in the present study with those used as adjuvant moieties by other groups (data not shown), which have obtained similar results in terms of induction of specific antibody and CTL activity in other systems [26,27]. Furthermore in the combination QH-A, -B and -C (ratio 7:0:3), used by Morein *et al.* [28], the QH-A fraction might contain QA-3 and the QH-C fraction might contain QA-22.

On basis of the collective data generated in these studies, it was decided to further study the potential of MV-iscom based on QA-3/22 (ratio 1:4), MV-BPL mixed with QA-3/22 (ratio 1:4) iscom-matrix or with free QA-22, to induce protective immunity in a macaque model for measles that was adopted by the WHO [9]. The recent demonstration that AMS can be reproduced in macaques with the original inactivated measles vaccine that predisposed infants of developing AMS [25], probably also offers the opportunity to study the safety of these preparations in this respect in macaques.

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Chapter 3.2

Use of cotton rats for preclinical evaluation of measles vaccines

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ABSTRACT

The continued prevalence and medical impact of measles worldwide has created interest in the development of new generations of measles vaccines. Monkeys can be used for preclinical testing of these vaccines. However, a more practical and less expensive animal model is highly desirable, particularly for initial vaccine development and evaluation. Cotton rats have been shown to support the replication of different strains of measles virus (MV), and thus may be useful for these purposes. To test this concept, the immunogenicity and protective efficacy of two standard (Moraten and trivalent measles, mumps, rubella) and four experimental (two recombinant ALVAC, one ISCOM subunit and live attenuated Edmonston-Zagreb) MV vaccines were evaluated in naïve cotton rats, and cotton rats with passively acquired MV-specific neutralizing serum antibodies. All of the test vaccines were immunogenic and protected naïve animals from pulmonary infection and viral dissemination. However, under the conditions utilized, only the Edmonston-Zagreb vaccine provided such protection to animals with significant levels of passively acquired MV-specific neutralizing antibodies. The results of these tests and the potential of using cotton rats as an animal model for preliminary testing of MV vaccines are discussed.

INTRODUCTION

Forty million cases of measles and nearly one million deaths due to this disease still occur worldwide annually, despite the presence of efficacious live measles vaccines and a vigorous measles virus (MV) eradication effort [1-3]. This can, at least in part, be attributed to the inability of live attenuated measles vaccines to induce protective immunity in the presence of maternally derived MV-specific antibodies. The continuing impact of measles has lead to the development of new generation measles vaccine candidates that may be effective in the presence of pre-existing immunity.

Monkeys, because they are susceptible to MV infection and develop a disease similar to measles in man [4-6], have been used to help develop and evaluate measles vaccines [4,7,8]. However, these primates can be difficult to obtain, expensive and exacting to work with, making even simple evaluation of the effects of vaccine composition, vaccine dose, inoculation schedule or ancillary adjuvants costly and problematic. Hispid cotton rats (*Sigmodon hispidus*) have recently been shown to support the replication of different strains of MV [9-11], including non-culture adapted wild-type isolates, and thus may be useful for initial preclinical testing of candidate measles vaccines. To evaluate this possibility, two licensed (Enders-Moraten [E-M] and trivalent measles, mumps, rubella [MMR]), and four experimental (two recombinant ALVAC, one ISCOM and one live attenuated [Edmonston-Zagreb; E-Z]) measles vaccines were compared for immunogenicity and protective efficacy in naïve hispid cotton rats and in hispid cotton rats with passively acquired MV neutralizing serum antibodies. The results of these tests and the potential of using these animals as a model for initial testing of measles vaccines are discussed below.

MATERIALS AND METHODS

Cell culture

B95-8 (Epstein-Barr virus-transformed marmoset leukocyte; cat. no. CRL 1612) and Vero (African green monkey kidney; cat. no. CCL 23) cells were obtained from the American Type Culture collection (ATCC; Rockville, MD). The latter cells were grown in minimum essential medium (MEM; Sigma Chemical Co., St. Louis, MO; cat. no. M4655) supplemented with 5% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 µg/ml), sodium bicarbonate (0.2%) and L-glutamine (2 mM/ml; 5% FCS-MEM). RPMI 1640 medium (JRH Biosciences, Lexana, KS; cat. no. 51501-78P), supplemented identically as the MEM, was used to grow the B95-8 cells. All of the media supplements were purchased from the Sigma Chemical Co. (St. Louis, MO).

Animals

Six to ten week old (60-110 g) cotton rats (*Sigmodon hispidus*) of either sex were used in these experiments. These animals were obtained from the Baylor College of Medicine (BCM) cotton rat colony that was started in 1984 using six pair of animals obtained from the Small Animal Section, Veterinary Research Branch, Division of Research Services, National Institutes of Health (Washington). All of the animals were housed in cages covered with barrier filters and were given food and water ad libitum.

Measles viruses and vaccines

The MO2 strain of MV that was used in these studies to challenge cotton rats or to act as a positive control in experiments evaluating the different MV vaccine candidates, was originally isolated in B95-8 cells in 1993 from a Zambian child hospitalized with measles. The virus was passaged three times in these cells before being sent to us by Dr. Hiroshi Suzuki (Department of Public Health, Niigata University, Nigata, Japan), with permission of his collaborators, Katsumi Mizuta (Virus Research Center, Sendai National Hospital, Sendai, Japan), and Mwila E. Mpabalwani (Virology Laboratory, The University Teaching Hospital, Lusaka, Zambia). The growth of this virus in lungs of cotton rats has been described in detail previously [11]. A working pool of MO2 MV was prepared by infecting monolayers of B95-8 cells with this virus and then placing the flasks containing the infected cells in a 368C (5% CO2) incubator. Six days later, when the cell monolayers exhibited >90% syncytia formation, the flasks were removed and sonicated six times (15 s each at 50/60 Hz) in a Branson model 220 sonicating water bath. The medium from each flask was then collected, pooled and centrifuged at 450 g for 20 min. The resulting supernatant fluids were passed through a 0.45 mm filter (Acrodisc, Gelman Scientific Inc., Ann Arbor, MI; cat. no. 4184; Ann Arbor, MI), portioned, labeled and frozen at –70°C. The median tissue culture infectious dose (TCID50) of this pool was determined as described below. The E-M and MMR virus vaccines were obtained from the Influenza Research Center BCM. These vaccines were produced by Merck, Sharp and Dohme and Merck and Co., respectively. Vials of both vaccines were stored at 4°C and were reconstituted with distilled water just prior to use. The E-Z MV vaccine was obtained from Dr. Lauri E. Markowitz, Division of Immunization, Centers for Disease Control, Atlanta, GA. Its derivation from the prototype Edmonston MV vaccine strain has been described in detail elsewhere [12]. In some experiments, cotton rats were injected with E-M or MMR MV vaccines inactivated by ultraviolet (UV) light. This inactivation was carried out by placing thin layers of reconstituted vaccine 35 mm below an activated 30 W UV light source (GTE Sylvania) for 7 min. Portions of the exposed suspensions were then collected and tested for virus viability as described below. The rest were used neat to inoculate cotton rats. Two avianpox virus vaccines, ALVAC-F,HA and ALVAC-F,HA,M,N were used in these studies. The former consists of a recombinant avianpox virus that expresses both the fusion (F) and haemagglutinin (HA) proteins of the Edmonston strain of MV. The latter vaccine is comprised of a recombinant avianpox virus that expresses four Edmonston MV proteins: the F, HA, matrix (M) and nucleoprotein (N). ALVAC, the parental virus vector used to derive these recombinant vaccines, is not a recombinant virus and does not express any MV proteins. It was included in these tests as a negative control preparation. All three ALVAC vaccines were acquired from Virogenetics Corp., Troy, NY. The engineering of the latter vaccines has been described in detail elsewhere [13,14].

Immune-stimulating complexes (ISCOM) manufactured in the absence (ISCOM-matrix) or presence of solubilized MV (Edmonston strain; ISCOM-F,HA) were prepared using HPLC-purified Quil A components as described previously [15]. At the same time beta-propiolactone (bpl)-inactivated MV (Edmonston strain) mixed with ALUM (aluminum hydroxide) and bpl-inactivated MV mixed with QA-3/22 (an HPLC-purified Quil A component) were also produced. Formalin-inactivated, monovalent influenza A/Texas subvirion virus vaccine (Connaught Laboratories Inc., Swiftwater, PA) mixed with (not deliberately incorporated into) ISCOM-matrix (ISCOM-matrix/Flu) was also prepared and included as a (negative) control in these experiments. Prior to the start of experiments testing ISCOMS, all of the preparations

containing MV were adjusted so that each cotton rat received approximately 0.25 μg of MV F protein per inoculation. The ISCOM-matrix/Flu suspension was adjusted so that each animal was injected with approximately 0.25 mg of influenza virus haemagglutinin (HA)/inoculation.

Virus quantification

Viable MV in the E-M and MMR vaccines was quantified using Vero cells and a standard plaque assay [16]. Viable MO2 virus in inocula, lung fluids (LF) and mediastinal lymph nodes (MDLN) was determined in 96-well tissue culture plates (Falcon 3071) using B95-8 cells and virus-induced cytopathic effects (CPE) as an endpoint. This CPE-based assay has been described previously [9]. The minimal amount of virus detectable in these assays was 100 $TCID_{50}$/g of lung. Titers were not usually determined for MDLN suspensions. Instead, the lymph node suspensions were generally recorded as being positive or negative for virus.

Quantification of virus neutralizing serum antibodies

Assays for MV-specific neutralizing (VN) antibodies were performed in 96-well tissue-culture plates (Falcon 3071) as described previously [9]. In all instances MV MO2 was used as the challenge virus. All assay plates were kept in a 36°C CO_2 incubator and observed daily. When the cells in the virus control wells (wells with cells and virus, but no antibody) exhibited ≥70% CPE, all of the wells in the assay were observed and scored for the presence of virus. Titers were expressed as the $\log_2$ of the reciprocal of the last dilution of anti-serum that completely inhibited virus-induced CPE.

Inoculation of animals

MV MO2 was always administered intranasally (i.n.). These inoculations were carried out by gradually introducing 50 μL of inoculum, containing approximately 10^4 $TCID_{50}$ of virus, into the nares of cotton rats anesthetized with Metofane (methoxyfurane, Pitman-Moore, Mundelein, IL). Delivery was made using a P-200 pipetting aid (Rainin Instruments Co. Inc. Woburn, MA). All live attenuated vaccine preparations were administered intramuscularly (i.m.) into the quadriceps of the animals. Immune serum globulin (ISG; Armour Pharmaceutical Co., Phoenix, AZ) was given intraperitoneally (i.p.). This preparation had a VN titer of 4096/0.05 ml against the MO2 strain of MV. It was always administered in 0.2 ml vols approximately 30 min prior to vaccine. Naïve cotton rats administered ISG in this manner generally had a VN titer between 5 and 6 $\log_2$ /0.05 ml of serum one hour after inoculation. For both i.m. and i.p. inoculations, the cotton rats were rendered unconscious using CO_2 prior to inoculation.

Collection of samples

Blood was collected from the retro-orbital sinus plexus of animals anesthetized with Metofane. Serum was collected from each sample, heat inactivated at 56°C for 30 min and stored at 4°C until assayed for virus-specific neutralizing antibodies. The lungs and MDLN were collected from cotton rats sacrificed using CO_2. Each set of lungs was rinsed in phosphate buffered saline, weighed and transpleurally lavaged as described previously [17]. The MDLN were teased into single-cell suspensions. The resulting lung lavage and lymph node cell suspensions were assayed for MV using the CPE-based $TCID_{50}$ assays described above.

Experimental designs

Vaccine and control suspensions were usually administered twice (3 or 4 weeks apart). The cotton rats were bled from the retro-orbital sinus plexus just prior to the start of the experiment, before administering the booster inoculation and just before inoculating the animals with challenge virus. The cotton rats were always challenged with MV MO2 1 week after the last administration of vaccine or control suspension. All animals were sacrificed 4 days after virus challenge, at a time when peak virus replication usually occurs in unvaccinated animals [11]. At that time the lungs and MDLN of the sacrificed animals were removed and tested for MV. In experiments comparing the immunogenicity and protective efficacy of different test vaccines in nonimmune animals and animals that were passively immunized with MV-specific antibodies (see Table 4 for an example), one-half of the cotton rats were inoculated i.p. with placebo (medium; 0.2 ml/animal) and one-half were inoculated similarly with human immune serum globulin (ISG). Thirty minutes later, five of the animals inoculated with placebo and five that received ISG were bled from the retro-orbital sinus plexus to obtain baseline VN titers. The animals given placebo were then divided into six groups of four animals, as were the cotton rats inoculated with ISG. One group in each of these sets was administered E-M vaccine, one group MMR, one group ALVAC-H,F and one group E-Z vaccine (all i.m.). Four animals in each set were inoculated i.n. with MO2 virus. Nothing was done at this time to the remaining animals (four in each set). Twenty-one days later each animal was bled and then challenged i.n. with 10^4 $TCID_{50}$ of MV MO2. Four days later, all of the cotton rats were sacrificed and the levels of virus present in the lungs of each were determined. Serum from each blood sample was assessed for VN antibody levels. In the experiment shown in Table 4, the geometric mean antibody titer (GMAT) of the sera collected from the animals bled 30 minutes after the administration of media was determined to be 0.5 ± 0.6 $\log_2$/0.05 ml of sera, while that of the cotton rats injected with ISG was established to be 5.6 ± 0.9 $\log_2$/0.05 ml of sera. By day 21 in this experiment, the level of MV-specific neutralizing antibodies present in the sera of the latter group of cotton rats (see Group 2, Table 4) diminished to background levels (i.e., to 0.4 ± 0.6), while the antibody titers in the group given medium remained about the same (0.5 ± 0.6; see group 1, Table 4).

Histology

Lobes of lung designated for histopathological studies were placed in Omnifix (Zymed Laboratories Inc., San Francisco, CA) for a minimum of 24 h. They were then embedded in low-melting point paraffin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin. The stained sections were observed in a blinded fashion for histopathology using a light microscope.

Statistics

Calculation of descriptive statistics (e.g., geometric mean titers and standard deviations) and all statistical analyses were performed using Instat (GraphPad Software, San Diego, CA), a software program for IBM compatible computers. Means obtained for the different test groups were compared using the Kruskal-Wallis nonparametric analysis of variance test ($\geq$three means compared). The Fisher Exact Test was used to compare the number of virus-positive versus the number of virus-negative MDLN in different groups. In all of these tests a p-value ≤ 0.05 was considered to be statistically significant.

RESULTS

Virus-specific neutralizing antibody responses and protective efficacy in cotton rats administered licensed measles vaccines

Cotton rats vaccinated with two doses of commercially available E-M or MMR measles vaccine produced significant levels of VN antibodies (Table 1, column 4). The mean VN titers seen in these groups were equivalent (i.e., 7.3-7.8 for the groups inoculated with E-M virus versus 7.2-7.8 $\log_2$/0.05 ml serum for the cotton rats vaccinated twice with MMR). These titers were about twice those measured in animals inoculated once with wild-type MO2 MV (i.e., 6.6 $\log_2$/0.05 ml serum). Following challenge, 1 week after the last immunization, all of the sero-converted cotton rats appeared to be solidly protected, since challenge virus could not be isolated from any lung or mediastinal lymph node collected from these animals (Table 1, columns 5 and 6).

Table 1. Measles virus-specific neutralizing antibody responses, pulmonary virus titers and virus dissemination in cotton rats administered commercially available Enders -Moraten (E-M) or measles, mumps and rubella (MMR) vaccines prior to challenge with wild -type measles virus[a].

Group no.[b]	Vaccine or virus	Challenge virus	Mean Ab titer ($\log_2$/0.05 ml serum)	Mean MV titer ($\log_{10}$ $TCID_{50}$/g lung	No. MDLN positive
Expt 1					
1	None (dH_2O)	MV MO2	0.8±0.5	3.5±0.4	3 of 10
2	E-M	MV MO2	**7.8±1.0**[c]	**0±0**	0 of 10
3	MMR	MV MO2	**7.2±1.0**	**0±0**	0 of 10
4	MV MO2	MV MO2	**6.6±0.8**	**0±0**	0 of 10
Expt 2					
1	None (dH_2O)	MV MO2	1.8±0.5	4.0±0.4	3 of 10
2	E-M	MV MO2	**7.3±0.5**	**0±0**	0 of 10
3	MMR	MV MO2	**7.8±1.0**	**0±0**	0 of 10
4	MV MO2	MV MO2	**6.6±1.3**	**0±0**	0 of 10

[a] In both Experiment 1 and 2, the cotton rats in Group 1 were inoculated intramuscularly (i.m.) on days 0 and 28 with placebo (distilled water), while those in Groups 2 and 3 were inoculated similarly with 10^4 plaque forming units (pfu) of monovalent Enders-Moraten measles or trivalent measles, mumps, rubella (MMR) virus vaccines. The cotton rats in Group 4 in both experiments were inoculated intranasally with 10^4 $TCID_{50}$ of MV MO2 only on day 0. On day 35, all of the animals were bled, sera was obtained and the animals were challenged intranasally with 10^4 $TCID_{50}$ of MV MO2. Four days later, the animals were sacrificed and their lungs and mediastinal lymph nodes were tested for MV.

[b] Abbreviations: no. = number; Ab = antibody; MDLN = mediastinal lymph nodes, dH_2O = distilled water.

[c] Bolded means and standard deviations indicate a statistically significant different mean virus-specific serum neutralizing antibody and mean pulmonary virus titers ($p < 0.05$) compared to these mean titers in Group 1 as determined using the Kruskal-Wallis nonparametric analysis of variance test; number of animals/group = 5.

In contrast to these findings, pulmonary virus was readily detectable in the lungs of animals taken from the control animals given placebo (i.e., distilled water). In experiment 1, the mean pulmonary virus titer for this group was 3.5 $\log_{10}$ $TCID_{50}$ of virus/g lung, and in experiment 2, 4.0 $\log_{10}$ $TCID_{50}$ of virus/g lung. These values were significantly higher than the titers seen in the vaccinated animals, as indicated by the bolded values in column 5 of Table 1. In neither experiment 1 nor 2 was there a significant difference in the mean number of virus-positive MDLN obtained from any group (i.e., p was >0.05 when any of the values obtained were compared using the Fisher Exact Test). However, if the data from the two experiments were combined, a significant difference in the mean number of virus-positive lymph nodes obtained from the control animals (i.e., 6 of 20) and the number of positive samples obtained from the other groups (i.e., 0 of 20) was seen ($p = 0.02$), indicating fairly frequent dissemination of virus from the lungs of unvaccinated animals.

Virus-specific neutralizing antibody responses and protective efficacy in cotton rats administered recombinant avianpox-measles vaccines

A single vaccination of cotton rats with 10^8 pfu of either of the recombinant avianpox-measles vaccines induced significant levels of virus-specific neutralizing antibodies (Table 2, column 4). The VN titers attained in both groups of vaccinated animals were similar (i.e., 5.4 $\log_2$/0.05 ml for ALVAC-H,F compared to 5.6 $\log_2$/0.05 serum for ALVAC-H,F,M,N). Significant levels of MV specific VN antibodies were not detected in the control animals that were inoculated with the were seen at this time in the animals vaccinated with MV mixed with ISCOM-matrix (i.e., 5.8 $\log_2$/0.05 ml serum). However, in this experiment, the highest antibody levels occurred in the groups of animals inoculated with bpl-inactivated MV adjuvated with ALUM (mean VN titer 6.6 ± 0.9) and those inoculated with bpl-inactivated MV mixed with free QA-3/22 (mean VN serum titer 8.2 ± 0.9). As in the previous experiments, all sero-converted cotton rats were protected upon challenge 1 week after the last vaccination: challenge virus was not isolated from any of the lungs or MDLN of these animals (Table 3, columns 5 and 6). In contrast, >4 $\log_{10}$ $TCID_{50}$ of MV/g lung was detected in the animals given two doses of inactivated influenza virus vaccine mixed with the ISCOM matrix, and virus was detected in the MDLN of two of the five cotton rats in this group.

Table 2. Measles virus-specific neutralizing antibody responses, pulmonary virus titers and virus dissemination in cotton rats administered recombinant avianpox viruses expressing measles virus proteins prior to virus challenge with wild-type measles virus[a]

Group no.[b]	Vaccine or virus	Challenge virus	Mean Ab titer ($\log_2$/0.05 ml serum)	Mean MV titer ($\log_{10}$ $TCID_{50}$/g lung	No. MDLN positive
Expt 1					
1	ALVAC	MV MO2	0.4 ± 0.5	3.9 ± 0.4	6 of 10
2	ALVAC-H,F	MV MO2	**5.4 ± 0.8**[c]	**0 ± 0**	**0 of 10**
3	ALVAC-H,F,M,N	MV MO2	**5.6 ± 0.8**	**0.5 ± 1.2**	**0 of 10**
Expt 2					
1	ALVAC	MV MO2	0.4 ± 0.5	3.9 ± 0.4	4 of 10
2	ALVAC-H,F	MV MO2	**5.4 ± 0.8**	**0 ± 0**	**0 of 10**
3	ALVAC-H,F,M,N	MV MO2	**5.6 ± 0.8**	**0 ± 0**	**0 of 10**

[a] The cotton rats were inoculated intramuscularly with 10^8 plaque forming units of the appropriate vaccine on day 0. Twenty-eight days later each was bled and challenged with approximately 10^4 median tissue culture infectious doses of measles virus (MV) strain MO2. Four days later, each animal was sacrificed. The lungs and mediastinal lymph nodes from each rat were collected and tested for MV.

[b] Abbreviations: no. = number; H = MV hemagglutinin protein; F = MV fusion protein; M = MV matrix protein; N = MV nucleoprotein.

[c] Bolded means and standard deviations indicate a statistically significant different mean virus-specific serum neutralizing antibody and mean pulmonary virus titers ($p \leq 0.05$) compared to these mean titers in Group 1 as determined using the Kruskal-Wallis nonparametric test; number of animals/group = 10.

Immunogenicity and protective efficacy of different measles virus vaccines in nonimmune cotton rats and cotton rats with passive antibodies to measles virus

To further test the value of cotton rats for preliminary evaluation of MV vaccines, four different MV vaccine candidates were given to naïve cotton rats or to cotton rats that were passively immunized with ISG with high titer of VN antibodies 30 min prior to vaccination. Four different MV vaccines (i.e., E-M, MMR, ALVAC-H,F and E-Z) were tested. In addition, an additional group of naïve and passively immunized animals were inoculated with MV MO2 to act as a positive control. As the data in Table 4 indicate, all of the vaccines tested induced

Table 3. Measles virus-specific neutralizing antibody responses, pulmonary virus titers and virus dissemination in cotton rats administered ISCOM measles virus vaccine prior to virus challenge with wild-type measles virus[a]

Group no.[b]	Vaccine or virus	Challenge virus	Mean Ab titer (log_2/0.05 ml serum)	Mean MV titer (log_{10} $TCID_{50}$/g lung	No. MDLN positive
Expt 1					
1	ISCOM+FLU	MV MO2	1.0±0.7	4.2±0.5	2 of 5
2	bpl-MV+ALUM	MV MO2	**6.6**±0.9[c]	**0**±0	0 of 5
3	Quil A+bpl-MV	MV MO2	**8.2**±0.9	**0**±0	0 of 5
4	ISCOM+MV	MV MO2	**5.8**±0.8	**0**±0	0 of 5
5	MV in ISCOM	MV MO2	**5.6**±0.9	**0**±0	0 of 5

[a] The cotton rats in Group 1 in each experiment were inoculated on day 0 intramuscularly with empty ISCOM mixed with formalin-inactivated whole influenza virus vaccine (2.5 μg influenza haemagglutinin (H) protein/inoculation). The animals in Groups 2-5 were inoculated similarly on this day with one of the measles virus (MV) preparations listed in the table above (each containing 2.5 μg of MV fusion (F) protein/inoculation). Twenty-eight days later each animal was bled and challenged with approximately 10^4 median tissue culture infectious doses of measles virus (MV) strain MO2. Four days later, each animal was sacrificed. The lungs and mediastinal lymph nodes from each rat were collected and tested for MV.

[b] Abbreviations: no.=number; H=MV haemagglutinin protein; F=MV fusion protein; M=MV matrix protein; N=MV nucleoprotein.

[c] Bolded means and standard deviations indicate a statistically significant different mean virus-specific serum neutralizing antibody and mean pulmonary virus titers ($p=0.01$) compared to these mean titers in Group 1 as determined using the Kruskal-Wallis nonparametric test; number of animals/group = 5.

Table 4. Virus-specific neutralizing antibodies and pulmonary virus titers in cotton rats administered measles virus vaccines 1 h after being given placebo or immune serum globulin[a]

Group	ISG[b]	Virus or Vaccine[c]	Mean serum Nt. antibody titer (log_2/0.05 ml±S.D.)[d]	Mean pulmonary MV titer (log_2/0.05 ml±S.D.)[e]
1	No	None	0.5±0.6	4.3±0.3
2	Yes	None	0.8±0.5	4.8±0.6
3	No	E-M	6.0±1.2	**0**±0
4	Yes	E-M	**2.0**±0	3.3±0.5
5	No	MMR	6.3±1.0	**0**±0
6	Yes	MMR	**1.8**±0.5	3.5±0.4
7	No	ALVAC-H,F	5.5±0.6	**0**±0
8	Yes	ALVAC-H,F	**1.8**±0.5	3.3±0.5
9	No	E-Z	8.3±1.0	**0**±0
10	Yes	E-Z	6.0±1.4	**0**±0
11	No	MO2	6.8±1.2	**0**±0
12	Yes	MO2	4.8±1.0	**0**±0

[a] On day 0, five cotton rats were bled. All of the animals in even numbered groups were then inoculated intraperitoneally (i.p.) with immune serum globulin (ISG), while those in the odd numbered groups were injected i.p. with medium. One hour later, five of the cotton rats injected with ISG were bled and the different vaccines or MV MO2 were administered intramuscularly or intranasally (i.n; doses indicated in footnote c). Twenty-one days later, each animal was bled, sera were collected and each animal was challenged i.n. with 10^4 $TCID_{50}$ of MV MO2. Four days later, all of the cotton rats were sacrificed and assessed for pulmonary virus.

[b] Abbreviations: CR=cotton rat; MV=measles virus, E-Z=Edmonston Zagreb; E-M=Enders-Moraten; ISG=immune serum globulin; Nt.=neutralizing; S.D.=standard deviation; ALVAC-H,F=avianpox virus expressing measles virus haemagglutinin and fusion proteins.

[c] Animals were inoculated with 10^4 plaque forming units (pfu) of E-M, MMR or E-Z, 10^4 $TCID_{50}$ of MV MO2 or 10^8 pfu of ALVAC-H,F.

[d] The antibody titers shown in this column are those obtained for sera collected on Day 21, just prior to virus challenge. The mean measles virus-specific antibody in the sera of the five animals bled at T0 was 12±0.8, while the titer of those bled at +1 h=was 5.6±0.9.

[e] Bolded means and standard deviations in column 4 indicate a statistically significant reduction in mean virus-specific serum neutralizing antibody titer ($p \leq 0.05$) in groups administered vaccine 1 h after ISG, compared to this titer in animals not inoculated with ISG but given the same vaccine. Bolded means and standard deviations in column 5 indicate a significant difference in mean pulmonary virus titer compared to the mean pulmonary virus titer in group 1.

significant levels of VN titers (i.e., ≥ 5.0 $\log_2/0.05$ ml of serum) in animals lacking such antibodies at the time of vaccination (column 4, groups 3, 5, 7 and 9). Moreover, no virus was detected in any of the lungs removed from these animals (column 5), indicating that these animals were protected from pulmonary MV infection. In contrast, the cotton rats administered the E-M, MMR and ALVAC-MV vaccines 30 min after administration of the ISG (i.e., Groups 4, 6, and 8) had significantly lower mean MV-specific neutralizing antibody titers in their sera on day 21 than the comparably vaccinated animals that had not been administered ISG prior to vaccination (i.e., titers ≤ 2.0 $\log_2/0.05$ ml), and they had significantly higher levels of MV present in their lungs (i.e., titers ≥ 3.3 $\log_{10}$ $TCID_{50}/g$ lung). Indeed, the mean antibody and virus titers in these groups were not significantly different ($p > 0.05$) from those determined for the untreated, nonvaccinated control animals (i.e., Group 1). Interestingly, the only two groups whose antibody responses were not significantly suppressed by the presence of passively acquired VN antibodies were 10, the group that contained animals vaccinated with E-Z vaccine 30 min after ISG was administered, and 12, the group containing cotton rats inoculated with MV MO2 subsequent to ISG administration. No virus was detected in the lungs of any of the animals in these two groups.

Histological findings

Lung sections obtained from sham-inoculated cotton rats, or on day 4 after virus challenge from cotton rats with levels of MV-specific neutralizing antibody titers ≥ 5.0 $\log_2/0.05$ ml serum, manifested little evidence of inflammation or pathology (see Fig. 1 which shows a section of lung obtained 4 days after challenge with MV MO2 from a cotton rat vaccinated with MV in ISCOM 28 days prior to virus challenge). In contrast, scattered foci of edema and inflammatory cells were observed scattered throughout hematoxylin and eosin-stained sections of lung prepared from unvaccinated cotton rats four days after these animals were challenged i.n. with MV MO2 (see Fig. 2 for an example). Similar numbers and kinds of foci were present in sections of lung taken from the animals vaccinated with influenza virus mixed with ISCOM-matrix QA-3/22 or those inoculated with the nonrecombinant ALVAC vector prior to challenge with MV MO2. Sections of lung from animals with lower VN titers (e.g., Groups 4, 6 and 8 in Table 4) showed intermediate levels of inflammation (fewer, smaller and more scattered foci of inflammatory cells and edema). Thus, the inflammation and pathology present in the lungs of the animals used in these tests appeared to correlate inversely with serum antibody titers, and directly with the levels of virus present in the lungs.

DISCUSSION

Six vaccines, representative of the major types of MV vaccines currently available (replicating recombinant [ALVAC-H,F and ALVAC-H,F,M,N], non replicating subunit vaccine [ISCOM-MV] and live attenuated [E-M, MMR, E-Z]), were used in these studies. The ALVAC-MV vaccines tested are just two of many recombinant avianpox virus vaccines that have been developed. These vaccines differ from many live recombinant virus vaccines in that although they are capable of good growth in avian host cells, their replication in nonavian cells is abortive, a potential safety advantage, particularly in immunocompromized individuals [18]. Despite this restricted replication, apparently there is sufficient antigen produced to induce immune responses, as attested to by the fact that ALVAC virus vaccines expressing rabies [19,20], cytomegalovirus (CMV) [21], canine distemper virus (CDV) [22], MV [13] and human

immunodeficiency (HIV) [23,24] virus antigens have all been shown to be immunogenic. In one study (most relevant to the present ones), dogs inoculated with ALVAC-H,F MV virus vaccine produced equivalent levels of virus-specific neutralizing antibodies as animals inoculated with a productive recombinant vaccinia virus vector expressing these same antigens [13]. These vaccines may also induce cell-mediated immune responses. Production of HIV-specific cytotoxic T lymphocytes (CTL) has been reported in seronegative human recipients of an ALVAC virus vector expressing multiple HIV genes [25]. In models where it is possible to do such studies, (i.e., feline leukemia-cat [26], rabies-ferret [27], CDV-ferret [22,28] and CDV-canine [14]), these vaccines have been shown to be protective against infection and disease. There was not evidence of any safety problems in any of these species. In the present studies, both of the recombinant ALVAC vaccines tested (i.e., ALVAC-H,F and ALVAC-H,F,M,N) were immunogenic and provided protection against pulmonary MV infection and viral dissemination (Table 2). Interestingly, the vaccine with four components appeared to be no more effective than the two component recombinant vaccine.

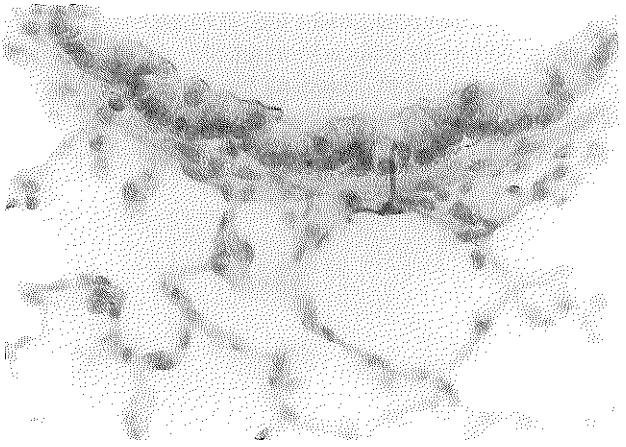

Figure 1. Hematoxylin and eosin stained section of lung from a cotton rat 4 days after intranasal inoculation with approximately 100 $TCID_{50}$ of measles virus strain MO2 and 32 days after receiving one dose of ISCOM-measles virus vaccine. Note that the tall columnar epithelial cells lining the bronchiole are intact and that there is virtually no cellular infiltration present in the section (Magnification 128x).

Like the ALVAC vaccines, numerous ISCOM virus vaccine candidates have been produced. All of them have in common a matrix consisting of a uniform stable complex of cholesterol, phospholipid and saponin containing entrapped antigen(s) [29]. The matrix has been shown to enhance antigen targeting, uptake and activity of antigen presenting cells [29], and thus has adjuvant activity. Interestingly, although nonreplicating, ISCOM vaccines, unlike most subunit vaccines, have been reported to induce class 1 virus-specific CTL [29-32]. ISCOM virus vaccines have been produced against Herpes simplex, pseudorabies, bovine herpes, cytomegalo, Epstein-Barr, hepatitis B, rubella, bovine diarrhoea, canine distemper, respiratory syncytial, rabies, influenza, human immunodeficiency and measles viruses (reviewed in Reference 33). All of these candidate vaccines have been shown to be immunogenic, and in models where prevention of infection and disease could be looked for, protective (i.e., pig-pseudorabies, bovine-bovine herpes type 1, bovine-bovine diarrhoea, canine-CDV, canine-

rabies, mouse- respiratory syncytial virus, mouse-influenza and mouse-MV). More recent studies have shown an ISCOM-HIV subunit vaccine to be immunogenic and protective in monkeys [34] and several ISCOM-influenza virus subunit vaccines to be effective in protecting mice from recently circulating influenza virus strains including H5N1 influenza virus [35-37]. Most pertinent to the present studies, MV-ISCOM subunit vaccines have been shown to induce virus-neutralizing, haemagglutination-inhibiting and/or fusion-inhibiting antibodies in mice and monkeys [8,15,30,38].

The ISCOM-MV vaccine used in the present studies contained only one MV component, MV F protein, a major constituent of the coat of MV, and a protein known to induce protective immune responses [38,39]. It was also the only vaccine used in these studies that did not replicate. Despite these limitations, the ISCOM-MV vaccine protected all of the challenged cotton rats from pulmonary virus infection and extra-pulmonary dissemination (Table 3). Interestingly, the two bpl-inactivated control preparations (i.e., (bpl-MV+ALUM and bpl-MV+Quil A) induced higher levels of virus-specific neutralizing antibodies than either of the ISCOM-MV preparation. However, both of the latter preparations contained not only the same amount of F protein as the ISCOM preparations (i.e., 0.25 μg F/0.1 ml of inoculum), but also additional virus antigens known to induce virus-specific neutralizing antibodies (i.e., MV haemagglutin) [39]. It is also noteworthy that both the MV in ISCOM (i.e., ISCOM-MV) and ISCOM-matrix mixed with MV (i.e., ISCOM+MV) were equivalently immunogenic and protective. These data indicate that virus antigen does not have to be incorporated in the ISCOM matrix to be effective.

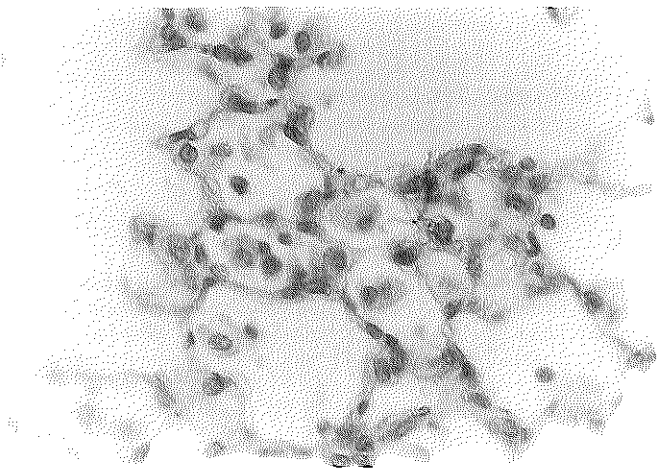

Figure 2. Hematoxylin and eosin stained section of lung from an unvaccinated cotton rat 4 days after intranasal inoculation with approximately 100 $TCID_{50}$ of measles virus strain MO2. Note foci of inflammatory cells, primarily mononuclear cells (Magnification 128x).

The E-Z vaccine strain used in these studies was derived by multiple passage in WI-38 tissue culture cells from the same Edmonston MV strain that has been used to make many other live attenuated MV vaccines [40]. In extensive trials, "high-titered" E-Z vaccine was shown to be consistently more immunogenic in infants with maternal antibodies than standard ("low-titered") MV vaccines [41-43], and thus was recommended by the World Health Organization for use in infants six months of age in developing countries where the risk of death by measles

was high [44]. However, testing of this vaccine was stopped when increased mortality was found to occur in recipients of the E-Z vaccine compared to infants who received standard doses of MV vaccines [45-47]. Although the excess mortality associated with the E-Z was subsequently found to be associated with its use as a high-titered vaccine, and not inherent properties [48], recommendation of use of this vaccine has not been reinstated. We have previously shown that this virus, unlike the E-M virus, can grow well in unstimulated human peripheral blood leukocytes and modulate leukocyte function antigen-1 (LFA-1), possibly contributing to both its untoward effects and increased immunogenicity [16]. E-Z virus was used in the present studies primarily to determine if cotton rats could be used to evaluate the immunogenicity and protective efficacy in animals with passive immunity. As the data in Table 4 indicates, under the conditions used, only this virus and the MO2 strain were immunogenic and protective in naive cotton rats and cotton rats with passively acquired immunity.

The MO2 strain of MV used in these studies is a relatively low passage level of a clinical MV isolate. It was used in these studies as a positive control in the different vaccine studies and as a challenge virus. Although it cannot cause overt disease in cotton rats, it has been shown to grow in the lungs of these animals following intranasal inoculation and to frequently disseminate from this organ [11]. It can also cause immunosuppression in these animals (unpublished data). Thus it appears to have maintained properties of wild-type MV. As the data in Tables 1 and 3 show, this virus was able to induce protective immunity against a second infection with this virus.

In these studies, only the appearance of virus-specific neutralizing antibodies was used as a measure of vaccine immunogenicity. The primary reason for choosing this immune response was because it is known to strongly correlate with protection from MV-induced infection and disease [41]. On the basis of the neutralizing antibody responses induced, all of the live virus vaccines were immunogenic in nonimmune animals and appeared to have nearly equivalent activity (excluding results obtained in passively immunized animals, the serum neutralizing titers measured in these tests ranged from only 5.4 ± 0.8 $\log_2$/0.05 ml [ALVAC-H,F in experiments 1 and 2, Table 2] to 8.2 ± 0.9 [bpl-MV+Quil A, Table 3]). In fact, none of these antibody responses was statistically different from each other when compared in the Kruskall-Wallis nonparametric analysis of variance). There also was no difference between any of these responses and those seen cotton rats inoculated i.n. once with the clinical MO2 MV isolate (e.g., 6.6 ± 0.8 and 6.6 ± 1.3 in experiments 1 and 2, Table 1, and 6.8 ± 1.2 in Table 4). Virus replication appeared to be required for at least some of the live virus vaccines, since cotton rats inoculated with UV-inactivated E-M, MMR or MO2 viruses did not mount significant antibody responses (i.e., virus-specific serum neutralizing titers remained < 2 $\log_2$/0.05 after two inoculations [data not shown]). Virus-specific CTL responses were not looked for in these studies because we currently lack validated CTL assays. However, the BCM cotton rat colony contains inbred cotton rats and we can obtain primary cells from these animals that can support MV replication [11]. Work is in progress to develop a MV-specific CTL assays using cotton rat target and effector cells. If this can be accomplished, we hope to revisit these vaccines and compare their potential to induce virus-specific CTL. The ability to measure virus-specific cell mediated immune responses as well as virus-specific antibody responses would significantly increase the value of the MV-cotton rat model for evaluating MV vaccines.

It is clear that cotton rats cannot replace nonhuman primates as the best experimental model for studying the pathogenesis of human measles. The latter animals remain the only species that manifest disease similar to humans following exposure to measles virus [50-52] or experimental inoculation [4-6]. Indeed, nonhuman primates remain the premier models for

final testing of candidate MV vaccines. Despite this, the data presented in this work suggest that cotton rats may fill a useful niche and provide a more practical animal model for preliminary screening and testing of MV vaccine candidates using virus-specific antibody responses, pulmonary virus titers, virus dissemination and pulmonary pathology as parameters of vaccine immunologic and protective efficacy. Supporting this view is the fact that there was a consistent inverse relationship between levels of virus-specific neutralizing antibody and pulmonary virus titers, extrapulmonary dissemination (to MDLN) and pulmonary pathology. In addition, all of the test vaccines had equivalent activity in naïve animals, but only the E-Z vaccine and MO2 virus were efficacious in animals passively immunized with ISG. (The ISCOM-MV vaccine was not tested for this property because of a limited supply of this vaccine; however, this vaccine has been reported to be immunogenic in monkeys in the presence of passively transferred MV-specific antibodies [49]). All of these findings are consistent with those seen clinically or in field trials. (It should be noted that ferrets, which are permissive for CDV, a virus closely related to MV, have been used to test Morbillivirus vaccine strategies [22,28]). It remains to be seen if cotton rats can be used to study atypical measles, a severe form of measles that is associated with the inactivated measles virus vaccine tested in the United States in the 1960s [53,54], or to evaluate new vaccines for their potential to induce this form of measles. We are currently preparing inactivated MV vaccine preparations to start these determinations.

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Chapter 3.3

Longevity of neutralizing antibody levels in macaques vaccinated with Quil A-adjuvated measles vaccine candidates

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ABSTRACT

Iscom-based candidate measles vaccines may induce protection in macaques in the presence of virus neutralizing (VN) antibody levels that precludes vaccination of infants with live attenuated vaccines. Here we studied the longevity of protective VN antibody levels induced in macaques with one dose of measles virus (MV) iscom. Inactivated MV adjuvated with iscom-matrix or with purified Quillaja saponin QA-22 were also tested in like manner. Within one month all animals developed high levels of VN antibody and MV-specific IFNγ-producing cells. Especially the high VN antibody levels induced by the latter two preparations showed virtually no decrease during the two year follow-up.

The possibility to induce long-term protection against measles with a "one shot – early in life" vaccination approach, would be a major step forward towards measles control and the envisaged eradication of measles virus (MV) [1]. Recently, we showed that both iscom- and MVA-based candidate measles vaccines may induce protection against MV infection in macaques, when administered in the presence of pre-existing virus neutralizing (VN) serum antibody levels that preclude successful vaccination of infants with live attenuated vaccines (LAV)[2-4]. It has been shown that VN antibody levels $\geq$0.2 international units (IU) per ml are protective in infants and macaques [2,3].

The development of Quil A-based vaccines has been hampered by problems related to toxicity of crude Quil A preparations. The safety of iscom- and Quil A component-based vaccines has largely been improved, since with highly purified Quil A components virtually non-reactogenic vaccines can be made [5-7]. Further identification of components and structures essential for the induction of an optimal immune response with MV-iscom, have focussed on the use of purified Quil A components [8]. In addition, the question was addressed whether formation of the characteristic iscom structure and the incorporation of antigen into this structure are required to induce cytotoxic T lymphocytes (CTL) *in vitro* and protective immune responses *in vivo* [8]. On the basis of the data generated in these studies three different preparations were selected for further evaluation: a MV-iscom preparation, inactivated MV adjuvated with iscom-matrix and inactivated MV adjuvated with QA-22 [8]. These three candidate vaccines induced protection against challenge with wildtype MV in cotton rats [9]. These results prompted us to evaluate the longevity of protective levels of MV neutralizing antibody in macaques induced by one dose of these Quil A-based vaccine candidates. To this end, a vaccination study was carried out in 15 captive-bred subadult healthy cynomolgus monkeys (*Macaca fascicularis*). Preparation and characterisation of the three vaccine candidates were described previously [8]. Briefly, MV-iscoms were prepared using the "dialysis method" with HPLC-purified Quil A components QA-3 and QA-22 (ratio 1:4). The iscom-matrix were prepared identically to the MV-iscoms with the omission of solubilized MV. The iscom-matrix and "free" QA-22 were mixed with BPL-inactivated MV so that the F-protein-to-Quil A ratio was 0.5. The macaques were vaccinated intramuscularly with one dose of the vaccines (5 animals per group), each containing 10 µg F-protein. Heparinized blood samples were collected at weeks 0, 2, 4, 8, 12, 37, 77 and 112, and both plasma and PBMC samples were cryopreserved.

The titres of MV-specific neutralizing antibody in plasma were measured and expressed in IU per ml as previously described [4]. High VN antibody levels (2-16 IU per ml) were induced within the first weeks after vaccination with all three vaccine candidates (Fig. 1). The groups vaccinated with inactivated MV mixed with iscom-matrix and adjuvated with QA-22 both showed a tendency of higher antibody levels that also persisted at higher levels than those of the MV-iscom group: the former two groups showed virtually no decrease in antibody levels during the two year follow-up period.

MV-specific cellular immune response was measured in a selection of the vaccinated macaques four weeks after vaccination with an interferon gamma (IFNγ) enzyme-linked immunospot (ELISPOT) assay for macaques (U-cytech, Utrecht, The Netherlands). PBMC were plated in 96-well V-bottomed plates (Greiner Labor Technik, Nürtingen, Germany) at a concentration of 1.5 x 10^5 cells/well. To these wells autologous herpes papio virus-transformed B cells, either or not infected with MV Edmonston 48 hours before, were added at a concentration of 3 x 10^4 cells/well. Plates were centrifuged for 10 seconds, incubated at 37°C for one hour and the co-cultured cells were then transferred to the ELISPOT plates (Silent Screen Plate 96 well w/Nylon,

Nalge Nunc International, USA). After six hours cells were removed and the plates processed according to instructions provided the manufacturer. In all the macaques tested, MV-specific IFNγ-producing cells were demonstrated in PBMC (57-500 per 150,000 cells) collected four weeks after the vaccination, with no significant differences between the groups (Fig. 2).

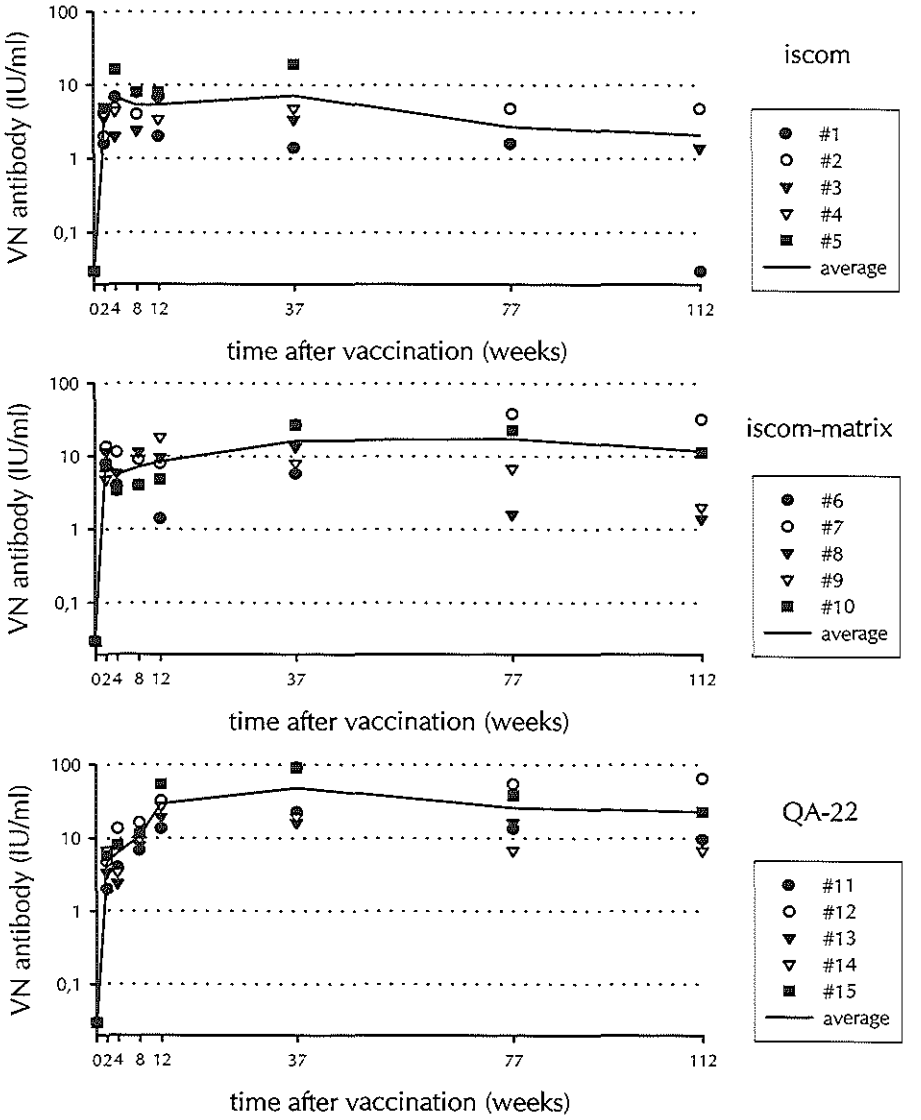

Figure 1. Development of MV-specific virus neutralizing antibodies in plasma collected at different time-points after vaccination.

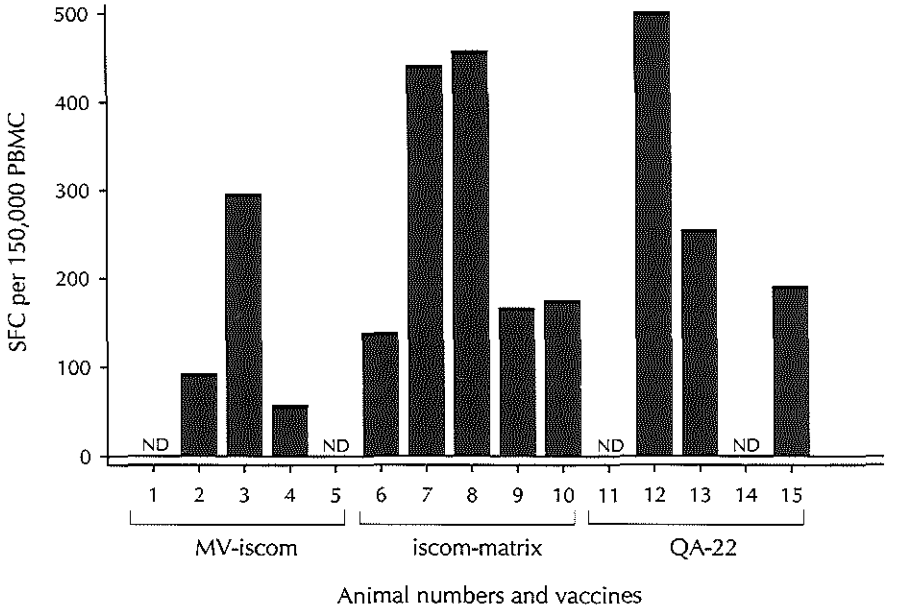

Figure 2. MV-specifc vaccine-induced spot forming cells (SFC) in PBMC at four weeks after vaccination. The results were corrected for SFC observed in wells with un-infected herpes papio virus-transformed B cells. ND, not done.

In conclusion, all three measles vaccine candidates tested induced longlasting protective levels of VN antibody in a "one-shot" regimen. Especially the two vaccines based on inactivated whole MV, adjuvated either with iscom-matrix or with QA-22, induced antibody levels that showed virtually no decline over the two year follow-up period. This would favor these two preparations for further efficacy and safety evaluation.

It should still be confirmed that the iscom-matrix- and the QA-22-adjuvated measles vaccine candidates, like the MV-iscom preparation [3], also induce protective immunity upon administration in the presence of VN antibody. A specific problem with the use of measles vaccines based on inactivated MV, as proposed here, is the legacy of the problem that arose in the late sixties: the use of tween/ether- and formalin-inactivated measles adjuvated with alum predisposed vaccines infants for developing atypical measles upon later infection with wild-type MV [10]. Therefore, before clinical trials can be considered, the safety of these Quil A-based candidate measles vaccines with respect to immunopathology should be addressed in the recently described macaque model [11].

Taken together, the data generated in this study justify further evaluation of the safety and efficacy of iscom-matrix- and QA-22 adjuvated measles vaccine candidates.

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Chapter 4.1

Protective immunity in macaques vaccinated with a MVA based measles vaccine in the presence of passively acquired antibodies

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ABSTRACT

Recombinant modified vaccinia virus Ankara (MVA), encoding the measles virus (MV) fusion (F) and haemagglutinin (H) (MVA-FH) glycoproteins, was evaluated in a MV vaccination-challenge model with macaques. Animals were vaccinated twice in the absence or presence of passively transferred MV-neutralizing macaque antibodies and challenged 1 year later intratracheally with wildtype MV. After the second vaccination with MVA-FH, all the animals developed MV-neutralizing antibodies and MV-specific T cell responses. Although MVA-FH was slightly less effective in inducing MV-neutralizing antibodies in the absence of passively transferred antibodies than the currently used live attenuated vaccine, it proved to be more effective in the presence of such antibodies. All vaccinated animals were effectively protected from the challenge infection. These data suggest that MVA-FH should be further tested as an alternative to the current vaccine for infants with maternally acquired MV-neutralizing antibodies and for adults with waning vaccine-induced immunity.

INTRODUCTION

Measles is a highly contagious infectious disease that continues to be a major cause of morbidity and mortality in infants, with an estimated number of one million deaths annually [8]. Inactivated whole virus vaccine preparations used in the 1960's did not induce long-lasting protection, and were shown to predispose for severe immunopathological complications, collectively referred to as the atypical measles syndrome [7,14]. In the 1970's live-attenuated MV vaccines were introduced, which proved to be safe and effective. Application of these vaccines, which are still being used, resulted in a significant reduction of the global numbers of measles cases, and largely abrogated the circulation of wildtype MV in the industrialized world. However, measles vaccination proved less effective in a number of developing countries, where measles continues to be endemic. Several factors are responsible for this reduced effectiveness, most of which are related to logistic problems like vaccination coverage and cold chain maintenance [3]. However, an important additional factor is that measles frequently occurs at an early age (< 9 months) in developing countries. At this age pre-existing MV-specific maternal antibody may interfere with the replication of live attenuated vaccine virus, resulting in suboptimal protection upon vaccination [16]. The WHO has proposed a global measles eradication strategy based on the current live attenuated MV vaccine [28]. However, it is uncertain if this vaccine will be able to achieve a sufficient level of herd immunity to completely abrogate circulation of MV. Several outbreaks of clinical and subclinical measles have been described amongst vaccinated populations [10], and in the final stages of an eradication campaign vaccines may be needed which are able to boost low levels of immunity.

In recent years a number of new generation candidate MV vaccines have been developed, including immune stimulating complexes (iscoms), DNA vaccines and recombinant poxviruses [17]. An iscom-based vaccine proved to be effective in inducing protective immunity in macaques even in the presence of passively acquired MV neutralizing antibodies [26]. In contrast, recombinant vaccinia viruses encoding the MV fusion (F) and hemagglutinin (H) proteins, although able to induce strong MV-specific virus neutralizing (VN) antibody and T cell responses, were only partially effective when used in the presence of passively acquired MV neutralizing antibodies [26]. In addition, concerns about the safety of vaccinia virus made this vaccine candidate less attractive. Recently, recombinant poxviruses were developed based on the replication-deficient Modified Vaccinia virus Ankara (MVA) [15]. This strain was proven safe for use in humans during its application in the late stage of the smallpox eradication campaign [23]. Compared to fully replication competent strains of vaccinia virus, MVA induced similar expression levels of the recombinant genes [24], and induced equal or better B- and T-cell responses in animals [11,21,25].

Here we describe the evaluation of a recombinant MVA based candidate vaccine encoding the MV F- and H-genes (MVA-FH), in a MV vaccination/challenge model in macaques. MVA-FH successfully induced MV-specific antibody and T cell responses, including $CD8^+$ T cells, both in the absence and presence of passively transferred MV-specific antibodies. All vaccinated macaques were still effectively protected from intratracheal challenge with wildtype MV, one year after vaccination. The use of non-replicating candidate measles vaccine is not likely to predispose for atypical measles-like immunopathology. Collectively these properties would favour MVA-FH as a candidate measles vaccine, that either alone or as part of a prime-boost strategy, could be used in a measles eradication programme.

MATERIALS AND METHODS

Macaques

The studies were carried out in eight captive-bred subadult healthy female cynomolgus macaques (*Macaca fascicularis*) which were all confirmed MV-seronegative. The animals were housed together except during the vaccination and challenge periods, when they were kept as pairs in separate cages.

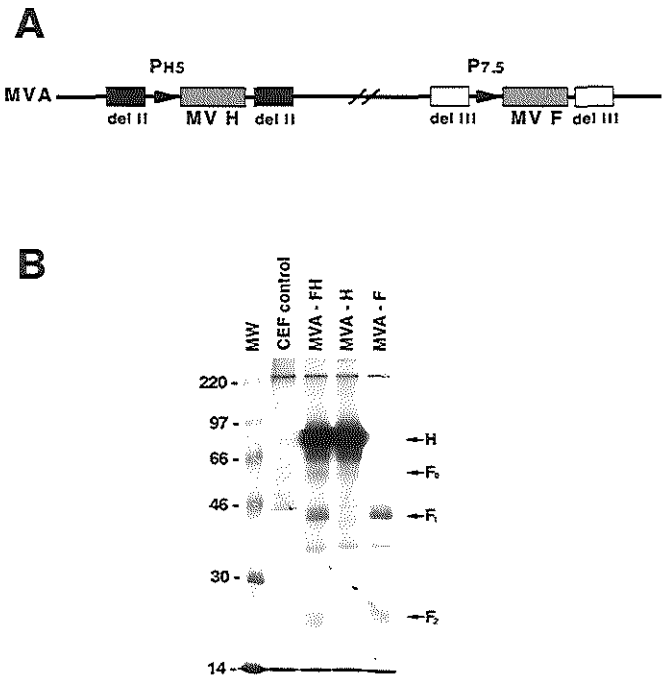

Figure 1. Expression of MV F- and H glycoproteins by MVA-FH. A. Diagram of the genome of MVA-FH. Abbreviations: del II, deletion II; del III, deletion III; PH5, modified H5 promoter; P7.5, 7.5 promoter. B. SDS-polyacrylamide gel electrophoresis of [^{35}S]methione-labeled proteins from CEF infected with MVA-FH and immunoprecipitated with measles polyclonal antibody. MW, molecular weigh ts of marker proteins in thousands. The positions of MV H, F0, F1 and F2 proteins are indicated by arrows.

Viruses

A recombinant MVA that expresses the Edmonston strain of measles virus F and H glycoproteins was made used previously described procedures [5]. The F and H genes, contained within plasmids pTM-F/MV and pTM-H/MV respectively [22], were excised by digestion with NcoI and StuI and the overhanging ends were filled in with Klenow enzyme. The H gene was inserted into the SmaI site of pLW-17 [29], a plasmid transfer vector which contains the modified H5 promoter [20] to express the recombinant foreign gene and inserts within deletion II of MVA. The F gene was inserted into the SmaI site of MVA vector, pLW-24, which contained the 7.5 promoter of vaccinia virus [13] and MVA flanks for insertion into del III of MVA [24]. Initial attempts to make recombinant MVA stably expressing the F protein

under stronger vaccinia virus promoters, modified H5 or the strong synthetic promoter, were unsuccessful.

Recombinant MVA viruses expressing the measles virus F or H proteins were made by transfecting pLW-17 or pLW-24 containing the measles virus H or F gene, respectively into chick embryo fibroblasts (CEF) that were infected with MVA as previously described [22]. Recombinant virus was obtained by immunostaining recombinant MVA foci utilizing polyclonal anti-measles virus rabbit serum (Accurate Scientific, Westbury, NY). A double recombinant MVA expressing both the F and H measles glycoproteins was made by transfecting the measles H containing plasmid into the single MVA recombinant virus expressing the F gene. Double recombinant MVA foci were obtained by staining with polyclonal anti-measles antiserum that had been absorbed with CEF cells infected with the single MVA-F recombinant. This double MVA recombinant was designated MVA-FH. Stocks of MVA and recombinant MVA-FH were prepared in secondary CEF as previously described [22]. The recombinant MVA was aliquoted and stored at –80°C. The samples were thawed and sonicated in ice for 30 seconds with a cup sonicator (Sonicor Instruments Corporation, Copiaque, NY) shortly before inoculation.

Immunization and samplings

Macaques were inoculated intramuscularly and intranasally (10^8 pfu at each site) at weeks 0 and 4. Six macaques were vaccinated with MVA-FH, of which three were naive (animal numbers H1, H5, H7) and three had been passively immunized with anti-MV serum (BMS94) 48 hours before vaccination (animal numbers UH3, UH4, K128) [26]. This serum, which has a specific virus-neutralizing (VN) antibody level of 40 IU/ml [6], was administered intravenously at a dosage of 0.7 ml per kg of body weight. This resulted in VN antibody levels at the time of the first vaccination of about 0.3 IU/ml, as was also observed in a previous study [26]. The two other macaques (animal numbers H4 and H6) were vaccinated with MVA-wt at identical dosages and vaccination routes. During the 1-year period between vaccination and challenge heparinized blood samples were collected at regular intervals, with initial intervals of a week but later less frequently. To be able to compare the data with those from previous vaccination experiments, plasma samples obtained from macaques vaccinated with MV-Schwarz or rVV-FH were reassayed [26].

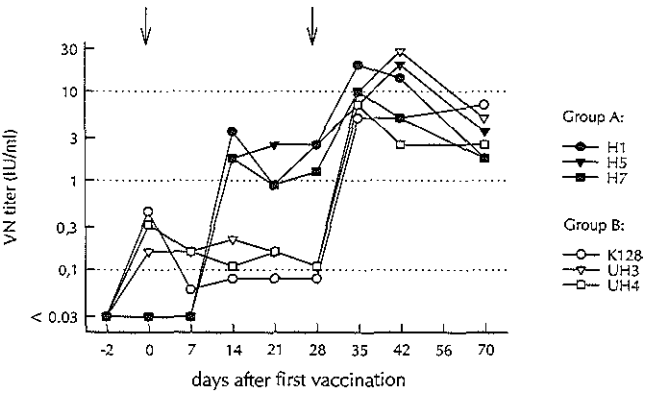

Figure 2. Development of virus neutralizing (VN) antibody responses in plasma of macaques vaccinated at weeks 0 and 4 (indicated with arrows) with MVA-FH in the absence (Group A) or in the presence (Group B) of passively transferred MV-specific VN antibodies.

Challenge and samplings

One year after vaccination, the macaques were challenged with wildtype MV as previously described [26,27]. Briefly, 1000 50% tissue culture infective doses ($TCID_{50}$) of the wildtype MV strain BIL was diluted in 5 ml of phosphate-buffered saline (PBS) and administered via the intratracheal route at day 0. Pharyngeal epithelial cells (PEC), lung lavage cells (LLC) and peripheral blood mononuclear cells (PBMC), as well as plasma, were collected at days 3, 6, 9, 13 and 17, and additional plasma and PBMC samples were collected at days 0, 24 and 41. PEC were collected using a Cytobrush® Plus™ (Medscand Medical AB, Sweden) that was applied into the throat using a laryngoscope. After sampling the brush was transferred into a tube that contained 2 ml RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), 2-mercaptoethanol (10^{-5} M), 10% FBS (referred to as culture medium: CM). The tube was vortexed and following removal of the brush, cells were pelleted by centrifugation (5 minutes at 400xg). The cell pellet was then resuspended in 1 ml CM. Lungs were lavaged with 10 ml PBS by a small catheter that was applied into the lungs using a laryngoscope. About 5 ml lavage fluid containing the LLC was recovered. This LLC suspension was transferred to a tube, pelleted by centrifugation (5 minutes at 400xg) and resuspended in CM. PBMC were isolated from heparinized blood by density gradient centrifugation (20 minutes at 600xg), using a gradient consisting of Optiprep (density: 1.32 g/ml, Nycomed), 6% Dextran (Sigma) in H_2O, and 10 times concentrated PBS (16.7% / 75% / 8.3%, v/v/v).

MV-reisolation

Following MV challenge, virus replication was monitored by cocultivating the LLC, PBMC and PEC (collected at days 3, 6, 9, 13 and 17) with a human EBV-transformed B-lymphoblastic cell line (hu.B-LCL). Four replicates of 100 µl PEC suspension were cocultivated with 2×10^5 hu.B-LCL in 1 ml CM in a 24-wells plate (Greiner Labor Technik, Nürtingen, Germany). MV cytopathic effect (cpe) in one or more of the PEC/ hu.B-LCL cocultivations was interpreted as positive reisolation. Virus isolations from LLC and PBMC were carried out by cocultivation of a 2-log dilution range of the macaque cells with a standard amount of the hu.B-LCL. Briefly, 3.2×10^5 LLC or PBMC were divided over eight wells of a 96-well round-bottomed plate (Greiner) in CM (200 µl/well). In case of the PBMC, 1 µg/ml PHA-L (Boehringer Mannheim, Almere, The Netherlands) was added to this medium and the cells were incubated at 37°C for 2 hours. Subsequently, a 2-log dilution range of the LLC or PBMC was prepared in the plate (ranging from 2^{-1} to 2^{-11}), and hu.B-LCL were added at an amount of 1×10^4 cells/well. After screening for MV cpe during the following week, the numbers of MV-infected cells were calculated using the formula of Reed and Muench [19].

Vaccinia virus specific antibody responses

Rabbit kidney (RK-13) cells were infected with wildtype vaccinia virus at a multiplicity of infection of 10. Seven hours after infection, the cells were trypsinized and used as target cells in a FACS-measured immunofluorescence assay. The cells (1×10^5 to 3×10^5 cells/100μl) were incubated with plasma samples diluted 1:100 in PBS supplemented with fetal bovine serum (FBS). After 1-h incubation on ice, cells were washed and subsequently incubated with fluorescein isothiocyanate (FITC)-labeled rabbit anti-human immunoglobulin G (IgG) [$F(ab')_2$ fragments, (DAKO, Denmark]. After another hour on ice, cells were washed twice with PBS and fixed with 1% paraformaldehyde for 20 minutes on ice. Fluorescence signals were measured using a FACScan (Becton Dickinson). Fluorescence intensity was quantified by determining the geometric mean of the fluorescence histograms.

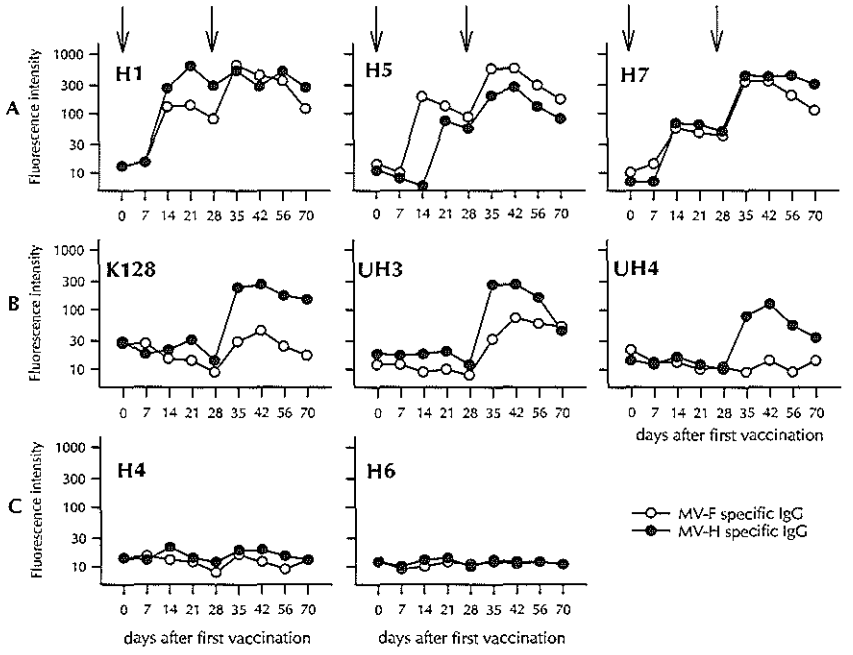

Figure 3. Development of MV glycoprotein specific plasma IgG (MV-F: open circles; MV-H: black circles) responses in macaques vaccinated at weeks 0 and 4 (indicated with arrows) with MVA-FH in the absence (group A) or in the presence (group B) of passively transferred MV-specific VN antibodies. The control macaques (group C) were vaccinated with MVA-wt.

MV-F and MV-H specific immunofluorescence assay

Antibodies directed against the F and H glycoproteins were detected by a FACS-measured immunofluorescence assay using transfected human melanoma cell lines as targets, as previously described [4]. Briefly, Mel-JuSo cells (wild type, MV-F and MV-H) were incubated with plasma samples diluted 1:100 in PBS supplemented with 2% FBS (1×10^5 to 3×10^5 cells/100 μl). After 1-h incubation on ice, cells were washed and subsequently stained with FITC-labeled rabbit anti-human IgG or IgM [$F(ab')_2$ fragments; DAKO]. Fluorescence signals were quantified by a FACScan.

MV-N specific IgM capture ELISA

IgM antibodies directed against the MV nucleoprotein (N) were measured in a capture ELISA using purified recombinant baculovirus-expressed N protein directly conjugated with peroxidase (N-HRP). ELISA plates (Greiner Labor Technik, Nürtingen, Germany) coated with rabbit anti-human IgM antibodies (Meddens Diagnostics, The Netherlands) were washed with demineralized H_2O containing 0.05% Tween 80, followed by incubation with plasma samples diluted 1:100 in ELISA buffer (Meddens Diagnostics). After 1 hour at 37°C, the plates were washed and incubated with N-HRP. Following another hour at 37°C, the plates were washed again and incubated with substrate solution (tetramethylbenzidin, Meddens Diagnostics). Results were expressed as the absorbance at 450 nm.

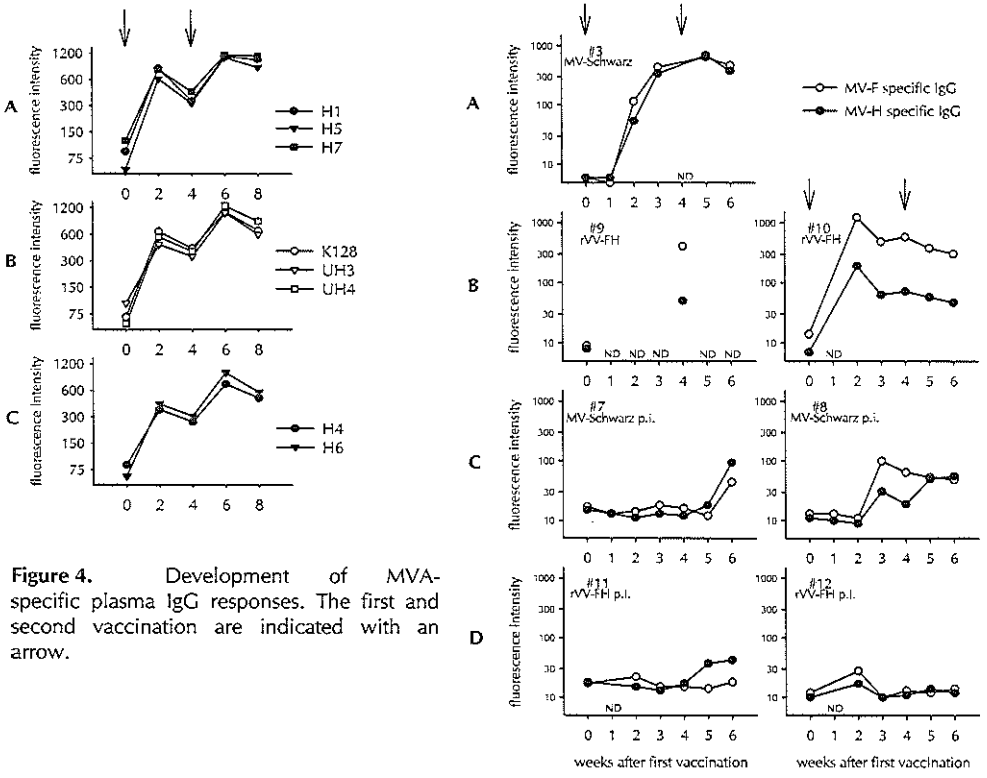

Figure 4. Development of MVA-specific plasma IgG responses. The first and second vaccination are indicated with an arrow.

Figure 5. Development of MV glycoprotein specific plasma IgG responses in macaques vaccinated at weeks 0 and 4 with MV-Schwarz or rVV-FH in the absence (group A and B, respectively) and presence (group C and D, respectively) of MV-specific VN antibodies. p.i., passively immunized; ND, not done (due to unavailability of historical plasma).

Virus neutralization (VN) assay

Serial 2-log dilutions (starting at 2^{-2}) of heat-inactivated (30 min at 56°C) plasma samples were incubated in duplicate with 60 $TCID_{50}$ of MV Edmonston for 1 hour at 37°C in 96-well flat-bottomed plates (Greiner) in DMEM supplemented with 2% FBS. Subsequently, trypsinized Vero cells were added at a concentration of 1 x 10^4 cells/well. Plates were incubated for 5 days at 37°C and visually monitored for MV cpe. VN antibody titers were calculated as the means of the highest plasma dilutions still giving 100% reduction of cytopathic changes. The titers were transformed to I.U./ml using the NIBSC reference serum (human anti-measles serum, 2nd International Standard 1990, National Institute for Biological Standards and Control; 5 I.U./ml) which in our assay had a VN antibody titer of $2^{8.5}$ [6]. The VN antibody level in a plasma sample expressed as IU/ml was calculated using the following formula:

VN level = $[\, 2^x / 2^{2.85} \,]$ **X** 5 I.U./ml, in which 2^x is the VN titer measured in that plasma sample.

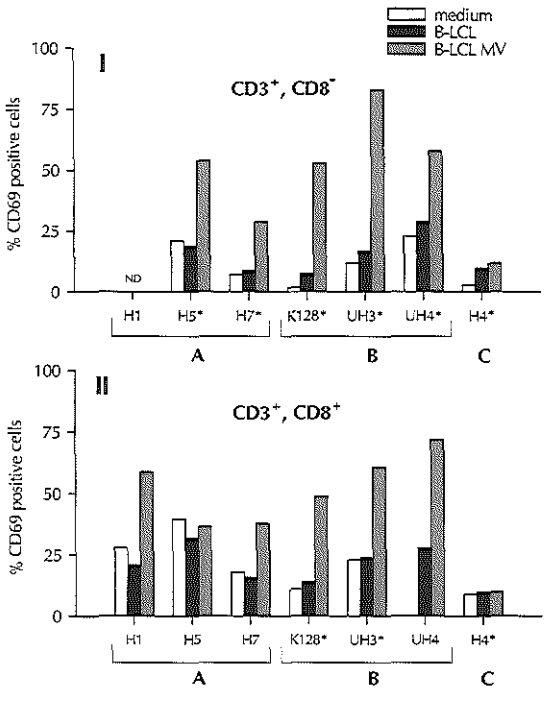

Figure 6. MV-specific T cell responses in PBMC bulk cultures of macaques 8 weeks after vaccination with MVA-FH in the absence (A) or presence (B) of passively transferred MV-specific VN antibodies, or with MVA-wt (C). PBMC were stimulated once *in vitro* with autologous MV-infected mac.B-LCL, and expanded in the presence of rhIL2 alone or in the presence of both rhIL-2 and rhIL-4 (indicated with an asterisk). After 12 to 14 days, cells were harvested and treated with chymotrypsin to strip pre-existing CD69 molecules from the membrane surface. Subsequently, cells were re-stimulated for 6 hours with UV-inactivated autologous MV-infected mac.B-LCL, uninfected mac.B-LCL or without mac.B-LCL, and the membrane expression of CD3, CD8 and CD69 was determined. The percentages CD69-positive cells in the $CD3^+$ lymphocytes, as gated on basis of a FSC/SSC plot, are shown for $CD8^+$ (I) and $CD8^-$ (II) cells. ND, not done (because no cells could be expanded).

MV-specific T cell responses

PBMC were isolated as described above. Cells were cultured in96-well round-bottomed plates (Greiner) in CM supplemented with 1% pooled macaque serum containing MV-specific antibodies, at a concentration of 1 x 10^5 cells/well. These cells were co-cultivated with UV-irradiated autologous herpes papio-transformed B cells (mac.B-LCL), which had been infected with MV Edmonston 48 hours before at an effector-to-target ratio of 0.1. After 3 days recombinant human interleukin-2 (rhIL-2) or a mixture of rhIL-2 and rhIL-4 was added, and cultures were maintained for 12 to 14 days. Cells were harvested and treated with chymotrypsin (type II, Sigma) in order to remove CD69 from the membrane [12]. Briefly,cells were washed with PBS, incubated with 0.1% (w/v) chymotrypsin in PBS for 10 minutes at 37°C, and subsequently washed with CM. Subsequently, cells were cultivated for 6 hours with UV-irradiated autologous MV-infected mac.B-LCL, uninfected mac.B-LCL or without mac.B-LCL. After this restimulation cells were stained with anti-CD3-FITC (BPRC, Rijswijk, The Netherlands), anti-CD69-PE (Becton Dickinson) and anti-CD8-RPE/Cy5 (DAKO), and fluorescence was measured using a FACScan. CD69 expression on $CD8^-$and $CD8^+$ cells was determined in the $CD3^+$ fraction of the lymphocytes as gated in a forward light scatter versus side light scatter diagram. During previous experiments we had observed that varying percentages (ranging from 5 to 25%) of cells continued to express low levels of CD69 after 2 to 3 weeks of bulk culture. Therefore, we removed any residual CD69 by using chymotrypsin, ensuring that CD69 levels measured in the assay were indeed induced during the 6-h restimulation. Background levels of CD69-expressing cells in the medium control of our assay represent cells that either produce CD69 endogenously or express CD69 from recycled membranes.

The animal study was approved by the Local Animal Ethics Committee of the National Institute of Public Health and the Environment, Bilthoven, The Netherlands and carried out according to animal experimentation guidelines.

RESULTS

Expression of F and H glycoproteins by recombinant MVA

Recombinant MVA that expressed either the MV F or H proteins or both F and H were constructed. The genome of the double recombinant virus is represented in figure 1A. Expression of the MV proteins was demonstrated by labeling MVA infected CEF with [^{35}S]methionine, incubating the lysates with measles polyclonal or monoclonal (not shown) antibodies and analyzing the bound proteins by SDS polyacrylamide gel electrophoresis (figure 1B). The H glycoprotein, from cells infected with MVA-H or MVA-FH, migrated as a single major band of the expected size. The F glycoprotein was resolved as F1 and F2 subunits. When monkey BSC-1 cells were infected with the double recombinant virus, but not with either of the single recombinants, syncytia formed indicating that F and H proteins were expressed on the cell surface and were functional (data not shown). The double recombinant MVA-FH was used for all vaccination studies.

MVA-FH vaccination induced antibody responses

Vaccination of MV-seronegative macaques with MVA-FH induced VN antibody, F-specific and H-specific levels (titers ranging from 2.0-2.3, 50-87 and 56-294 respectively), which were boosted in all three animals by a second vaccination four weeks later (titers ranging from 2.9-3.5, 340-649 and 198-523, respectively; figures 2 and 3A). All animals remained MV seropositive until challenge a year later. Initial vaccination of macaques passively immunized with MV-specific VN antibodies induced lower MV-specific antibody responses when compared to the vaccinated naive animals (VN titers of about 0.1 IU/ml), but after the booster vaccination similar levels of VN antibodies were induced in these animals (titers ranging from 5.0-10) (figure 2B). Note that at the moment of the second vaccination all three macaques demonstrate VN titers of 0.1 IU/ml. VN antibody titers measured one year after vaccination were in the same order of magnitude in all six MVA-FH vaccinated macaques (titers ranging from 2.1-2.7 respectively). The H antibody titers of the passively immunized monkeys were also boosted to levels comparable to those of the naïve animals whereas the F-specific antibodies remained lower. No MV-specific antibody responses were detected in the control animals vaccinated with MVA-wt (figure 3C). The kinetics of vaccinia virus-specific antibody responses were similar in all eight macaques upon primary and secondary vaccinations (figure 4), indicating that the passively acquired MV-specific antibodies did not prevent infection of cells by MVA.For comparison of the immunogenicity of MVA-FH with those of MV-Schwarz and rVV-FH, we re-assayed plasma samples from a previous experiment [26] in which the same vaccination regimen had been used. The levels of MV-neutralizing, as well as F- and H-specific, antibodies induced by MVA-FH in seronegative macaques were similar to those induced by rVV-FH, but slightly lower than those induced by MV Schwarz (figure 5A and 5B) [26]. However, when comparing the animals vaccinated in the presence of passively transferred MV-specific antibodies, the responses measured in the MVA-FH vaccinated animals were substantially higher than those of macaques vaccinated with either MV Schwarz or rVV-FH (figure 5C and D) [26].

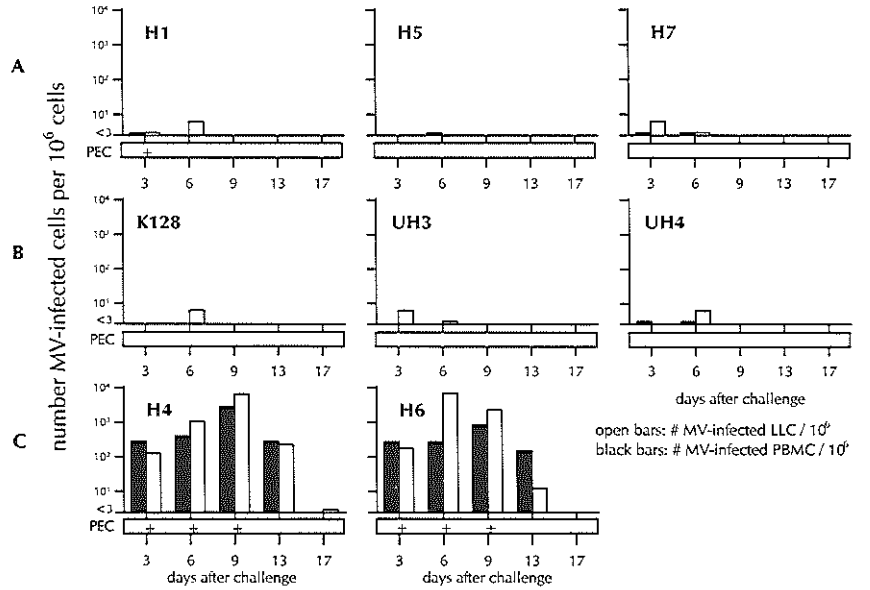

Figure 7. Number of MV-infected cells/10^6 LLC (open bars) or PBMC (black bars) at different times after intratracheal challenge with 10^3 $CCID_{50}$ MV-BIL. Macaques had been vaccinated with MVA-FH in the absence (A) or in the presence (B) of passively transferred MV-specific VN antibodies, or with MVA-wt (C). +, time point at which MV could be reisolated from PEC.

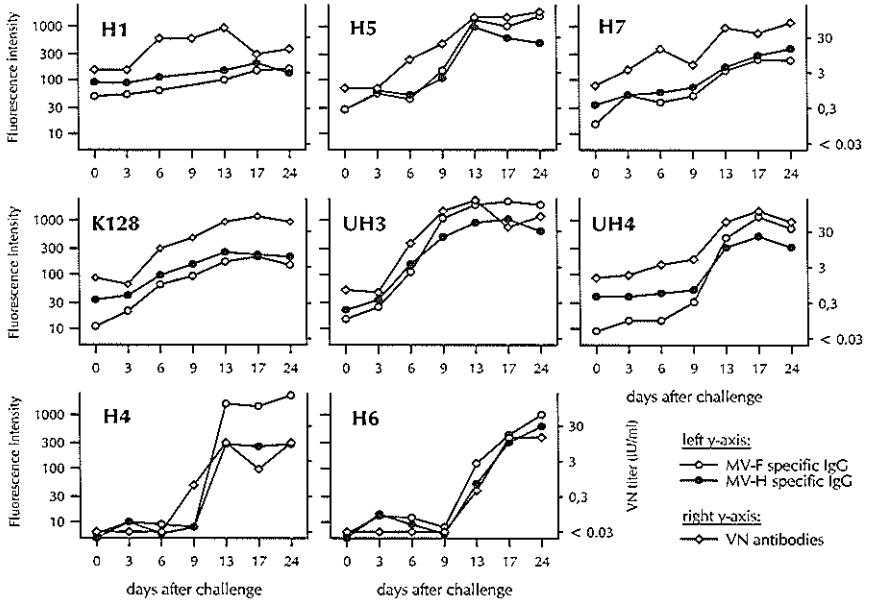

Figure 8. Development of MV glycoprotein-specific plasma IgG and VN antibody responses in macaques at different times after intratracheal challenge with 10^3 $TCID_{50}$ of MV-BIL. Macaques had been vaccinated with MVA-FH in the absence (A) or in the presence (B) of passively transferred MV-specific VN antibodies or with MVA-wt (C).

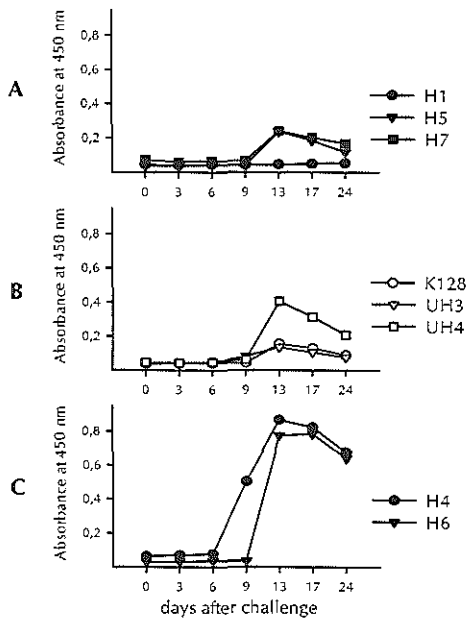

Figure 9. Development of MV-N specific plasma IgM responses in macaques at different times after intratracheal challenge with 10^3 $CCID_{50}$ of MV-BIL. Macaques had been vaccinated with MVA-FH in the absence (A) or in the presence (B) of passively transferred MV-specific VN antibodies or with MVA-wt (C).

MVA-FH vaccination-induced MV-specific T cell responses

Eight weeks after the second vaccination, the phenotype of the vaccinationinduced MV-specific T cells was determined. Following MV-specific bulk stimulation of PBMC with MV-infected autologous mac.B-LCL, the presence of MV-specific $CD8^-$ and/or $CD8^+$ T cells could be demonstrated in the MVA-FH vaccinated macaques. In animal H1 no $CD3^+CD8^-$ cells had been expanded, while in animal H5 no specific $CD3^+CD8^+$ cells could be demonstrated (figure 6).

Protection from MV challenge infection

All six MVA-FH vaccinated macaques proved to be effectivelly protected from challenge with wildtype MV. Low MV loads ($<10^1/10^6$ cells) could be demonstrated in the PBMC and/or LLC of the MVA-FH vaccinated macaques, while significant levels of MV were isolated from PEC, LLC and PBMC of the MVA-wt vaccinated animals (figure 7). The three animals from which no virus could be reisolated all showed a rise in MV-specific antibody levels, suggestive of a low level challenge virus replication (figure 8). Only one of the six vaccinated animals (H1) did not show a serological booster response following challenge. As an additional parameter of infection, IgM antibodies specific for MV-N (not included in the vaccine construct) were measured following challenge. The observed levels proved to correlate well with the serological booster responses of the F- and H-specific and VN antibodies: no N-specific IgM could be measured in macaque H1, low levels of N-specific IgM were measured in the other five MVA-FH vaccinated animals, and high levels of N-specific IgM were measured in the wildtype MVA vaccinated animals (figure 9).

DISCUSSION

In the present study, we have shown that macaques are effectively protected from intratracheal challenge with wildtype MV 1 year after vaccination with MVA-FH irrespective of the presence of passively transferred homologous MV-specific antibody. These experiments were carried out with a macaque model for MV infection, with which the same parameters had been studied upon vaccination with MV-Schwarz, rVV-FH and MV-iscom by using essentially the same regimen [26]. The level of passively transferred homologous MV-specific antibodies used was also identical and corresponded to levels of serum VN antibodies that in epidemiological studies have been shown to interfere with the outcome of measles vaccination of infants [2]. These levels relate to serum antibody levels that may be expected in infants of 6 to 9 months of age. In a cohort of 160 Sudanese infants aged about 6 months, we found levels below 0.1 IU/ml in 22% (n=35), levels between 0.1 and 0.2 IU/ml in 45% (n=72), and levels above 0.2 IU/ml in 33% (n=53) of the cohort (unpublished data). This level had been shown to completely abolish the induction of MV-specific antibodies by MV-Schwarz vaccination and almost completely abolish the induction of this response by rVV-FH vaccination [26]. In contrast, a candidate MV-iscom vaccine was shown to induce high titers of MV-specific serum antibody both in the presence and absence of passively transferred homologous MV-specific antibody [26]. In the present study, MVA-FH was shown to induce higher levels of MV-specific antibodies than rVV-FH when administered in the presence of passively transferred neutralizing antibodies. We hypothesize that this is related to the relatively high MVA-FH doses used for vaccination ($1x10^{6.2}$ pfu per animal for rVV-FH versus 1x108 PFU per animal for MVA-FH). For safety reasons [15], lower doses of rVV-FH could be administered in the previous experiment [26]. The level of VN antibodies present at the time of the second vaccination (about 0.1 IU/ml, figure 2), which may at least in part be attributed to the passive immunization, has been shown to interfere with the replication of MV [26]. The serological data also showed that the vaccinia virus-specific immune response induced by the first MVA-FH vaccination does not have a major impact on the immunogenicity of the second vaccination: it did not prevent a clear booster effect in the serological responses against either MV or MVA (figure 2, 3 and 4). Eight weeks after the second vaccination, all the vaccinated macaques showed a pronounced MV-specific T cell response, as evidenced by MV-specific induction of CD69 expression by $CD3^+CD8^-$ and of $CD3^+CD8^+$ bulk cultured cells (figure 5). This observation is of particular interest since in previous experiments we have shown that also in the absence of MV-neutralizing antibodies, vaccinated macaques may still be largely protected from challenge infection, indicating a protective effect of thus induced specific T cell responses [26].

One year after vaccination, all the vaccinated macaques were intratracheally challenged with MV-BIL [1,26,27]. All the vaccinated macaques proved to be effectively protected from MV infection. Only low cell-associated virus loads could be demonstrated in lung lavafes and peripheral blood, whereas, as expected, full-blown infection was demonstrated in the MVA-wt sham-vaccinated macaques. The increase in MV-neutralizing as well as F- and H-protein-specific antibody levels after challenge observed in all the macaques, and the induction of N-protein specific IgM antibodies in five of the six vaccinated macaques, confirmed that in all vaccinated macaques, low-level virus replication had still occured upon challenge.

Table 1. Average virus neutralizing (VN) antibody titers in vaccinated macaques

Vaccine	Passively immunized[a]	VN titers (IU/ml) at the time of:			
		Prime	Booster[b]	Challenge[c]	4 weeks post-challenge
MVA-FH	YES	0.2	0.05	0.2	3
	NO	<0.08	0.5	0.5	10
rVV-FH[d]	YES	0.16	0.05	0.1	100
	NO	<0.08	1.7	1	80
MV-ISCOM[d]	YES	0.16	0.2	3	100
	NO	<0.08	0.5	2	300
MV-Schwarz[d]	YES	0.16	0.1	0.2	90
	NO	<0.08	8	3	2

[a] Passively transferred MV-specific antibody (0.2 IU/ml) 48 hours before prime.
[b] Second vaccination 4 weeks after the prime.
[c] Intratracheal challenge with wild-type MV (10^3 $TCID_{50}$ of BIL strain).
[d] Data were taken from the work of Van Binnendijk et al. [26].

Collectively, our data show that vaccination with MVA-FH in a two-dose intramuscular-intranasal regimen in the presence of passively acquired MV-neutralizing antibodies induces long-lasting protective immunity against challenge with wildtype MV. In previous experiments this was also achieved with MV-iscom, but not with live attenuated measles vaccine (MV-Schwarz), or with low doses of rVV-FH (Table 1). A clear advantage of MVA-FH over rVV-FH is its documented safety profile, since we have recently shown that even in severely immunosuppressed macaques neither virus replication nor any adverse side effects occurred upon MVA infection (Stittelaar et al., Vaccine 2001;19:3700-9). Furthermore, our data suggest that MVA-FH may be used to boost low levels of vaccine-induced immunity more efficiently than live attenuated MV vaccine, which might be of importance during the final stages of the MV eradication campaign. This could become of major importance during the final stages of the MV eradication campaign.

Finally, it is important to note that a live non-replicating vaccine candidate such as MVA-FH may not be expected to be associated with the use of inactivated MV vaccines [7,9,18]. We conclude that our data favor the further exploration of the value of the MVA-FH as a candidate replication-deficient vaccine as an alternative to the present vaccine for infants with maternally acquired MV-neutralizing antibody and for adults with waning vaccine-induced immunity.

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Chapter 4.2

Safety of Modified Vaccinia virus Ankara (MVA) in immune-suppressed macaques

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ABSTRACT

Modified Vaccinia virus Ankara (MVA)-based recombinant viruses have been shown to be potent vaccine candidates for several infectious and neoplastic diseases. Since a major application of these live, replication-deficient vectors would be their use in immunocompromised or potentially immunocompromised individuals, a preclinical safety study was carried out. Macaques were inoculated with high doses of MVA (10^9) via various routes, after immune-suppression by total-body irradiation, anti-thymocyte globulin treatment, or measles virus infection. No clinical, haematological or pathological abnormalities related to MVA inoculation were observed during a 13-day follow-up period. The presence of MVA genomes was demonstrated by nested PCR during the course of the experiment in all macaques, but from none of these animals replication competent MVA could be reisolated. These data suggest that MVA can safely be used as a basis for recombinant human vaccines, and that it is also safe for use in immunocompromised individuals.

INTRODUCTION

Modified vaccinia virus ankara (MVA) is a highly attenuated vaccinia virus (VV), which has successfully been used as a smallpox vaccine during the end stage of the smallpox eradication program [1]. MVA replicates well *in vitro* in avian cells, but is largely replication deficient in human and other mammalian cells; limited replication has been demonstrated in certain mammalian cell lines, like BHK and BS-C-1 cells [2-5]. MVA-based recombinant viruses (rMVA) have, either alone or in prime-boost regimens, been shown to be promising vaccine candidates for certain infectious and neoplastic diseases of human and animals [6-13]. Recombinant gene expression by rMVA proved to be as efficient as by replication competent recombinant VV (rVV). Furthermore, levels of rMVA-induced antibody and T cell responses, and protection proved to be at least equal to that induced by rVV in the same systems. The enhanced immunogenicity of rMVA may largely be attributed to the deletion of certain immune evasion genes [2,14].

Recently, we have shown that rMVA encoding the surface glycoproteins of measles virus (MV) - induced protective immunity in macaques, both in the presence and absence of passively acquired MV-specific antibodies [15]. We and others have also shown that rMVA encoding simian immunodeficiency virus (SIV) proteins can induce protection in macaques against SIV infection, either used alone or in prime-boost regimes [8,12,16]. These findings raise the question, whether the systematic use of rMVA vaccines would be safe in immunocompromised or potentially immunocompromised target individuals. These would include infants, the elderly [17,18] and individuals with human immunodeficiency virus (HIV) infection. Generalized VV infections have been observed in HIV seropositive individuals, vaccinated with VV vaccines [19]. Similarly, patients with neoplastic disease, for which rMVA immunotherapy is also considered, are often immunocompromised.

MVA has been used succesfully in more than 100,000 people in the smallpox eradication campaign, without documentation of any of the safety problems associated with other VV vaccines [1]. Furthermore, rMVA-based vaccines have been used without apparent safety problems in a limited number of melanoma patients [1]. Also, it has been shown that MVA is rapidly cleared after intraperitoneal inoculation of immunocompetent mice, and that irradiated adult mice given up to $10^{6.2}$ infectious units of MVA parenterally do not develop any untoward effects [1,20]. In addition, irradiated rabbits did not develop any signs of disease after intravenous inoculation with $5x10^8$ infectious units of MVA [21]. In immunocompetent rabbits and monkeys, only a minor inflammatory reaction was detected after intramuscular administration [22]. However, no systematic preclinical safety studies have been carried out with MVA in deliberately immune-suppressed non-human primates, which may be expected to be as sensitive to adverse side-effects as immune-suppressed humans. Therefore, we have inoculated macaques with high doses of MVA via various routes, after they had been immune-suppressed by total-body irradiation (TBI), antithymocyte globulin (ATG) treatment or MV infection. In these animals clinical, haematological, and pathological parameters were studied and it was attempted to show the presence of MVA genomes or replicating virus in tissues of the animals, by PCR and virus isolation, respectively.

MATERIALS AND METHODS

macaques

The study was carried out in eight captive-bred adult (four to six years) healthy female cynomolgus macaques (*Macaca fascicularis*), which were all confirmed MVA-seronegative by an MVA-specific FACS-measured immunofluorescence assay (see below) [15]. The macaques were housed in pairs in negatively pressurized glove boxes. Before MVA infection the animals were immunocompromised (animals #1, 2, 3, 4, 5 and 6). Animals #7 and 8 were healthy immunocompetent controls.

anesthesia

Where indicated, the macaques were anaesthetised with a ketalin-rompun-atropine cocktail (2.5% *(w/v)* ketalin® (Apharmo BV, Duiven, The Netherlands) + 0.25% *(w/v)* rompun® (Bayer AG, Leverkusen, Germany) + 0.025% *(w/v)* atropine (Bela-Farm gmbh & Co, Germany) at an intramuscularly (im) dose of 0.4 ml/kg body-weight (bw).

Anti thymocyte globulin treatment (ATG)

Macaques #1 and #2 were treated with 60 mg/kg-bw rabbit anti-human thymocyte immunoglobulin (ATG: Thymoglobuline, Pasteur Mérieux, Lyon, France) subcutaneous (sq) two days before and at the day of MVA infection (day 0). At days 3 and 6 doses of 30 mg/kg-bw and 10 mg/kg-bw were given, respectively. The ATG dosage was based on FACS-measured $CD3^+$ cell numbers and the Sysmex™-measured hemoglobin (HGB) concentration, white blood cell (WBC) count and platelet (PLT) numbers.

Immune-suppression induced by ATG was demonstrated by the impaired proliferative responses to the mitogens PHA, Con A PWM and LPS (Figure 1A). At day 0 the effect of the first ATG administration was demonstrated by an acute drop of WBC numbers (Figure 2A). Between day 0 and day 4 no $CD3^+$ (T-cells) or $CD20^+$ (B-cells) lymphocytes were detactable in peripheral blood, and although $CD20^+$ lymphocytes were recovered thereafter, $CD3^+$ lymphocytes remained undetectable until the end of the experiment (Figure 2A).

Total-body irradiation (TBI)

Macaques #3 and #4 were exposed to 5 Gy (^{173}Cs-source, Gammacell 40, Atomic Energy of Canada Limited) at a dose rate of 0.88 Gy/min at day 0. This irradiation dose is approximately the LD50/30d for rhesus macaques [23,24]. Fourteen days before the TBI was carried out, an im dose of Ivomec®Plus (Merckshарp&Dohme BV, Haarlem, The Netherlands; anti-parasiticide) had been given, which was repeated at day 0. From day –2 to day 10 anti-microbial prophylaxis was carried out daily by oral (intubation of the oesophagus) administration of: 10 mg/kg-bw trovafloxacine (Trovan™, Pfizer Limited, Kent, Great-Britain); 6 mg/kg-bw fluconazol (Diflucan®, Pfizer Limited). After TBI blood samples were monitored for the presence of aerobic- and anaerobic bacteria, using Bactec™ culture vials -anaerobic and -aerobic (Becton Dickinson) that were incubated at 35°C. In a blood sample of macaque #4 collected at day 1 the presence of a *Staphylococcus epidermidis* and *Staphylococcus aureus* was demonstrated. This prompted us to immediately start with anti-microbial therapy in these two animals: 50 mg/kg-bw ceftazidimpentahydrate (Fortum™, Glaxo Wellcome B.V.) im and 20 mg/kg-bw vancomycin (Vancocin®, Eli Lilly Nederland B.V., Nieuwegein, The Netherlands) intravenously (iv) daily. The isolated bacteria appeared to be sensitive to vancomycin, and were probably

originating from contamination during blood sampling. No bacteria were isolated from subsequent EDTA-blood samples.

The ability of peripheral blood cells to proliferate in response to mitogens was measured in PBMC cultures established at day 0, taken 10 minutes before and 10 minutes after TBI (though before MVA application). The proliferative responses to the different mitogens were considerably impaired after TBI (Figure 1B). In all subsequent blood samples the effect of the TBI was demonstrated by the decreasing amounts of WBC (Figure 2B).

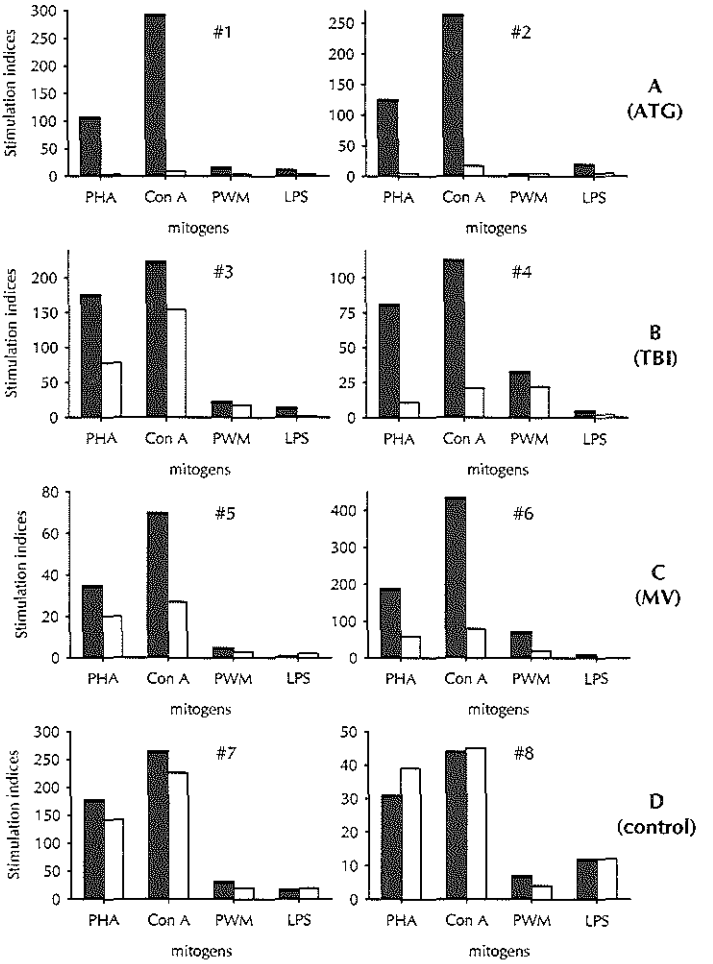

Figure 1. Proliferation of PBMC following stimulation with different mitogens. PBMC were cultured in triplicates at a concentration of $5x10^4$ cells/well in the absence or presence of PHA, Con A, PWM or LPS. After three days ([^{3}H]Tdr) was added to the wells and the cultures were maintained for one more day. Subsequently, cultures were harvested and the ([^{3}H]Tdr) incorporation was measured. Results are expressed as stimulation indices that were calculated as mean $cpm_{mitogen}/cpm_{medium}$. (A) ATG-treated macaques: responses at day –2 *versus* day 13 (black- and open bars, respectively); (B) macaques subjected to TBI: responses at day 0, 10 minutes before *versus* 10 minutes after TBI (black- and open bars, respectively); (C) MV-infected macaques: responses at day –20 *versus* day 0 (black- and open bars, respectively); (D) control macaques: responses at day 0 *versus* day 4 (black- and open bars, respectively).

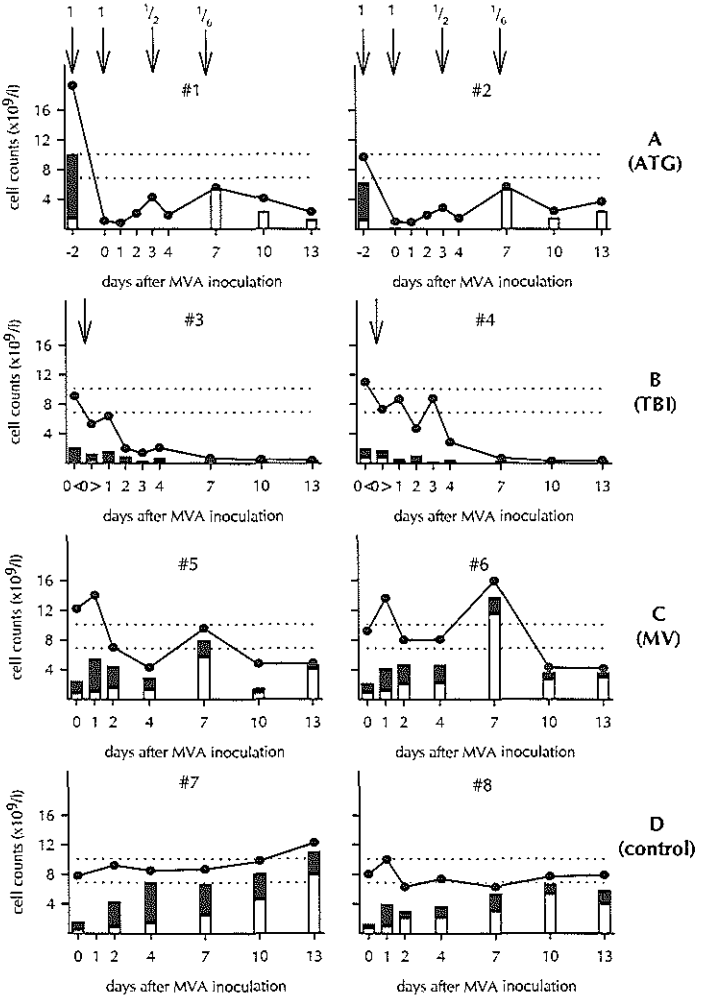

Figure 2. White blood cell numbers (lines) and phenotypes of PBMC (stacked bars) in EDTA-blood samples collected at different days after inoculation with MVA. Using flow-cytometry the absolute numbers of $CD3^+$ (gray bars) and $CD20^+$ (black bars) cells were determined. (A) ATG treated macaques, the size and the moment of ATG-doses is indicated with arrows; (B) macaques subjected to TBI, the moment of irradiation is indicated with an arrow; (C) MV-infected macaques; (D) Control macaques.

Measles virus (MV) infection

Macaques #5 and #6 (both confirmed MV-seronegative) were infected intratracheally with 1000 $TCID_{50}$ of two recently isolated wild-type MV strains 20 days prior to MVA inoculation: animal #5 was infected with a strain isolated from a Sudanese measles patient (sm32, clade B3) [25], while animal #6 had been infected with a strain isolated from an outbreak in a Dutch hospital (MVi/Amsterdam.NET/49.97, clade G2) [26]. Both infections were monitored using

virological and serological methods as described previously [27], and both strains were shown to have similar or higher pathogenicity for macaques as compared to strain BIL.
The MV infection induced immune-suppression in macaques #5 and #6 was demonstrated by the impaired proliferative responses to the mitogens PHA, Con A, PWM and LPS (Figure 1C) measured in PBMC cultures established at day –20 and day 0 (day of infection with MV and day of inoculoation with MVA, respectively). WBC counts showed a decrease from normal values at day 20 after MV infection to about half the normal values at day 30 after MV infection (Figure 2C). This is in line with the generally observed decrease in WBC counts three weeks after infection of macaques with MV (unpublished observations).

MVA inoculation
Virus was obtained from the laboratory of Dr. Anton Mayr (Munich, Germany), and had been further plaque purified by limiting dilution at passage 574 by Dr. Gerd Sutter (designated MVA F6). MVA was grown in chick embryo fibroblasts (CEF), purified by sedimentation through a cushion of sucrose, and stored at –80°C. Virus was thawed and sonicated in ice for 30 seconds with a cup sonicator (Sonicor Instruments Corporation, Copiaque, NY) shortly before inoculation. Each macaque was inoculated with $1x10^9$ pfu divided over three different sites: intradermal (id; $1x10^8$ pfu), intranasal (in; $4.5x10^8$ pfu) and im ($4.5x10^8$ pfu). The id and im injection sites were shaved before inoculation.

Sampling procedures
EDTA-blood samples and throat-swabs were collected at days –2, 0, 1, 2, 3, 4, 7, 10 and 13 after MVA inoculation. At day 7 two inguinal lymph nodes, draining the sites of i.m. administration, were collected surgically. Single cell suspensions were prepared by forcing the lymph nodes through a filter (Braun NPBI Medical B.V., Oss, The Netherlands). The cells were washed, counted microscopically and used immediately for virus isolation, phenotypic analysis and proliferation experiments (see below).

Isolation of peripheral white blood cells (WBC)
One ml EDTA-blood was mixed with 5 ml erythrocyte lysis buffer (8.29 g/l NH_4Cl, 1.0 g/l $KHCO_3$ and 0.037 g/l Na2EDTA.$2H_20$ in destilled H_20) for 15 minutes at 4°C. Subsequently, WBC were washed, counted microscopically and used immediately for virus isolation, phenotypic analysis and proliferation experiments.

Isolation of peripheral blood mononuclear cells (PBMC)
Approximately 3 ml EDTA-blood was centrifuged at 800xg for 15 minutes after which the plasma was removed and stored at -70°C until use. The pellet was reconstituted with PBS and loaded on a gradiënt (4.5% (v/v) dextran and 16.7% (v/v) Opti-prep (Nycomed) in PBS), that was subsequently centrifuged at 600xg for 25 minutes. PBMC were washed, counted microscopically and used immediately for virus isolation, phenotypic analysis and proliferation experiments.

Haematology and Clinical chemistry
Standard haematologic parameters were measured in every EDTA-blood sample taken from day –2 using an automated haematology analyzer (Sysmex™, MYCO Instrumentation Source, Inc., Renton, Washington). Kidney- and liver-function parameters and C-reactive protein (CRP) were measured in plasma samples taken at days 0 (or day –2 in case of the ATG group), 7 and 13.

Pathology

All macaques were euthanized by exsanguination under anesthesia on day 13 after inoculation, and examined for gross lesions. Samples of muscle and skin at the injection sites as well as lung, heart, liver, kidney, salivary gland, stomach, thymus, spleen, and submandibular, axillary, and inguinal lymph nodes were collected for histologic examination. After fixation in 6% neutral-buffered formalin and embedding in paraffin, 5-nm-thick, hematoylin-and-eosin-stained sections were prepared by routine methods and examined by light microscopy.

MVA isolation

MVA isolation procedures were carried out in confluent primary and secondary CEF monolayers. CEF were obtained from 10- to 12-day-old embryos and grown at 37°C in minimal essential medium supplemented with penicillin (100 U/ml), streptomycin (100 ng/ml), L-glutamine (2 mM), 2-mercapto-ethanol (10^{-5}M) and 5% foetal bovine serum (FBS). Numbers of MVA-infected cells in freshly isolated pharyngeal epithelial cells (PEC), PBMC, WBC and lymph node cells were determined by plating serial dilutions of the respective cells in confluent primary CEF monolayers grown in 96-well flat-bottomed plates as replicates of eight ($3x10^5$ cells per plate). After seven days, a second passage was carried out by transferring the cultures into fresh confluent secondary CEF monolayers. For virus isolation 1-5 gram samples of lungs, heart, liver, spleen and kidneys were freeze-thawed, minced, forced through a filter (see sampling procedures), alliquoted and stored at -70°C until use. Two freeze-thaw steps have not been shown to reduce infectivity titers of MVA in organ homogenate measurably (unpublished observation). Thawed organ-homogenates were pooled per macaque and tested for the presence of MVA in 25cm^2 culture-flasks in which confluent primary CEF monolayers were grown. Again, after seven days a second passage was carried out by transferring the cultures into fresh CEF monolayer cultures. All cultures were visually monitored daily for cytopathic changes. Sensitivity of the CEF cells for MVA replication was confirmed by titrating a stock of MVA on these cells separately.

MVA-specific polymerase chain reaction (PCR)

The presence of MVA genomes was demonstrated as described elsewhere [28]. Briefly, samples were subjected to a nested PCR using the outer primers FP1 and FP2 (ATGGACGGAACTCTTTTCCC and TAGCCAGAGATATCATAGCCGC, respectively), and then a pair of internal primers, FP3 and FP4 (CTGAATT-TTTCTCTACAAAGGCTGCTAA and TCAGCGTGATTTTCCAACCTAAATAG, respectively).

Proliferation assay

Proliferative responses of PBMC upon stimulation with mitogens or MV-antigen were measured by [^{3}H]-thymidine ([^{3}H]-Tdr) incorporation. Briefly, cells were plated in 96-well round-bottomed plates in RPMI-1640 supplemented with antibiotics, 10% FBS and 1% pooled macaque serum, at a concentration of $5x10^4$ cells/well. Cells were cultured in the presence of: 16 ng/ml phytohemagglutinin (PHA)-L, 1 ng/ml pokeweed mitogen (PWM), 10 ng/ml concanavilin A (Con A), or 100 ng/ml lipopolysaccharide from *Salmonella typhimurium* (LPS). After three days of culture [^{3}H]-Tdr was added and cells were harvested 20 hours later to measure the incorporated radioactivity. Results are expressed as stimulation indices, which were calculated as the ratio of mean counts per minute (cpm) obtained in the presence of mitogen to the cpm obtained in the absence of mitogen.

Phenotypic analysis
To determine the numbers of B- and T-cells and their subtypes in PBMC, WBC and lymph node cells, FACS-measured fluorescence was performed on freshly isolated cells. Cells were stained with anti-CD3 (FN18-FITC, U-Cytech BV, Utrecht, The Netherlands) and anti-CD19 (B1-RD1-PE; Coulter, Miami, USA), and fluorescence was measured with a FACScan (Becton Dickinson).

MVA-specific serum IgG responses
MVA-specific IgG antibodies were measured by a FACS-measured immunofluorescence assay using vaccinia virus (VV) infected rabbit kidney (RK-13) cells as targets, as previously described [15]. Briefly, RK-13 cells were infected with wild-type VV at a moi of 10. Seven hours after infection the cells were trypsinized and used as target cells in a FACS-measured immunofluorescence assay. Fluorescence intensity was quantified by determining the geometric means of the fluorescence histograms. An arbitrary cut-off level was chosen at two times the background, as indicated in Figure 3.

RESULTS

Clinical signs
All eight macaques showed a red spot of about 1 cm in diameter at the intradermal injection site by day 2 after MVA inoculation, which disappeared during the following two days. No changes were noted at the sites of im or in inoculation. Clinical signs that could be related to replication of MVA were not observed during the thirteen days of follow-up.

MVA re-isolation
No MVA could be reisolated from any of the samples (plasma's, throat swabs, WBC, PBMC, draining lymph node cells and organ homogenates) collected from the macaques during and at the end of the experiment.

MVA-specific serum IgG
The development of MVA-specific IgG responses in the MV infected macaques appeared to be slightly lower as compared to that of the control-group (Figure 3C and 3D). In the macaques treated with ATG, MVA-specific serum IgG responses were completely abolished (Figure 3A). Also, in the macaques subjected to TBI the development of MVA-specific IgG responses was largely abrogated (Figure 3B).

Pathology
No gross lesions were detected in any of the eight macaques. Histologically at the site of intradermal injection, mild epidermal hyperplasia and orthokeratosis with mild fibrosis and perivascular aggregates of few mononuclear cells in the underlying dermis was observed in all eight macaques (Figure 4). At the muscle injection site, there was myocyte degeneration, necrosis, and regeneration, with moderate numbers of mononuclear cells in the adjacent interstitium in all eight macaques (Figure 5). Eosinophilic intracytoplasmic inclusion bodies, typical for vaccinia virus infection [29], were not detected.
Lymphocytic depletion was detected histologically in thymus, spleen, and lymph nodes of all the six immune-suppressed macaques (Table 1). The degree of depletion was most marked in

the TBI macaques and the mildest in the MV-infected macaques. No significant histological lesions were found in the other tissues examined.

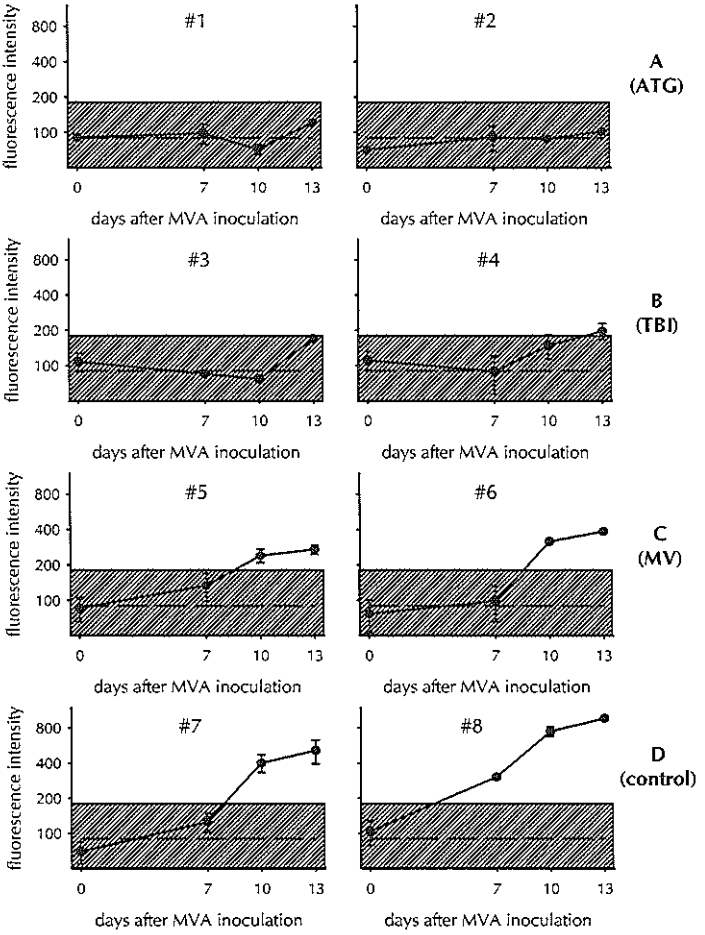

Figure 3. Development of MVA-specific plasma IgG responses in samples collected at different days after inoculation with MVA. Rabbit kidney cells (RK-13) infected with VV wild-type served as target cells in a FACS-measured immuno fluorescence assay. The average values +/- standard deviations of the antibody levels are expressed as fluorescence intensity. An arbitrary cut-off level was chosen at two times the background, as indicated by hatching. (A) ATG treated macaques; (B) macaques subjected to TBI; (C) MV-infected macaques; (D) Control macaques.

MVA-specific PCR

The presence of MVA genomes could be demonstrated in plasma through day 4 after MVA inoculation in three out of six macaques, including one of the control macaques (Table 2). In PBMC, positive PCR signals were largely confined to day 1. Five out of eight macaques showed positive PCR reactions in PEC at day 4. Two of these monkeys still showed positive PEC at day 10 (including one of the control macaques). The draining lymph node of the im injection site removed at day 7 was positive in six macaques, including one of the control macaques. The draining lymph node of the im injection site removed at day 13 was negative in all the eight

macaques. Both ATG-treated macaques and one of the control macaques were PCR positive in the lung at the end of the experiment, whereas all the other organs were negative.

DISCUSSION

In the present paper we have shown that severely immune-suppressed macaques, inoculated with high doses of MVA via various routes, did not exhibit any clinical, haematological or gross pathological or histoathological abnormalities related to progressive vaccinia, and that no MVA could be reisolated from their tissues.

Progressive VV infection, a complication of smallpox vaccination in immune-suppressed human beings, is characterized pathologically by tissue necrosis that spreads from the inoculation site, and may result in metastatic vaccinial lesions in the skin. Progressive vaccinia is defined as failure of the primary vaccination site to heal due to continued virus replication with progressive infection of the surrounding normal skin. Secondary lesions are due to viremia and appear in uninoculated parts of the body. Extensive infection of practically every tissue and organ may occur ultimately. Usually a papule develops within three to four days followed by vesiculation in five to six days and evolvement into a larger umbilicated pustule with a maximum reaction by eight to ten days [29]. Crusting of the lesion begins shortly after the height of the reaction and proceeds to complete drying and extrusion by 14 to 21 days. Virus may be recovered from the earliest papule through the crusting stage. The absence of any such lesions in the eight macaques used in these experiments, corresponds with the failure to re-isolate VV from their tissues. Besides a small red lesion at the sites of inoculation in all animals, no clinical signs were observed. This localized lesion was seen within 48 hours after inoculation and lasted for about 5 days in all the animals.

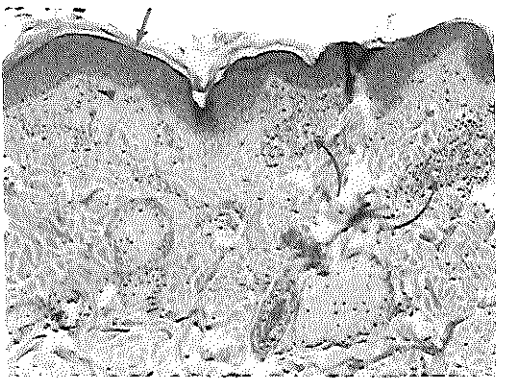

Figure 4. Skin of macaque #2 at site of intradermal injection. Note mild epidermal hyperplasia and orthokeratosis (arrow), mild fibrosis (arrowhead), and perivascular aggregates of mononuclear cells in the dermis (curved arrows). Haematoxylin and eosin. Original magnification 25x.

These studies were prompted by our recent observations that rMVA may, either alone or in prime-boost regimes, be potent vaccine candidates for measles and AIDS [15,16]. The use of rMVA in young infants, who may have an immature immune system, potentially immunocompromised individuals and individuals with an increased risk for HIV infection, highlighted the safety concerns associated with the use of MVA in immunocompromised individuals. Also their potential of rMVA as vaccine candidates for diseases in the elderly and in individuals with neoplastic disease, further motivated us to address this issue in a preclinical non-human primate study.

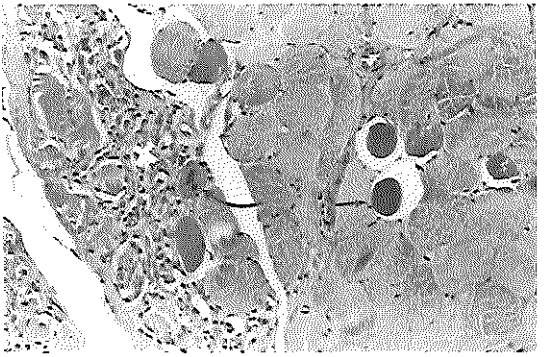

Figure 5. Striated muscle of macaque #3 at site of intramuscular injection. Note degenerate myocyte (arrow) with hypereosinophilic sarcoplasm and pyknotic nuclei, presence of mononuclear inflammatory cells (cross), and regenerative myocyte with central nuclei (curved arrow)

Table 1. Lymphocytic depletion in lymphoid organs of cynomolgus macaques subjected to different methods of immune-suppression

Treatment group	ID no.	Tissue[a]					Total individual score	Total group score
		Thymus	Spleen	Inguinal ln.	Axillary ln.	Submand. ln.		
ATG	1	0	2	0	2	2	6	14
	2	3	2	3	0	0	8	
TBI	3	3	3	n.d.	3	3	12	21
	4	3	3	0	0	3	9	
MV	5	0	1	3	0	0	4	5
	6	0	0	1	0	0	1	
Control	7	0	0	0	0	0	0	0
	8	0	0	n.d.	0	0	0	

[a]Lymphoid organs were arbitrarily scored from 0 (no lymphocytic depletion) to 3 (marked lymphocytic depletion) based on microscopic examination of hematoxylin and eosin-stained tissue sections. n.d.: not done

The three immune-suppressive treatment regimes were chosen on basis of documented evidence of their different immune-suppressive effects in macaques. TBI (5 Gy) and ATG treatment are generally used in experimental organ or tissue transplantations [30,31]. MV-induced immune-suppression [32-35] was chosen to mimic the natural situation which may be expected in MV infected individuals. As expected TBI and ATG treatment caused the most pronounced immune-suppression, as judged from the lymphocytic depletion in lymphoid organs and the immediate decline in WBC counts which was largely caused by the disappearance of both B and T lymphocytes from the circulation. The effect of MV infection was less pronounced in this respect, although it did have a significant effect on functional B and T lymphocyte parameters. Both B and T lymphocyte mitogen induced proliferative responses (LPS vs. PHA and Con A induced responses) also proved to be more severely impaired in the TBI and ATG-treated macaques, with their effects being present within hours after start of the treatment.

No haematological, clinical chemical or pathological abnormalities that could not be attributed to the immune-suppressive treatment or inoculation procedure were observed in any of the animals during the 13 day observation period.

Table 2. PCR detection of MVA in materials of cynomolgus macaques subjected to different methods of immune-suppression.

			Treatment group							
			ATG		TBI		MV		Control	
Material/Tissue	Day	ID no.:	1	2	3	4	5	6	7	8
Plasma	1		+[a]	+	+	+	+	+	+	+
	2		+	+	+	+	+	-	+	-
	4		-[b]	+	+	-	n.d.	n.d.	+	-
	7		-	-	-	-	-	-	-	-
	13		-	-	-	-	-	-	-	-
PBMC	1		n.d.	n.d.	+	n.d.	+	+	+	+
	2		n.d.	-	-	-	-	-	-	-
	4		-	-	-	-	-	-	-	-
	13		-	-	-	-	+	-	-	-
PEC	1		+	+	+	+	+	+	+	+
	2		+	+	n.d.	+	n.d.	n.d.	n.d.	n.d.
	4		-	-	+	-	+	+	+	+
	10		-	-	-	-	+	-	+	-
Inguinal Ln.	7		+	+	+	+	+	-	-	+
	13		-	-	-	-	-	-	-	-
Heart	13		-	-	-	-	-	-	-	-
Lung	13		+	+	-	-	-	-	+	-
Liver	13		-	-	-	-	-	-	-	-
Kidney	13		-	-	-	-	-	-	-	-
Spleen	13		-	-	-	-	-	-	-	-

[a]positive in MVA-specific PCR
[b]negative in MVA-specific PCR and subsequent nested PCR. n.d.: not done

The presence of MVA genomes was demonstrated using a sensitive nested PCR, in plasma, PBMC, PEC and draining lymph nodes, through days 4, 13, 10 and 7 after MVA inoculation, respectively, and in the lungs of both ATG-treated macaques and one of the control macaques at day 13. The immune-suppressed macaques did not show more positive PCR signals than the control macaques, and all attempts to re-isolate MVA in CEF cells from any sample were negative. We therefore conclude that upon inoculating a massive amount of MVA (10^9 pfu) via various routes no detectableor MVA replication had taken place in any of the macaques. The observed PCR signals can most probably be attributed to residual inoculated MVA, having spread from the inoculation sites.

Collectively, these data show that MVA is safe in macaques with different levels of immune-suppression, and suggest that MVA can indeed safely be used as basis for recombinant vaccines, intended for immunocompromised individuals.

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Chapter 5

Enteric administration of a live attenuated measles vaccine does not induce protective immunity in a macaque model

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Submitted

ABSTRACT

To test the option of oral vaccination with a live attenuated measles vaccine, we have evaluated the potential of an orally administered enteric-coated tablet containing a candidate live attenuated measles vaccine (strain Leningrad-16, MV-L16). To this end three groups of two cynomolgus macaques each were vaccinated via different routes with $10^{3.8}$ $TCID_{50}$ MV-L16 vaccine: intramuscularly (im), intra-intestinally (ii) upon laparotomy and via enteric-coated tablets. Upon vaccination, MV-L16 could only be isolated from one of the im-vaccinated monkeys and not from any of the other five. Both the im-infected monkeys and one of the ii-infected monkeys developed a MV-specific serum antibody response. Also, MV-specific $CD8^+$ IFNγ-producing T-cells could be demonstrated in all three monkeys that had seroconverted. Upon challenge with wild-type MV one year after vaccination, only these three monkeys proved to be protected. These data do not support the viability of the concept of oral vaccination with live attenuated measles vaccines.

INTRODUCTION

The currently used live attenuated measles vaccines (LAV) are quite successful in controlling measles in those countries where adequate vaccination strategies have been implemented [1,2]. However, each year almost one million fatal measles cases continue to occur, and the global control and eventual eradication of measles virus (MV) will be extremely difficult. The majority of these fatal measles cases occur in developing countries, where vaccination coverage and the effectiveness of vaccination are insufficient to prevent endemic circulation of MV. Even in populations with a relatively high vaccine coverage, incidental measles outbreaks continue to occur [3-6]. Furthermore, there is increasing evidence for the occurence of asymptomatic or subclinical courses of MV infection, potentially allowing the continuous circulation of MV in a partially immune population [7-10]. As LAV are administered by injection via the subcutaneous route, the current LAV protects against measles but not necessarily against MV infection. Vaccination strategies that would allow the induction of adequate mucosal immunity might be more effective in this respect. If this could be combined with the easy and cheap administration of a stable vaccine, e.g. by oral vaccination, the eradication of MV would probably become easier to accomplish. Here we describe the evaluation of the concept of oral measles vaccination using enteric-coated tablets containing LAV (MV-L16) in the cynomolgus macaque model for MV infection [11,12].

MATERIALS AND METHODS

Macaques

The studies were carried out in seven captive-bred subadult healthy cynomolgus macaques (*Macaca fascicularis*), which were all confirmed MV-seronegative. The animals were housed in separate rooms, without the possibility of cross-infection.

Vaccination and sampling

The standard Russian vaccine (MV-L16) was injected into the thigh-muscle (0.5 ml/leg) of two monkeys (#1 and #2). Direct intestinal application was done by laparotomy: the virus (1.5 ml) was directly injected into the lumen of the small intestine of two monkeys (#3 and #4). The tablets were given once with a special administration device, which applied the tablet into the oesophagus to the third group of monkeys (#5 and #6). To confirm the amount of infectious MV, a tablet from the same batch was ground to powder using a mortar and pestle, suspended in 1 ml PBS and centrifuged (15,000 g for 10'). The supernatant, together with the standard Russian vaccine and the ii-administered vaccine were tested for MV infectivity in serial four-fold dilutions in Vero cells. Cells were screened for cpe until approximately 7 days and the numbers of MV-infected cells were calculated by using the formula of Reed and Muench [13]. All vaccines used, contained the same amount of MV ($10^{3.7}$-$10^{3.9}$ $TCID_{50}$/dose). Sampling procedures were carried out essentially as previously described [11]. Heparinized blood samples were taken before vaccination, and at days 0, 3, 5, 7, 9, 12, 17, 26, 33, 47, 68, 124 and 188 after vaccination. Lung lavages were performed at days 3, 5, 7, 9 and 12 after vaccination. Peripheral blood mononuclear cells (PBMC) and lung lavage cells (LLC) were isolated as described previously [11].

Challenge with MV-BIL

One year after vaccination all monkeys were challenged intratracheally with 10^3 $TCID_{50}$ of the wild-type MV strain MV-BIL [11]. One MV-seronegative monkey (#7) was included as a non-vaccinated control in this challenge study. Historical controls were available from previous experiments [14]. At days 3, 6, 9, 13, 17 and 24 after infection with MV-BIL, lung lavages and heparinized peripheral blood samples were taken for MV isolations and serology as described below.

Virus isolations

Virus isolations were performed on PBMC and LLC as described previously [11,12,14]. Briefly, PBMC were first incubated in RPMI/5%FCS with PHA-L (1 μg/ml) for 1h at 37°C. Subsequently, serial two-fold dilutions of $3.2x10^5$ PBMC or LLC were co-cultivated in 96-wells plates with Vero cells ($2x10^4$ cells/well) or EBV-transformed human B-lymphoblastoid cell lines (B-LCL, $2x10^4$ cells/well) upon vaccination or challenge, respectively. The remainder of the PBMC was frozen at −135°C in 10% DMSO. Cells were screened for cytopathic effect (cpe) microscopically until approximately 7 days after sampling. Cell culture infectivity calculations of 50% end point ($TCID_{50}$) were performed as described above.

Cocultivations showing doubtful cpe were frozen at -20°C and later tested for the presence of MV-F using an inhibition ELISA. Thawed samples were incubated with anti MV-F monoclonal (F7-21) diluted in PBS/1%Triton X-100/1%BSA/0.1%Tween-20 (1h at 37°C), and transferred to MV-coated plates. After incubation (1h at 37°C) plates were washed, and incubated with horseradish peroxidase (HRP) polyclonal rabbit anti mouse Ig conjugate (1h at 37°C). After substrate reaction, the absorbance at 450 nm was read.

Serological assays

Plasma samples were screened for the presence of MV-specific IgG and IgM antibodies by a FACS-measured immunofluorescence method as described previously [15]. Briefly, human melanoma cell lines (Mel/JuSo), transfected with the MV Edmonston F- or H gene, were used as target cells. These transfected cell lines show a high cell surface expression of the MV-F- or MV-H proteins in their native conformation. The cells were incubated with plasma samples diluted as indicated in PBS/2%FCS (1h at 4°C), washed, and subsequently stained with FITC-conjugated polyclonal rabbit anti human IgG or IgM conjugates. Fluorescence intensity was quantified using a FACScan (Becton-Dickinson).

Plasma IgG antibodies directed against the MV nucleoprotein (N) were measured in an enzyme-linked immunosorbent assay (ELISA). ELISA plates (Greiner, Nürtingen, Germany) coated with purified recombinant baculovirus-expressed N protein (kind gift of T. F. Wild, Lyon, France) were washed with 0.05% (v/v) Tween 80 in PBS, followed by incubation with plasma samples diluted 1:900 in 5% (v/v) normal goat serum (NGS) in PBS. After 1h at 37°C, the plates were washed and incubated with HRP polyclonal goat anti human IgG conjugate (BioSource, Nivelles, Belgium). Following another hour at 37°C, the plates were washed again and incubated with substrate solution (tetramethylbenzidin; Meddens Diagnostics, Vorden, The Netherlands). Results were expressed as the absorbance at 450 nm.

Detection of MV-specific T cells in PBMC

MV-specific T-cell responses were measured essentially as previously described [14]. Briefly, PBMC were cocultivated with UV-irradiated autologous herpes papio (HP)-transformed B cells (mac.B-LCL). This culture was maintained for 12-14 days in the presence of recombinant

human IL-2. Subsequently, cells were treated with chymotrypsin (type II; Sigma) and cocultivated for 6 h with UV-irradiated MV-infected autologous mac.B-LCL, UV-irradiated autologous mac.B-LCL, or without mac.B-LCL. After this restimulation cells were labelled with anti-CD3-FITC (FN18-FITC, U-Cytech BV, Utrecht, The Netherlands), anti-CD69-PE (Becton Dickinson) and anti-CD8-Cy5 (DAKO). Fluorescence signals were quantified by a FACScan (Becton Dickinson). CD69 expression on $CD8^-$ $CD3^+$ and $CD8^+$ $CD3^+$ cells was determined.

Detection of cytokine producing cells

Stimulations of PBMC were carried out as described above, in the presence of 1μM monensin (Sigma) during the 6 hour incubation. Cells were labelled with anti-CD69-Cy5 (Coulter, Miami, USA) in FB for 1 hour on ice, washed twice with PBS and fixed with 4% PFA in PBS for 15 min. Following two quenching steps of 15 min. with 0.2 M glycin in PBS, cells were permeabilized in 0.1% saponin, 1% NMS, 1% FBS, % Na-azide (saponin buffer). After 30 min. on ice, anti-IFNγ-FITC (Pharmingen) and anti-IL-4-PE in saponin buffer was added. Cells were incubated for 1 hour on ice, washed with PBS and measured using a FACScan. $IFN\gamma^+$ and/or $IL\text{-}4^+$ cells were quantified in the $CD69^+$ fraction of the lympho-gate, as defined on basis of a FSC/SSC plot.

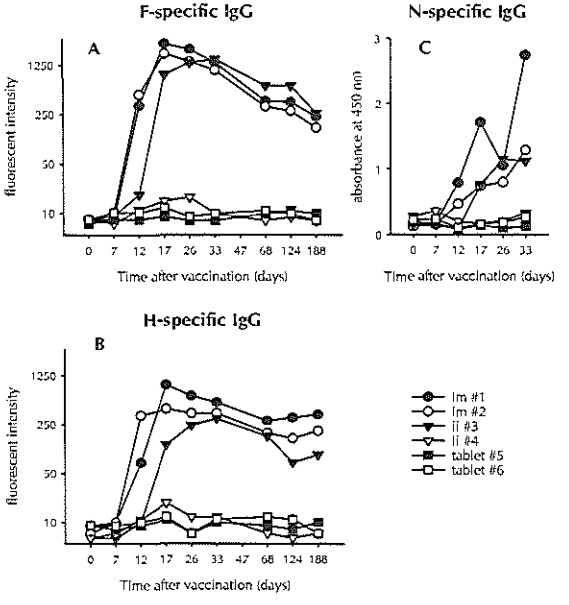

Figure 1. Development of MV glycoprotein and N specific plasma IgG (MV-F: A, MV-H: B and MV-N: C) responses in macaques upon vaccination with $10^{3.8}$ $TCID_{50}$ of MV-L16: intramuscularly (#1 and #2), intra-intestinally (#3 and #4) or orally with enteric-coated tablet (#5 and #6).

RESULTS

Virus isolation and serological response after vaccination

With the exception of one PBMC sample taken at day 7 post vaccination from monkey #1, all the PBMC and LLC samples collected between vaccination and challenge, were negative in the virus isolation assay (not shown).

Both im-vaccinated monkeys (#1 and #2) and one ii-vaccinated monkey (#3) developed MV-F, MV-H and MV-N specific IgG responses (Figure 1), indicating that the vaccine virus had replicated in these animals. They also developed MV-F and MV-H specific serum-IgM and -IgA

antibody responses (not shown). The other monkeys showed no, or only borderline and transient MV-specific antibody responses.

T cell responses in peripheral blood after vaccination

Four months after vaccination, following an *in vitro* restimulation with autologous MV-infected mac.B-LCL, MV-specific $CD3^+CD8^+$ cells were only demonstrated in monkeys #1; #2 and #3, which had seroconverted upon vaccination (Figure 2A). In monkeys #1 and #3 also MV-specific $CD3^+CD8^-$ cells could be demonstrated (Figure 2B). The MV-specific cells produced predominantly IFN and no IL-4 (Figure 2C and D). Interestingly, in two (#5 and #6) of the three monkeys that had not seroconverted (#4 to #6), MV-specific IL-4-producing cells were found.

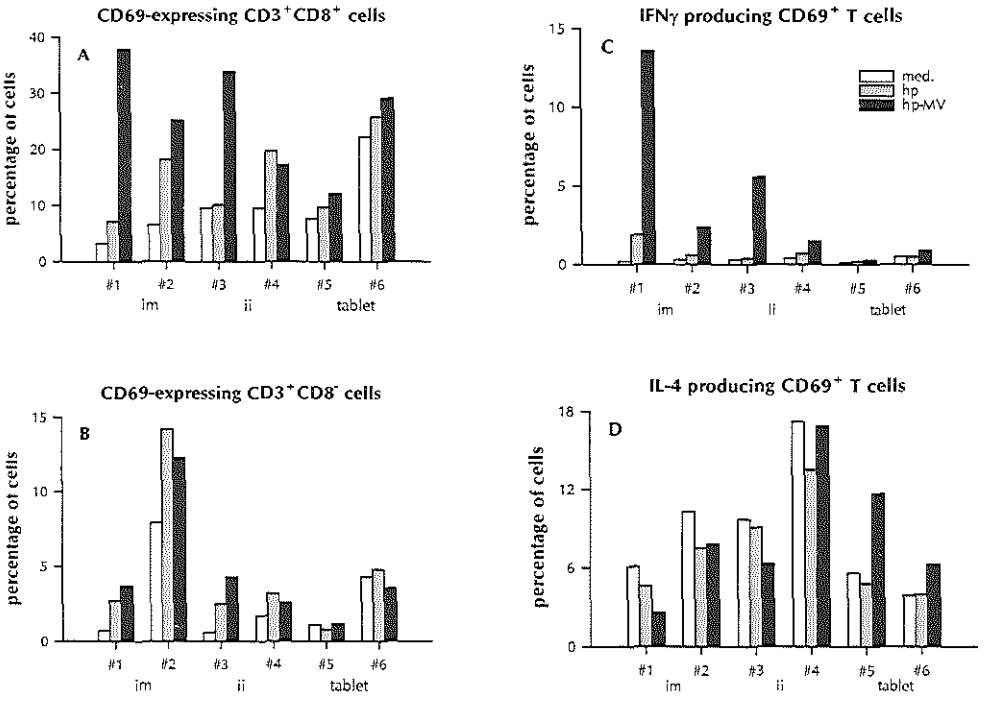

Figure 2. MV-specific T cell responses in PBMC bulk cultures of macaques four m onths after vaccination. PBMC were stimulated once *in vitro* with autologous MV-infected mac.B-LCL, and expanded in the presence of rhIL2. After 12-14 days, cells were harvested and treated with chymotrypsin. Subsequently, cells were restimulated for six ho urs with UV-inactivated autologous MV-infected mac.B-LCL (black bar), uninfected mac.B-LCL (grey bar) or without mac.B-LCL (med.; white bar)), and the membrane expression of CD3, CD8 and CD69 was determined. The percentages CD69 - positive cells in the $CD3^-$ lymphocytes, as gated on basis of a FSC/SSC plot, are shown for $CD8^+$ (panel A) and $CD8^-$ (panel B) cells. Using the same restimulation protocol, though with addition of monensin during the six hour co-cultivation period, the amount of IFNγ- and IL-4 producing CD69+ T cells were measured. The percentages of IFNγ-positive cells (panel C) and IL-4-positive cells (panel D) in the $CD69^+$ lymphocytes, as gated on basis of a FSC/SSC plot, are shown.

Protection against challenge with wild-type MV

The two im-vaccinated monkeys (#1 and #2) and one of the ii-vaccinated monkeys (#3) proved to be completely or almost completely protected from challenge with wild-type MV. This was demonstrated by the inability to isolate MV from LLC and PBMC of these animals after challenge (Figure 3). The two tablet-vaccinated monkeys (#5 and #6) and one of the ii-vaccinated monkeys (#4) showed MV isolation profiles similar to the control monkey (#7) and historical controls [14]. In agreement with this result, these four monkeys all showed clear MV-specific IgM serum responses, which were not found in the three protected monkeys upon challenge infection (Figure 4).

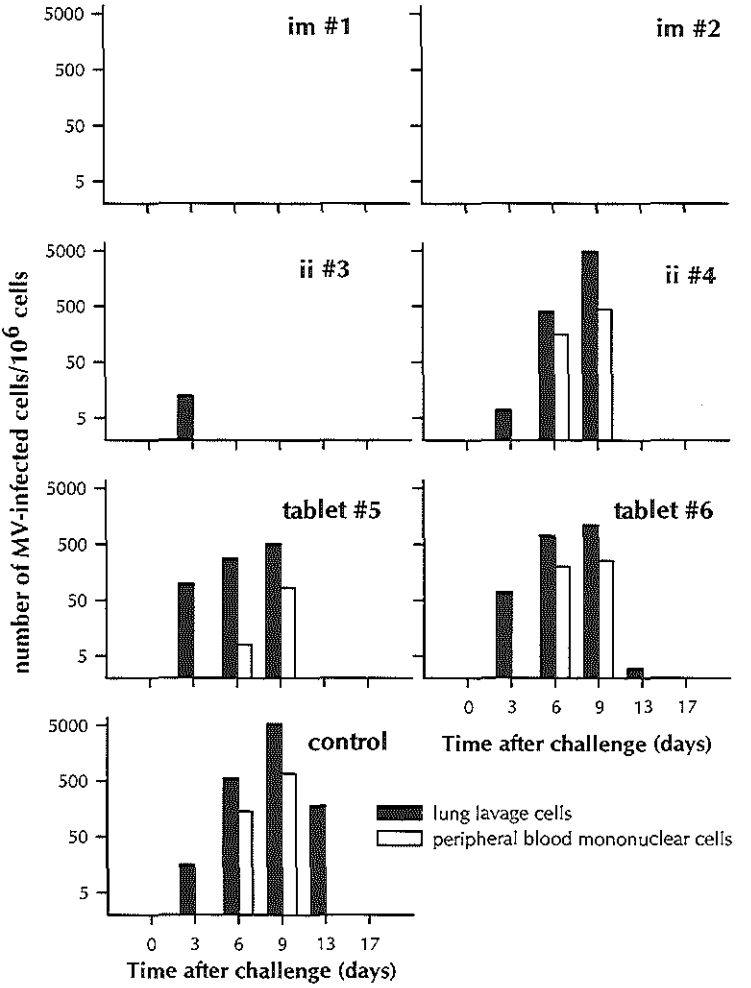

Figure 3. Estimated numbers of MV-infected cells/10^6 LLC (black bars) or PBMC (open bars) at different times after intratracheal challenge with 10^3 $TCID_{50}$ MV-BIL of macaques that had been vaccinated with $10^{3.8}$ $TCID_{50}$ of MV-L16: intramuscularly (#1 and #2), intra-intestinally (#3 and #4) or orally with enteric-coated tablet (#5 and #6). One MV-seronegative monkey (#7) was included as a non-vaccinated control.

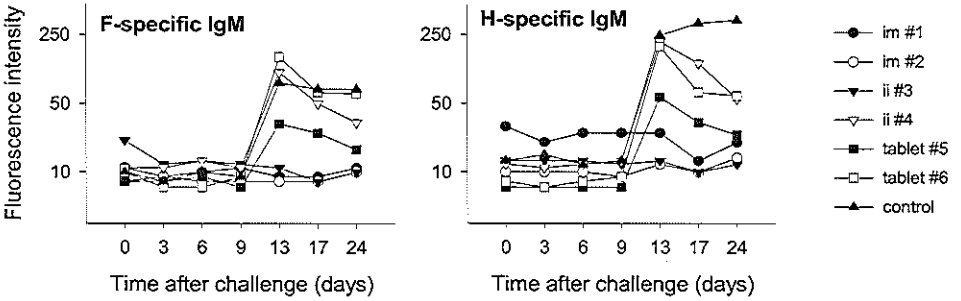

Figure 4. Development of MV glycoprotein specific plasma IgM (MV-F: upper graph; MV-H: lower graph) responses after intratracheal challenge with 10^3 $TCID_{50}$ MV-BIL of macaques that had been vaccinated with $10^{3.8}$ $TCID_{50}$ of MV-L16: intramuscularly (#1 and #2), intra-intestinally (#3 and #4) or orally with enteric-coated tablet (#5 and #6). One MV-seronegative monkey (#7) was included as a non-vaccinated control.

DISCUSSION

In the present paper we have shown that oral vaccination with an enteriecoated tablet containing MV-L16 does not induce protective immunity towards wild-type MV infection in cynomolgus macaques. In addition, we have shown that direct application of LAV into the lumen of the small intestine, does not result in replication of the vaccine virus. One of the i-i vaccinated monkeys (#3) showed a good MV-specific antibody response. Interestingly, we observed a delay in the antibody response in this animal, as compared to monkeys #1 and #2. This suggested infection with a lower infectious dose, which could probably be explained by only a small part of the administered virus actually infecting target cells. This may have resulted from the unintended infection of target cells in the intestinal wall during the inoculation procedure. The cell mediated immunity measured after vaccination in monkeys #1, #2 and #3, which also had seroconverted, was demonstrated by the presence of MV-specific IFNγ-producing cells (Figure 2C). This indicates the induction of typel T cells, probably the most desired T cell phenotype with regard to induction of antiviral immunity[16]. In contrast, in the two monkeys which had received the enteriecoated tablets (#5 and #6), the presence of MV-specific IL-4 producing cells was demonstrated (Figure 2D). This is probably associated with the administration of non-replicating MV antigen, since the induction of antigen-specific IL-4 producing cells is generally observed after orallyadministrated non-replicating antigen [17,18]. Taken together, these data indicate that MV does not replicate in the small intestine of primates. All the data concerning virus isolation, serology and T-cell responses upon im vaccination with MV-L16 (monkeys #1 and #2) and subsequent wild-type MV challenge (monkeys #1, #2 and #7) were in complete agreement with those of previous experiments with other live attenuated measles vaccines[11,12,14].

In conclusion, our data do not support the viability of the concept of oral vaccination with LAV against measles.

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Chapter 6

Priming of measles virus-specific humoral- and cellular immune responses in macaques by DNA immunisation

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Submitted

ABSTRACT

Although the currently used live attenuated measles vaccines are safe and effective, they are dependent on cold chain maintenance and are often ineffective in young infants due to interference by maternal antibody. Therefore, besides vector-based vaccines, different new generation non-replicating candidate measles vaccines are being considered, including nucleic acid vaccines. We have immunised cynomolgus macaques transdermally with DNA plasmids encoding measles virus proteins. Following two immunisations, low serum antibody responses were detected. Wild-type measles virus challenge one year after vaccination showed reduced viraemia in some animals. However, accelerated humoral and cellular immune responses were observed in all macaques, demonstrating successful priming by the DNA vaccines.

Measles is a highly contagious disease, which despite the existence of effective live attenuated vaccines still causes high morbidity and mortality among infants in developing countries. Research aiming at the development of alternative vaccines that would allow an improved vaccination strategy is conducted on a broad front [1]. Besides adjuvated subunit vaccine candidates (like iscoms) and recombinant viral vectors (like Modified Vaccinia virus Ankara), nucleic acid vaccination has been shown to induce measles virus (MV)-specific humoral and cellular immune responses in different animal models [2-4]. Intramuscular immunization of mice with DNA plasmids expressing the nucleoprotein (N) and haemagglutinin (H) induced humoral and cellular immune responses [5,6], whereas intramuscular immunization of young mice with only a DNA plasmid expressing the N protein did not show protection against intracranial challenge with a rodent-adapted MV strain [Fooks 1996 Viral Immunol]. Mucosal vaccination using a DNA plasmid carrying the H gene elicited MV-specific CTL in mice both with and without the co-administration of cholera toxin [7]. In addition, mucosal immunization in which DNA plasmids expressing the N, H and fusion protein (F) were delivered using a candidate-attenuated *Shigella* vaccine vector showed the induction of humoral- and cellular immune responses [8]. Cotton rats intradermally injected with DNA plasmids expressing the N, H and F proteins were protected against intranasal MV infection [9]. Besides these MV protein-based DNA vaccines, also minigene- or epitope-based DNA vaccines have been constructed and evaluated [Hsu 1998 Int Immunol]. A measles DNA vaccination experiment carried out in rhesus macaques (*Macaca mulatta*) showed induction of specific virus neutralizing (VN) antibodies after gene gun vaccination and a reduced viraemia following challenge infection, which coincided with a boosting of VN antibody levels [10]. We now performed an experiment in cynomolgus macaques (*Macaca fascicularis*), immunising animals with different combinations of DNA plasmids expressing the N, F or H proteins. Macaques were immunised transdermally at the abdominal site at weeks 0 and 9 using the Helios™ gene gun system (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) with 7μg DNA per dose [11]. Gold particle bombardment has the advantage of direct intracellular delivery of small DNA amounts into cells of the epidermis (for review see [12]). Plasmid DNA is very stable and the transdermal delivery, avoiding the use of needles, may improve overall compliance levels.

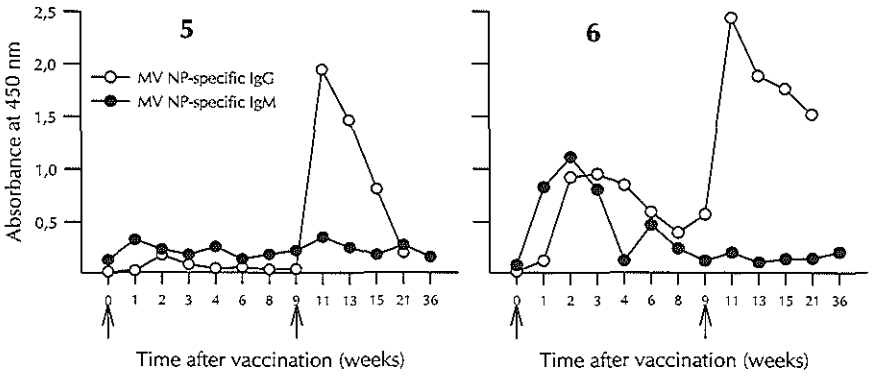

Figure 1. Development of MV N-specific plasma IgG- and IgM serum antibody in macaques after vaccination with DNA plasmids expressing the F-, H- and N protein of MV. Macaques were immunized transdermally at weeks 0 and 9 (arrows) using a gene gun system.

Two macaques were vaccinated with plasmids encoding H (#1 and #2), two macaques were vaccinated with H and F (#3 and #4) and two macaques were vaccinated with H, F and N (#5 and #6). Two other macaques (#7 and #8) served as challenge controls. During the one-year period between vaccination and challenge heparinized blood samples were collected at regular intervals.

Serological assays (VN assay, MV-F and MV-H specific immunofluorescence assays, MV-N specific IgM capture and IgG ELISA) were carried out as previously described [4,13,14]. DNA plasmids encoding the H- or F-protein induced no or very low levels of H- or F-specific or VN antibody (data not shown). In contrast, the macaques that were vaccinated with the N-encoding DNA plasmid showed the development of NP-specific antibodies (Figure 1). Although initially macaque #5 did not show significant antibody levels, upon the second vaccination high levels of N-specific IgG were mounted in the absence of N-specific IgM. Macaque #6 showed a classic antibody production profile. The N-specific IgG antibodies remained detectable in this macaque until the time of challenge.

One year after vaccination all macaques were challenged with wild-type MV as previously described [15]. Briefly, 10^3 $TCID_{50}$ of the wildtype MV strain BIL were administered via the intratracheal route at day 0. Lung lavage cells (LLC) and peripheral blood mononuclear cells (PBMC) and plasma were collected at days 3, 6, 9, 13 and 17, and additional plasma and PBMC samples were collected at days 0, 24 and 41. Virus was re-isolated by co-cultivation of macaque cells with a human EBV-transformed B-lymphoblastic cell line in an infectious center assay (log2-titration as replicates of eight) as previously described [4].

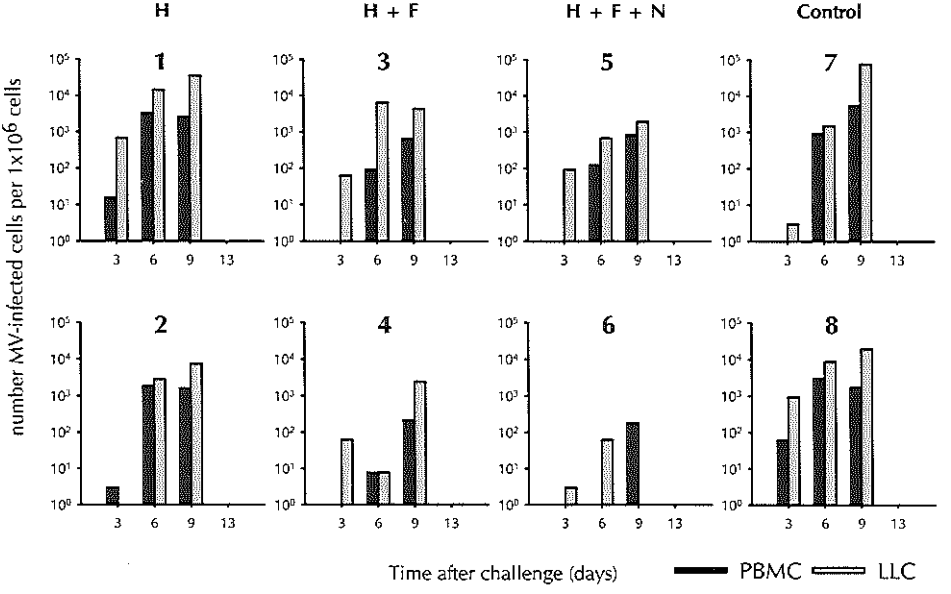

Figure 2. Number of MV-infected cells/10^6 PBMC (black bars) or LLC (grey bars) at different days after intratracheal challenge with 10^3 $TCID_{50}$ of MV-BIL.

Even though statistical analysis on the kinetics of viral loads and small animal numbers is difficult to perform, the challenge infection showed reduction of cell-associated viral loads in macaque #4 and #6 (Figure 2). Following the challenge infection all macaques showed a boosting of the MV-specific humoral responses as demonstrated by MV-specific VN antibody and IgG production (Figure 3). All DNA-vaccinated macaques showed an accelerated MV-specific IgG response as compared with the naïve challenge control animals.

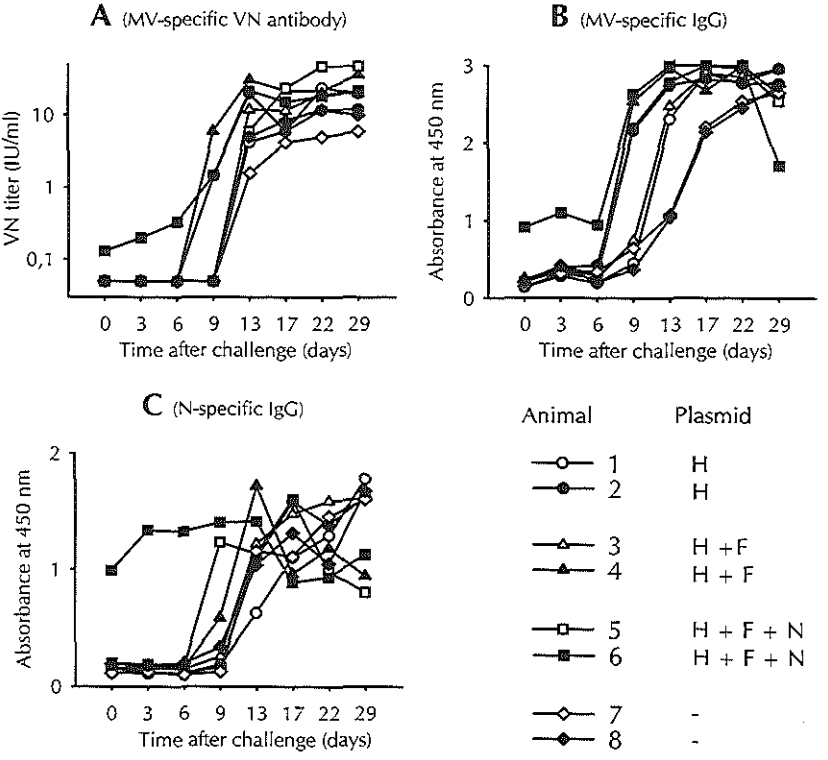

Figure 3. Development of MV-specific VN antibody (A), MV-specific IgG (B) and MV N-specific IgG (C) upon intratracheal challenge with wild-type MV. Macaques were vaccinated with different combinantions of DNA plasmids expressing the F-, H- or N protein as indicated.

The MV-specific cellular immune response was measured using an interferon gamma (IFNγ) enzyme-linked immunospot (ELISPOT) assay for macaques (U-cytech, Utrecht, The Netherlands). PBMC were plated in 96-well V-bottomed plates (Greiner Labor Technik, Nürtingen, Germany) at a concentration of 1.5 x 10^5 cells/well. To these wells autologous herpes papio virus-transformed B cells, either or not infected with MV Edmonston 48 hours before, were added at a concentration of 3 x 10^4 cells/well. Plates were centrifuged for 10 seconds, incubated at 37°C for one hour and the co-cultured cells were then transferred to the ELISPOT plates (Silent Screen Plate 96 well w/Nylon, Lalge Nunc International, USA). After six hours cells were removed and the plates processed according to instructions provided the

manufacturer. In samples collected after priming MV-specific IFNγ-producing cells (referred to as spot forming cells, SFC) could not be demonstrated (data not shown). In five of the six DNA-vaccinated macaques (#1, #3, #4, #5 and #6) the presence of MV-specific SFC was demonstrated in PBMC after challenge (Figure 4A). These cells were first detectable at day 9, ranging in numbers from 130 to 1800 per million PBMC, and remained detectable at least until day 29. In naïve control macaques MV-specific SFC appeared from day 13 after challenge onwards (Figure 4B).

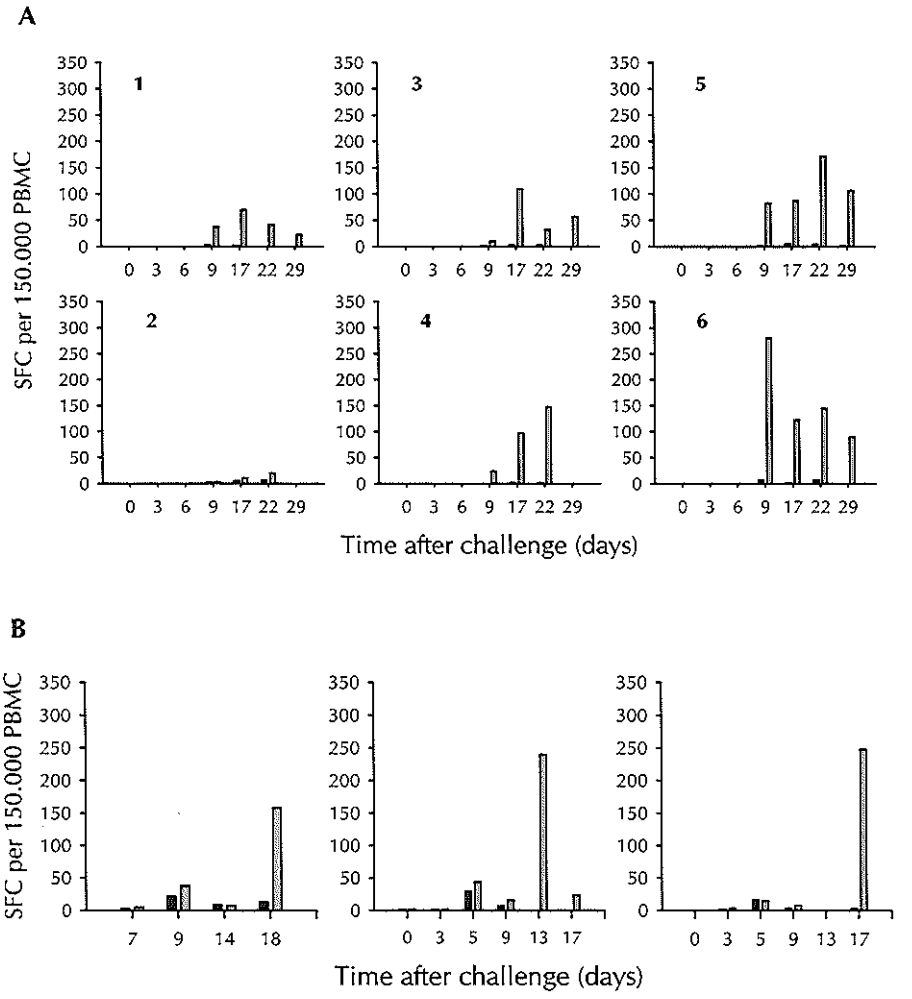

Figure 4. Number of spot forming cells (SFC) per 150.000 PBMC as measured by an IFN γ-specific ELISPOT assay. PBMC were co-cultured during six hours with herpes papio virus -transformed B cells that were either (grey bars) or not (black bars) infected with MV. PBMC were isolated from macaques that were vaccinated with different combinantio ns of DNA plasmids expressing the F-, H- or N protein (panel A) or from control macaques (panel B).

This accelerated appearance of MV-specific IFNγ–producing cells in peripheral blood of DNA-vaccinated compared to MV-naive macaques indicated successful priming of these cells by the vaccination. This observation is in agreement with a Sendai virus gene gun DNA vaccination experiment in which accelerated appearance of CTL activity was observed [16]. Similar results were also obtained from influenza virus and lymphocytic choriomeningitis virus (LCMV) plasmid DNA vaccination experiments in mice [17-19]. It has previously been shown in different systems, like in animal models for influenza, tuberculosis and malaria, that immune responses induced by DNA-priming can be upgraded to protective immunity by boosting with MVA-based vaccines [20,21]. Thus, although DNA vaccination against measles does not induce sterile protection, it may be further potentiated in heterologous prime-boost vaccination regimens.

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Chapter 7

Summarizing discussion

In part taken from:
MVA: a cuckoo in the vaccine nest? K.J. Stittelaar and A.D.M.E. Osterhaus
Vaccine, 2001;19:v-vi

The development of more efficacious vaccines and vaccination strategies for human and animal virus infections is subject of considerable effort [1]. In the present thesis several approaches to develop a new generation of measles vaccines have been addressed. A major theme was related to studies aiming at the induction of both VN antibodies and HLA class I-restricted cytotoxic T lymphocyte (CTL) responses. The latter are considered to play a major role in the clearance of MV [2]: their role in the elimination of MV infected cells during infection is considered essential. To induce CTL or activate memory CTL, MV antigens have to enter the endogenous Ag processing and presentation pathway in an APC, which generally requires *de novo* Ag synthesis [3]. Certain vaccine formulations, however, may allow non replicating, exogenous Ag to enter this pathway. Developments in organic chemistry, biochemistry and molecular biology in the past decades have boosted efforts to formulate new generations of vaccines, which indeed allow the efficient induction of both VN antibodies and HLA class I-restricted CTL responses.

Chapter 2 describes the *in vitro* processing and presentation of a lipidated cytotoxic T cell epitope derived from MV fusion protein [4,5]. Lipopeptidic formulations have been described as efficient activators of CTL and have been classed into the category of synthetic vaccines. To better understand the pathway via which lipopeptides reach the MHC class I molecules we studied the intracellular processing and presentation of a MV-derived CTL epitope, to which a palmitoyl moiety was added synthetically. The palmitoyl group was conjugated to the N-terminus either directly or via a spacer sequence. The use of single or double fluorescent-labeled lipopeptides allowed the visualization of intracellular processing of these antigens by confocal microscopy [4]. Our data indicate that the spacer composition influences internalization of the conjugate into the cell, proteasomal degradation, translocation into the ER by the transporter associated with antigen processing (TAP), and the intracellular trafficking of lipopeptides. This emphasizes the importance of a careful evaluation of the spacer composition in the development of lipopeptide immunogens. However, as a marginal note, it has been reported that the presentation of an antigen *in vitro* was strictly TAP dependent, it was presented in a TAP-independent manner *in vivo* [5,6]. It has been shown that T cell help is required for optimal induction of both CTL and B cell responses [7-9]. Therefore, an effective lipopeptide-based measles vaccine would probably need to be composed of a pool of B- and T cell epitopes. Although in different systems and with different presentation forms, like Pam3Cys constructs [10,10], synthetic vaccines have been shown to induce protective immune responses in animal models, such vaccines will most likely not be suitable for vaccination of large populations because they would have to be "tailor made". Epitope vaccines are designed for the individual on basis of MHC restriction, which might be a stumbling block. In this light the use of 'long' lipopeptides, which cover multi-epitope-spanning-regions derived from selected MV proteins, may help to overcome problems like epitope liberation and MHC restriction [11]. So far, a considerable number of B cell epitopes have been described on MV proteins but only a relatively small number of human MV T cell (Th and CTL) epitopes were identified [12]. Thus, the use of a candidate lipopeptide based measles vaccine is still far away, also when additional choices that need to be made are considered. These include the mode of delivery, i.e. incorporation into an antigen presenting particle, like a liposome or iscom, or other adjuvant systems and the route of application. Despite their intrinsic adjuvant properties lipopeptides are weak immunogens that will require further immunopotentiation if they are to be effective *in vivo*. The use of adjuvants may also reduce the amount of purified antigen required for successful immunization, thus making vaccine production more economical and practically

feasible. At present the only adjuvants registered for human use are still aluminum hydroxide and aluminum phosphate. Synthetic vaccines would of course have several advantages, like the option that their effectiveness would not necessarely be hampered by pre-existing immunity against measles [13,14,15], the option to orchestrate the type of immune responses (immunomodulation), and good possibilities to be produced under GMP conditions. Furthermore, they are relatively stable and cheap, and sequence variations can easily be implemented whenever required. Although VN antibodies induced by different MV strains are known to be cross-reactive, the reductionistic approach of a peptide-based vaccine may lead to mismatch between the vaccine and the wildtype MV. Besides the direct albeit limited implications for measles vaccine application, the observations described in chapter 2 may have much wider applications. These include studies related to the basics of antigen processing and presentation in the rational design of constructs aimed to target antigens or drugs to specific intracellular compartments, allowing them to elicit the desired immune response or drug-induced effect.

Another approach to deliver exogenous Ag into the endogenous processing and presentation pathway takes advantage of Quil A-derived adjuvant systems. An added value of this approach is that e.g. iscoms based on semi-purified Quil A have been shown to induce both strong VN antibody and CMI responses both in the absence and presence of pre-existing VN antibody [16]. Chapter 3.1 describes a series of experiments in which measles vaccine candidates, prepared with purified Quil A components are tested for their capacity to stimulate CTL *in vitro* and immune responses *in vivo* [17]. Variation in the relative amounts of MV-antigen and Quil A-components did not influence the induction of antibody responses in mice, but had a pronounced effect on the capacity to stimulate CTL activity of a CD8+ MV F-protein specific human CTL clone *in vitro*. A characteristic MV iscom preparation based on the combined use of HPLC-purified Quil A-components QA-3 and QA-22 (QA-3/22) efficiently stimulated CTL activity *in vitro*. Since only the transmembrane MV glycoproteins were incorporated in the preparation, the MV iscom used can be classified as subunit vaccine. Comparable results were obtained by mixing BPL-inactivated MV with iscom-matrix QA-3/22 or free QA-22. These two preparations containing whole inactivated virus should be classified as inactivated vaccines. On the basis of the data generated it was concluded that these three preparations are interesting MV vaccine candidates. The data were largely confirmed by experiments carried out in the cotton rat- MV model also described in Chapter 3 [18]. The results of those experiments showed that the three Quil A-adjuvated vaccine candidates but also LAV and recombinant ALVAC vaccine candidates were immunogenic in naive cotton rats, and that the animals were protected from pulmonary infection and viral dissemination upon vaccination. These experiments, in which also the effect of passively acquired MV-specific VN antibody was addressed, further strengthened the use of cotton rats for pre-clinical evaluation of measles candidate vaccines. Subsequently we focussed in Chapter 3 on the evaluation of these three vaccine candidates in the cynomolgus macaque- MV model, paying with special attention to the longevity of the vaccine-induced immune responses [19]. The data showed that 2 years after one single immunization significant levels of VN serum antibody were still present, indicating that these MV vaccine candidates are highly immunogenic. This shows that BPL-inactivated whole MV, when adjuvated with iscom-matrix QA-3/22 or free QA-22, does not exhibit the same characteristics as the formalin-inactivated, alum-precipitated vaccine, i.e. induction of short-lived MV-specific antibodies that soon fail to neutralize MV. In this respect it is important to note that protective antibody elicited in children by three immunizations with

the formalin-inactivated vaccine in the past dropped below protective levels within 2½ years in 43.2% [20].
QS-21 [21], a saponin fraction that closely resembles QA-22 (unpublished observation), has been tested in humans showing good adjuvant properties. Side effects like pain and erythema at the injection site and systemic reactions did, however, occurr [22]. Quil A-adjuvated candidate vaccines may increasingly comply with safety and quality standards of the currently used human vaccines. By further HPLC purification, components have now been identified with good adjuvant capacity and little or no toxicity [23-25]. Taken together, the currently available data on candidate vaccines based on Quil A-derived adjuvants, warrant further preclinical testing of the efficacy and safety of candidate measles vaccines based on this type of adjuvants in primate models.

A completely different approach to induce a broadly reactive immune response, including VN antibody and CTL responses, is the use of bacterial and viral vectors [26,27], each with their own advantages and disadvantages. Among the currently most interesting viral vectors for this purpose is undoubtedly a new generation of poxviruses [28], like avian poxviruses (ALVAC)[29] and viruses derived from vaccinia virus, like NYVAC [30] and modified vaccinia virus Ankara (MVA). We have chosen to explore the potential of the MVA vector on basis of the currently known advantages of this vector. One of the greatest achievements of modern medicine is the global eradication of variola virus, the causative agent of smallpox, about 20 years ago by a worldwide vaccination effort orchestrated by the WHO [31]. Variola virus, which has killed millions of people over the last centuries, has now been locked up in a couple of freezers in Russia and the USA. The effort was based on the concept of using vaccinia virus – an animal orthopoxvirus closely related to the human variola virus – which principle had been introduced about 200 years earlier by Edward Jenner [32]. It is interesting to highlight that by that time Jenner, founder of "modern vaccinology", had already attracted the attention of the scientific community by having discovered the breeding parasitism of the cuckoo, which he published in 1788 [33].
After the eradication of variola virus, it was decided to discontinue vaccination. This may have created niches in the human population for the introduction of other orthopoxviruses, resulting in the spillover from animals to humans of monkey pox in Africa and cowpox in Europe in recent years [34,35]. The vaccinia viruses used in the smallpox eradication campaigns were highly efficacious and relatively safe. However, the incidence of serious adverse events of about 1/500,000 immunocompetent individuals, and much higher figures in immunocompromised people, rendered vaccination with these viruses less acceptable towards the completion of the eradication. This prompted the development of more attenuated vaccinia viruses, like the Ankara strain that was extensively characterized by Anton Mayr [36]. By the time variola virus was eradicated, the Ankara strain had been used in more than 120,000 persons and had developed an impressive efficacy and safety record. This virus was therefore used by Bernard Moss and Gerd Sutter as the basis for the development of a highly effective and safe vector system for the delivery of antigens to induce protective immunity. The resulting modified vaccinia virus Ankara (MVA) proved to deliver these antigens in a highly immunogenic way, stimulating both antibody and T cell responses, even in the presence of pre-existing immunity against the vector [37]. Therefore, MVA could also be applied in individuals who had a history of smallpox vaccination. MVA is not able to complete a full replication cycle in most mammalian cells. Apparently, this does not preclude the adequate expression and immunogenic presentation of the foreign genes and gene products in mammals

[38]. The large coding capacity, of over 25 kb, allows poxviruses to simultaneously express foreign genes of several other viruses.
In Chapter 4 the evaluation is described of a recombinant MVA expressing the F- and H-glycoproteins of MV (MVA-FH), was evaluated as a vaccine candidate in the cynomolgus macaque MV model [39]. Animals were vaccinated twice in the absence or presence of passively transferred MV neutralizing macaque antibodies, and one year later challenged intratracheally with wildtype MV. This experimental design was chosen, since it largely coincided with that of a previous study in which the LAV, a recombinant vaccinia virus (rVV) and a MV iscom candidate vaccine were tested [16]. After the second vaccination with MVA-FH all the animals developed MV-neutralizing antibodies and MV-specific T cell responses. Although MVA-FH was slightly less effective in inducing MV neutralizing antibodies in the absence of passively transferred antibodies than the currently used LAV, it proved to be more effective than LAV in the presence of these antibodies. All vaccinated animals were effectively protected from the challenge infection one year later. These data suggest that MVA-FH is an interesting candidate measles vaccine that should be further tested for its potential to overcome the problems with measles vaccination caused by maternal or otherwise pre-existing antibodies in infants and adults with waning measles-specific immunity, respectively.

The favorable data obtained with this system prompted us to further address the safety of MVA as candidate vaccine vector. In this light it is important to consider that measles vaccines will in the future also have to be used extensively in the immunocompromised host, like children infected with HIV [40][1999 Pediatrics 103:1057-60]. Therefore, we carried out a safety experiment in immunocompromised macaques, as also described in Chapter 4 [41]. Macaques were inoculated with high doses of MVA (10^9 pfu) via various routes, after immune-suppression by total-body irradiation, anti-thymocyte globulin treatment, or MV infection. No clinical, haematological or pathological abnormalities related to MVA inoculation were observed during a 13-day follow-up period. Although the presence of MVA genomes was demonstrated by nested PCR during the course of the experiment in all macaques in several organs, from none of these animals replication-competent MVA could be reisolated in CEF. These data suggest that MVA can safely be used as a basis for recombinant human vaccines, and that it is also safe for use in severely immunocompromised individuals.
Our experiments do not provide a clue concerning the best route of administration, since we administered the vaccine both via the intranasal- and the intramuscular routes. Prompted by a HIV vaccination experiment in which a single intrarectal immunization with a recombinant MVA (expressing HIV-1 89.6 Env protein) was able to elicit effective CTL responses in mice [42], we performed a pilot experiment in which we vaccinated macaques with a single dose of 10^8 pfu MVA-FH using the rectal route. Although significant humoral immune responses could not be demonstrated after vaccination one macaque showed reduced viraemia upon intratracheal challenge with MV-wt 18 months later (unpublished data). Despite this limited success with rectal application, it would be interesting to test the immunogenicity of recombinant MVA vaccines upon mucosal delivery, using aerosol technologies that are currently being tested for measles LAV delivery (see below).

In Chapter 6 a nucleic acid vaccination (also referred to as DNA vaccination) experiment is described [43]. We immunised cynomolgus macaques transdermally with DNA plasmids encoding F, H or N protein using a "gene gun". Following two immunisations via the abdominal skin, low serum antibody responses were detected, but no specific cellular

responses could be demonstrated. Wild-type MV challenge one year after immunisation showed reduced viraemia in some of the vaccinated animals. However, accelerated humoral and cellular immune responses were observed in all macaques, demonstrating successful priming by the DNA constructs. Collectively, the data generated suggest that immunisation with DNA plasmids expressing N, F and H may limit or even prevent disease, but probably need to be incorporated in a prime-boost strategy to elicit protection against MV infection in humans. In this study we explored 'gold particle bombardment of the skin' as administration route but DNA constructs may also be applied by injection or by a bacterial vector. Targetting the DNA to mucosal tissues using a highly attenuated *Shigella flexneri* vector yielded promissing results in mice [44]. Gold particle bombardment has the advantage of direct intracellular delivery of small DNA amounts into cells of the epidermis [45]. Plasmid DNA is very stable and the transdermal delivery, avoiding the use of needles, may improve overall compliance rates. A point of concern with regard to DNA vaccination is the possibility that plasmid DNA integrates into genomic material of the host [46]. The targeted cell type may determine the chance of incorporation, i.e. myocytes (muscle cells) are terminally differentiated and do not undergo further cell division, which reduces the possibility of integration, whereas actively dividing cells have a higher probability to have plasmid DNA integrated into their genome. Whether or not plasmid DNA integration is a real safety issue remains elusive. Another drawback of DNA vaccination may be the induction of anti-DNA antibodies similar to those associated with auto-immune diseases, but sofar studies in non-human primates have failed to demonstrate such antibodies upon DNA vaccination [47,48].
Other strategies for the development of candidate measles vaccines that are being developed using recombinant DNA technology but are not addressed with in this thesis include: transgenic plant-derived measles vaccines [49], gene deleted viruses and chimeric viruses. Gene deleted and otherwise modified MV have been constructed, initially not for measles vaccination purposes but rather for pathogenicity studies or to identify antigenic determinants [50-56]. Chimeric viruses, combining the replication machinery of one virus with the envelope (determines the host range) of another have been constructed and evaluated [57-59]. MV-based chimeric constructs with envelopes of closely related morbilliviruses such as CDV or more distantly related viruses such as mumps virus, appear both feasible and promising. Interestingly, recombinant MV have been constructed to vaccinate against other pathogens by serving as a vaccine vector [60,61] and for transferring genes into neurons [62]. A disadvantage is that repeated application is not possible. In contrast to MV, reinfections with parainfluenza virus (PIV) and respiratory syncytial virus, two other human viruses of the family Paramyxoviridae, in humans are possible (for review see) [63], and recombinant PIV can induce immunity in animals immune to PIV [64].

The currently used LAV, when parenterally administered, has proven to be quite successful. However, there are several drawbacks associated to this vaccination strategy as listed in Chapter 1. More apparent vaccine failures may, at least in part, be attributed to an inadequate vaccine-induced mucosal immunity: the current vaccine protects against measles but not necessarily against MV infection [65]. Vaccination strategies that would allow the induction of adequate mucosal immunity would have obvious advantages in this respect. If this could be combined with the easy and cheap administration of a stable vaccine, the efforts to eradicate MV would considerably be facilitated. Besides the development of new generations of MV vaccines, the question has been raised whether it would be feasible to apply the existing LAV via mucosal routes instead of the currently used parenteral routes. This could lead to a better

immune response at the site of virus entry and might allow more efficient vaccination in the presence of pre-existing MV neutralizing antibody. Using a recombinant adenovirus vector expressing a glycoprotein of herpes simplex virus that mucosal immunisation resulted in long-lived CTL memory in mucosal tissue whereas systemic immunisation did not [66]. Mucosal immunization is thought to circumvent the barrier of pre-existing immunity against the inoculated antigen to a large extent [67]. It is hypothesized that immunization via non-mucosal sites leaves the inductive sites of the mucosal immune system naïve [42]. Actually, for measles vaccines this phenomennon has been reported years ago by Okune et al [68] and Ueda et al [69]. They found that subcutaneously injected LAV was neutralized in the presence of low levels neutralizing antibody, whereas LAV inhaled as aerosol was not. Since then, the concept of mucosal vaccination using the current LAV has been studied frequently. Different routes of administration have been explored (for review see [70], i.e. inhalation of nebulized reconstituted vaccine [71], inhalation of dry powder aerosols [72,73]. In Chapter 5 the evaluation of a candidate LAV (strain Leningrad-16, MV-L16) administered orally as an enteric-coated tablet is described [74]. To this end two monkeys were inoculated intramuscularly (im) with the standard Russian attenuated MV-L16, two monkeys were inoculated intraintestinally (ii) with MV-L16 and two monkeys were vaccinated orally with MV-L16 in tablet form. Upon vaccination, vaccine virus could be isolated from only one of the im-vaccinated monkeys and from none of the other five animals. Both the im-vaccinated monkeys and one of the ii-infected monkeys developed a MV-specific antibody response whereas, no MV-specific antibody response was detected in the other ii-infected monkey, or in the two orally-vaccinated monkeys. MV-specific $CD8^+$ IFNγ-producing T-cells could be demonstrated in both im-infected monkeys and in the ii-infected monkey that had seroconverted. Upon challenge with wild-type MV one year after vaccination, only the monkeys that had seroconverted proved to be protected. These data do not support the concept of oral or enteric vaccination with LAV.

Despite the fact that ample experience has been obtained with the current LAV via the subcutaneous route, the same vaccine but administrated via an alternative route will be considered a new vaccine according to current regulations [75]. Thus, like for new vaccine formulations, LAV administered via an alternative route would have to go through a complete process of registration and licensing.

The future of measles and measles vaccination.

Can MV, like smallpox virus and hopefully soon also polio viruses be eradicated? Lately, global health organizations recognized that at present the best thing to aim for is reaching high levels of measles vaccine coverage worldwide using the current LAV applied via the subcutaneous route. The coming years will show whether a global control of measles can be accomplished by more extensive vaccination campaigns (1^{st} opportunity), achieving routine vaccination coverage $\geq$95% with 1 dose LAV at 9 months of age. In addition, catch-up vaccination campaigns, a one-time vaccination campaign targeting all children aged 9 months to 14 years in a defined area reaching previously unvaccinated children (2^{nd} opportunity), should contribute to a significantly decreased morbidity and mortality. This concept has been proven largely effective in the Americas. At this moment it is probably the best choice, it is time to act, and there is no alternative vaccine or vaccination strategy available yet [76]. "We need a massive effort to use existing tools more widely and wisely" (quote D.L. Heymann, WHO). It may turn out that catch-up vaccination later in childhood leads eventually to a general waning immunity in adults making this population more susceptible to measles. This may change the

overall epidemiology of measles and cause measles outbreaks, which will therefore necessitate even a 3rd opportunity: vaccination of adults.
Finally we may contemplate the global eradication of MV. Although the complete elimination of MV from whole continents was achieved with the currently used LAV, global eradication might demand alternative vaccination strategies, like those being effective in the presence of maternal antibody and waning vaccine-induced immunity. Since the major burden of measles is in developing countries, the vaccination strategy must be able to overcome major logistical problems. A strategy that is based on two doses, or prime-boost regimens, will probably not be possible in certain areas. A first immunization that only primes the immune system, which probably prevents disease, will be sufficient to reduce mortality and morbidity but may allow the virus to continue to circulate. Optimally, at very young age one vaccine dose should induce long-lasting protective immunity that may not even have to be boosted later in childhood. Due to the HIV epidemic, which is ongoing in some of the target populations, safety in immunocompromised individuals must be guaranteed [77]. In this respect inactivated vaccine candidates, like the Quil A-adjuvated preparations described in Chapter 3 and poxvirus vectors as described in Chapter 4 hold promise. Since one dose human neonatal vaccines have not been described before, future experiments should address the potential of such vaccine candidates in an immature immune system in the absence or presence of variable amounts of maternal-acquired measles antibody. In addition the effectiveness and safety of this candidate measles vaccine should be addressed in LAV-vaccinated individuals and people that have had measles. Purified inactivated MV adjuvated with QA-22 may be used in combination with other inactivated vaccines like diphteria, pertussis, tetanus and polio.

The 'niche' after measles virus eradication.

More than twenty years after the global eradication of variola virus and the discontinuation of smallpox vaccination, the postulated niche of immunologically naïve people may lead to a potentially explosive situation. The major concerns are the reintroduction of variola virus through e.g. bioterroristic acts, and of animal poxviruses through contacts with infected animals. A similar concern may also arise after the discontinuation of measles vaccination once MV has been eradicated. The interspecies transmission of animal morbilliviruses, which under the current circumstances do not have the chance to cross the species barrier into humans, due to the presence of immunity to MV, could lead to catastrophic disease outbreaks. This was e.g. shown by the numerous introductions of different animal morbilliviruses into populations of aquatic and terrestrial mammals (for reviews see) [78,79]. Along the same line it has been suggested MV has originated from rinderpest virus after an interspecies transmission event that took place some millennia ago [80].
How to respond to these apparently emerging niches for animal viruses in naïve, non-vaccinated human populations? Jenner's good old "hobby-horse" - vaccinia virus - transformed into MVA, may now not only be used to immunise people against orthopoxviruses, but also against a number of other viruses, some of which are future candidates for eradication. In this way, the recombinant MVA would deprive the current vaccines against these viruses of their existence, thus playing the cuckoo's role in the Jennerian way.

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Samenvatting

Mazelen

Mazelen is een zeer besmettelijk infectieziekte, veroorzaakt door het mazelenvirus (MV). De ziekte kenmerkt zich door koorts, hoesten, neusverkoudheid, oogbindvliesontsteking en huiduitslag die ongeveer een week aanhouden. Iemand die hersteld is van een natuurlijke infectie met MV is waarschijnlijk levenslang beschermd tegen herinfectie. Met name in de ontwikkelingslanden is er een hoge mortaliteit en morbiditeit ten gevolge van mazelen. In de zestiger jaren werd gestart met vaccinatie tegen mazelen met een onzuiver met formaline geïnactiveerd viruspreparaat. Maar nadat bleek dat gevaccineerde kinderen na contact met het virus een atypische, ernstige vorm van mazelen konden krijgen werd dit vaccin van de markt genomen en in de zeventiger jaren vervangen door een levend verzwakt mazelen vaccin (LAV).

Toepassing van het LAV heeft tot een geweldige afname geleid van het aantal zieken en dodelijke slachtoffers ten gevolge van mazelen. Toch bleken uiteindelijk de mogelijkheden met dit vaccin beperkt, ten aanzien van de wijze van toepassing maar ook door eigenschappen van het vaccin zelf. Zo'n dertig jaar na de introductie van het LAV sterven jaarlijks wereldwijd nog ongeveer een miljoen kinderen aan de gevolgen van mazelen. Deze slachtoffers vallen voornamelijk in ontwikkelingslanden, maar ook in geïndustrialiseerde landen komen nog steeds mazelenuitbraken voor. Hiervoor zijn een aantal verklaringen. Ten eerste, is LAV niet werkzaam in de aanwezigheid van MV neutraliserende antistoffen, in de pasgeborene afkomstig van de moeder, of op latere leeftijd verkregen door vaccinatie. Ten tweede, moet LAV gekoeld bewaard en vervoerd worden, hetgeen lastig is in ontwikkelingslanden. Ten derde, niet iedereen die wordt gevaccineerd sero-converteert. Zelfs onder optimale omstandigheden sero-converteert niet meer dan 95% van de gevaccineerden. Hierdoor blijft een groep mensen gevoelig voor MV infectie en kan het MV onder bepaalde omstandigheden blijven circuleren. Het aanvankelijk behaalde succes na de introductie van het LAV werd in zekere mate een remmende factor voor financiële steun en verder wetenschappelijk onderzoek aan mazelen en vaccins tegen mazelen. Doordat het MV wereldwijd verantwoordelijk bleef voor een hoog sterftecijfer werd door gezondheidsorganisaties een nieuwe impuls gegeven aan het ontwikkelen van nieuwe mazelenvaccins en vaccinatiestrategieën

Het onderzoek

Dit proefschrift beschrijft de bestudering van verschillende kandidaat mazelen vaccins en hun toedieningswijze.

Peptide- of epitoop vaccin

In hoofdstuk 2.1 werd een lipopeptide, een peptide waaraan een vetzuur covalent gekoppeld was, bestudeerd. Het peptide bevatte een reeds beschreven epitoop, voorkomend in het Fusie eiwit van het mazelen virus, dat door een cytotoxische T cel (CTL) kloon werd herkend in de context van het MHC klasse I molecuul HLA-B27. Omdat van tevoren rekening werd gehouden dat het vetzuur door de antigeen presenterende cel (APC) mogelijk losgekoppeld moet worden van de epitoop werd een serie lipopeptiden gesynthetiseerd variërend in extra aminozuren tussen het epitoop en het vetzuur, de zgn. spacers. Omdat het fysisch chemische karakter van een lipopeptide aanleiding kan geven tot het ontstaan van micellen werd eerst vastgesteld dat de lipopeptiden in opgeloste toestand verkeren tijdens de experimenten. Als

testmethode werd het specifiek vrijkomen van radioactief chroom (^{51}Cr) uit de APC gemeten, als maat voor het cytolytisch sneuvelen van APC, of de specifieke productie van tumor necrose factor-alfa (TNF-α) door de CTL kloon werd gemeten, als maat voor de cytolitische activiteit van de T cel kloon. Uit de experimenten bleek dat de samenstelling van de spacer bepalend is voor de intracellulaire gebeurtenissen die nodig zijn voor een goede presentatie in de context van HLA-B27 aan de CTL kloon. Verder werd in deze studie gebruik gemaakt van fluorescerende analoga van de lipopeptiden, waarvan de synthese is beschreven in hoofdstuk 2.2, om het vetzuur- dan wel het peptidedeel zichtbaar te kunnen maken. Een dubbel fluorchroom gelabeld lipopeptide, een fluorchroom aan het vetzuur en een fluorchroom aan het peptidedeel, bleek pas te gaan fluoresceren na enzymatische splitsing tussen de twee labels. Gebruikmakend van confocal fluorescentie microscopie bleek dat een lipopeptide zich kan ophopen in het Golgi-apparaat. Dit kan duiden op een alternatieve presentatie door de MHC klasse I presentatie route. Geconcludeerd kon worden dat een lipopeptide inderdaad tot aktivatie van CTL kan leiden maar dat de presentatie van een lipopeptide erg gevoelig is voor sequentievariaties. Het feit dat een effectief op synthetische peptide gebaseerd mazelen vaccin zal moeten bestaan uit meerdere peptiden die gepresenteerd kunnen worden door meerdere MHC klasse I moleculen maakt dat een eventuele praktische toepassing van deze data niet eenvoudig zal zijn.

Subunit en geïnactiveerd vaccin

In hoofdstuk 3 werd de immunogeniteit en het beschermend vermogen van met Quil A-geadjuveerde kandidaat mazelen vaccins in verschillende model-systemen beschreven. Quil A is een mengsel van glycosiden met adjuverende eigenschappen en wordt geïsoleerd uit de boomschors van *Quillaja saponaria Molina*. Met Quil A, fosfolipiden en cholesterol kunnen zgn. iscoms ("immune stimulating complexes") worden gemaakt. Reeds eerder werd beschreven dat vaccinatie in de aan- en afwezigheid van MV neutraliserende antilichamen met iscoms, die werden gemaakt in de aanwezigheid van opgeloste MV eiwitten, bescherming gaf tegen mazelen. Echter het oorspronkelijk gebruikte Quil A-preparaat gaf ongewenste bijwerkingen. Verdere zuivering en betere scheidingstechnieken resulteerden in Quil A-componenten die hier bestudeerd werden op hun vermogen om iscoms te vormen en om ingebouwd antigeen af te leveren voor presentatie door MHC klasse I moleculen aan CTL. QA-22 bleek over de gewenste eigenschappen te bezitten maar voor het vormen van karakteristieke iscoms moest hieraan QA-3 in een juiste verhouding toegevoegd worden. Drie verschillende kandidaat vaccins, gemaakt op basis van deze twee gezuiverde Quil A-componenten, werden geselecteerd voor verder preklinisch onderzoek: iscoms met daarin het fusie-eiwit en het heamagglutine van MV; lege iscoms gemengd met gezuiverd geïnactiveerd MV; vrij QA-22 en QA-3 gemengd met gezuiverd geïnactiveerd MV. Deze drie kandidaat vaccins beschermden gevaccineerde katoenratten tegen MV infectie en één dosis was genoeg voor het induceren van hoge MV-specifieke antilichaam titers die jarenlang aanwezig bleven in cynomolgus apen. In dit hoofdstuk is tevens beschreven dat de katoenrat, een relevant model systeem is voor de evaluatie van kandidaat mazelenvaccins, mede omdat dit dier intranasaal geïnfecteerd kan worden met wildtype MV.

Recombinant vaccin

In hoofdstuk 4 is de evaluatie van een recombinant kandidaat mazelenvaccin beschreven. De genen die coderen voor het fusie-eiwit en het haemagglutinine van MV waren geplaatst in een sterk verzwakt pokkenvirus. Dit pokkenvirus, modified vaccinia virus Ankara (MVA) heeft door

honderden passages in kippen embryo fibroblasten (CEF) het vermogen verloren om te repliceren in de meeste zoogdier cellen. MVA kan nog wel zoogdiercellen infecteren, waardoor MVA eiwitten en in dit geval ook MV eiwitten tot expressie komen en gepresenteerd worden door MHC klasse I moleculen, maar er worden geen nieuwe infectieuze MVA virussen gemaakt. Hierdoor kan een hoge vaccindosis worden toegepast die dan ook in staat is beschermende immuniteit tegen mazelen op te wekken in de aan- en afwezigheid van MV neutraliserende antilichamen. De door het vaccin geïnduceerde immuneresponsen bestonden uit MV neutraliserende antilichamen en CD4+- en CD8+ MV-specifieke T cellen. De gunstige resultaten uit dit experiment en het feit dat de doelgroep voor mazelenvaccinaties in toenemende mate bestaat uit HIV- (human immunodeficiency virus) besmette individuen, waren aanleiding voor het uitvoeren van een veiligheidstest met MVA in cynomolgus apen met een sterk verzwakt immuunsysteem. Hiertoe werden apen onderworpen aan een totale lichaam bestraling (TBI) of behandeld met anti-thymocyten globuline (ATG), of, ter verkrijging van een mildere verzwakking van het immuun systeem, geïnfecteerd met wildtype MV. Hoewel met gevoelige PCR methoden DNA van MVA kon worden aangetoond in diverse organen, was het niet mogelijk om MVA te kweken uit verschillende orgaanmaterialen verzameld in een periode van dertien dagen na toediening van de zeer hoge dosis MVA. Hieruit werd geconcludeerd dat de vaccin-vector MVA veilig toegepast kan worden in individuen met een verzwakt immuun systeem.

Levend verzwakt MV, anders toegediend

Hoewel in dit proefschrift voornamelijk experimenten met alternatieve vaccins zijn beschreven, is in hoofdstuk 6 een vaccinatie-experiment beschreven op basis van een bestaand levend verzwakt MV maar toegediend als tablet in plaats van subcutane injectie. Met deze toedieningswijze werd beoogd om het vaccin af te leveren op het darmslijmvlies waardoor een locale mucosale immuunreactie zou moeten worden opgewekt. Er was reeds aangetoond dat mucosale vaccinatie op andere slijmvliezen bestaande immuniteit kan omzeilen. Het toedienen van een vaccin als tabletje zou veel voordelen hebben ten opzichte van een vaccin dat eerst moet worden gereconstitueerd en vervolgens met behulp van een injectienaald moet worden toegediend. Een bestaand vaccin, op een alternatieve manier toegediend, wordt door regelgevingen beschouwd als een nieuw vaccin en moet weer uitgebreid getest worden. Vermeerdering van het aldus toegediende MV in de darm kon niet worden aangetoond. Helaas werden dan ook geen MV-specifieke antistof responsen en MV-specifieke T cellen gedetecteerd na vaccinatie en waren de dieren niet beschermd tegen mazelen. Geconcludeerd werd dat locale vaccinatie met levend verzwakt MV in de darm, waarschijnlijk geen levensvatbare optie is.

DNA vaccin

Tenslotte werd in hoofdstuk 7 het testen van een vorm van DNA vaccinatie tegen mazelen beschreven. Hierbij werd gebruik gemaakt van DNA plasmiden die voor het fusie-eiwit, het haemagglutinine en het nucleoproteine coderen. Een DNA-vaccin kan relatief gemakkelijk worden ontwikkeld, op grote schaal worden geproduceerd en is in principe zeer stabiel. Verder leidt toediening van een DNA-vaccin tot een endogene eiwit synthese waardoor antigeen presentatie door MHC-klasse-I moleculen mogelijk is. Na twee vaccinaties, uitgevoerd met een zgn. "gene-gun", werden geen duidelijke immuun responsen gemeten tegen het fusie-eiwit en het haemagglutinine maar wel een antistof response tegen het nucleoproteine. Na challenge infectie met MV bleek dat het merendeel van de gevaccineerde dieren wel waren

geprimed. Er werd een versnelde MV-specifieke antistof- en cellulaire response waargenomen ten opzichte van niet gevaccineerde controle dieren. Deze resultaten geven aan dat een DNA-mazelen vaccin waarschijnlijk wel de mortaliteit en morbiditeit ten gevolge van mazelen kan reduceren maar dat voor het opwekken van solide bescherming een booster nodig is met een ander vaccin.

Toekomstig MV vaccin

Als uit de hier beschreven kandidaat mazelenvaccins het toekomstige MV vaccin zou moeten worden geselecteerd, lijken, op basis van de lang aanwezigblijvende MV neutraliserende antisoffen na 1 vaccinatie, de in hoofdstuk 3 beschreven QA-22/QA-3 preparaten een goede keus. Hiervan is waarschijnlijk het preparaat dat bestaat uit vrij QA-22 en QA-3 gemengd met gezuiverd geïnactiveerd MV om productietechnische redenen het meest aantrekkelijk. Toekomstig onderzoek zal moeten aantonen dat dit preparaat inderdaad veilig is, en effectief is in de aanwezigheid van verschillende hoeveelheden MV neutraliserende antilichamen en dit tevens in een immatuur immuun systeem. Tenslotte werd gespeculeerd over de mogelijkheden van wereldwijde beheersing van de mazelenproblematiek en de uiteindelijke complete eradicatie van het MV.

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Zo, nu is het wel welletjes. Koert

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 10 oktober 1968 te Schoonhoven geboren. In deze plaats doorliep hij de kleuterschool en de lagereschool. In 1987 werd het diploma HAVO behaald aan het Willem de Zwijger College aldaar. In datzelfde jaar werd een aanvang gemaakt met de studie Laboratoriumonderzoek aan de Hogeschool Utrecht te Utrecht. In 1988 werd de propedeuse behaald en in 1992 behaalde hij het diploma medische microbiologie. Van 1992 tot 1994 was hij aangesteld als tweejarige assistent in opleiding bij veterinaire immunologie, Rijksuniversiteit Utrecht (dr. E.J. Hensen). In deze periode was hij gedetacheerd bij de unit bacteriële vaccin ontwikkeling en pathogenese onderzoek, Rijksinstituut voor de Volksgezondheid en Milieu (RIVM) te Bilthoven (dr. J.T. Poolman en dr. E.J.H.J. Wiertz). Hier werkte hij mee aan onderzoek dat gericht was op het identificeren van T cell immuniteit tegen buitenmembraan eiwitten van *Neisseria meningitidis* groep C. In 1994 werd gestart met het hier beschreven promotie-onderzoek, aanvankelijk gedetacheerd bij het Laboratorium voor Vaccinonderzoek, RIVM (dr. L.J.W. van Alphen), en vanaf november 1997 bij het Instituut voor Virologie, Erasmus Universiteit Rotterdam te Rotterdam (prof.dr. A.D.M.E. Osterhaus en dr. R.L. de Swart).

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