INFLUENZA

New Vaccines and Antiviral Immunity

INFLUENZA

Nieuwe vaccins en antivirale immuniteit

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus

Prof. dr. ir. J.H. van Bemmel

en volgens besluit van het college voor promoties.

De openbare verdediging zal plaatsvinden op
donderdag 21 december 2000 om 11:00 uur

door

Josephus Theodorus Maria Voeten

geboren te Bosschenhoofd

Promotiecommissie:

Promotor:	Prof. dr. A.D.M.E. Osterhaus
Overige leden:	Prof. dr. R. de Groot Prof. dr. H.A. Verbrugh Prof. dr. E.H.J.H.M. Claassen
Copromotor:	Dr. G.F. Rimmelzwaan

ISBN nummer: 90-9014164-2

The studies described in this thesis were conducted at the Institute of Virology of the Erasmus University Rotterdam.

Printing of this thesis was financially supported by: Solvay Pharmaceuticals BV, Abbott BV, Glaxo Wellcome BV, Clean Air Techniek BV, Clindia Benelux BV, Merlin Diagnostic Systems BV, Promega Benelux BV, Dépex BV, Eurogentec BV.

Cover illustration: Pieter van Gageldonk

Aan hen die aan de totstandkoming
van dit proefschrift hebben bijgedragen.

CONTENTS

ABBREVIATIONS

APC:	antigen presenting cell
B-LCL:	B lymphoblastoid cell line
CTL:	cytotoxic T lymphocyte
ELISA:	enzyme-linked immunosorbent assay
FCS:	fetal calf serum
HA:	haemagglutinin
HI:	haemagglutination inhibition
HPS:	human pool serum
IL-2:	interleukin 2
ISCOM:	immunestimulating complex
LST:	lymphocyte stimulation test
MA:	matrix protein
MBP:	maltose binding protein
MDCK:	madin darby canine kidney
MHC:	major histocompatibility complex
NA:	neuraminidase
NP:	nucleoprotein
PHA:	phytohaemagglutinin
RT-PCR:	reverse transcriptase polymerase chain reaction
Th:	T helper

CHAPTER 1

General introduction

History and classification of influenza viruses

Influenza viruses are the causative agents of outbreaks of acute respiratory disease, known as influenza or ,shortly, 'flu' which has afflicted humans since ancient times. Although the earliest report of an outbreak of respiratory disease by Hippocrates dates back to 412 BC, the first recorded epidemic with characteristics of influenza occured in 1173-1174 while the first recognized influenza pandemic occured in 1580[62,123]. In 1933, the first influenza virus, later classified as influenza virus A, was isolated from a human followed in 1940 and 1947 with influenza virus B and C, respectively[37,94,155,162].

Influenza viruses are classified as members of the family *Orthomyxoviridae* and are divided into two genera: influenza virus A and B, and influenza virus C. Influenza virus A, B and C can be distinguished on the basis of antigenic differences between their nucleoprotein (NP) and matrix (M) protein. Influenza A viruses are further divided into subtypes based on antigenic differences in their surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). At present, 15 different HA (designated H1 to H15) and 9 different NA (designated N1 to N9) can be distinguished. This introduction mainly focusses on influenza A viruses.

Virus morphology

Influenza A viruses, in general, have a spherical morphology and are 80 to 120 nm in diameter[64,65]. The lipid membrane, or envelope, of influenza viruses is derived from the host cell in which the virus replicated[70]. Within the envelope are two membrane-spanning proteins, the glycoproteins HA and NA, which project outward and appear as rod-shaped and mushroom-shaped spikes, respectively[86] (Figure 1). Approximately 500 HA and 100 NA spikes cover the envelope with the HA molecules anchored in the membrane as trimers and the NA molecules as tetramers[107,171,182,183,185]. A third integral membrane protein, the matrix protein M2, is found in only a few copies per virus[84,192]. Underneath the lipid membrane a shell of matrix M1 protein, the most abundant protein, can be found[14,39,110,114,146]. Inside the virus, ribonucleoprotein (RNP) complexes are found consisting of viral RNA segments, NP and the polymerase proteins PB2, PB1 and PA which appear as rods[25,55,76,121]. The NP is relatively abundant in the virus while the polymerase proteins are present in 30 to 60 copies per virus[66,82]. Finally, the

nonstructural NS2 protein is found within the virus. Approximately 130 to 200 molecules are present that associate with the M1 protein[128,189].

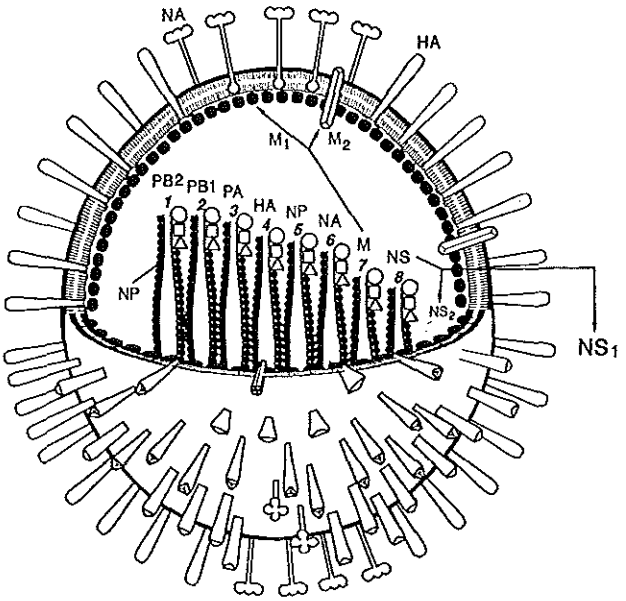

Figure 1. Schematic representation of influenza A virus.
(Reproduced with permission from *Fields Virology*, Third Edition, 1996, Lippincott Williams and Wilkins, Philadelphia)

Genome organization and protein function

The genome of influenza A viruses is composed of eight separate single-stranded RNA molecules with negative polarity which together encode a total of 10 proteins (Table 1). The RNA strands vary in length between 2341 and 890 nucleotides and are numbered 1 to 8 starting from the longest strand. Except for strand 7 and 8, each RNA strand encodes only one protein. RNA strand 1 encodes the PB2 polymerase protein which functions in the initiation of transcription by binding 5' cap structures and subsequent cleavage from host cell mRNAs which are then used as primers for viral mRNA synthesis[12,16,150,168]. Strand 2 encodes the PB1 polymerase protein which is responsible for elongation of primed nascent viral mRNA and also for complementary and viral RNA synthesis in the process of replication[16,135,168]. Strand 3 encodes the PA polymerase protein whose role in transcription and replication is not completely clear yet, but is at least

required for viral RNA synthesis[108]. The envelope glycoprotein HA is encoded by strand 4. HA is responsible both for binding of the virus to the host cell receptors and, after endocytosis, for fusion of the viral membrane with the membrane of the endosome[181]. HA undergoes extensive post-translational processing with the final step being its cleavage into HA1 and HA2 which remain connected by disulfide linkages. Cleavage is required for the virus to be infectious[85]. RNA strand 5 encodes NP which encapsidates viral RNA to form RNP complexes to which the three polymerase proteins (PB2, PB1 and PA) associate. NP is believed to play a role in the switching of viral RNA polymerase activity from mRNA synthesis to complementary and viral RNA synthesis. The second envelope glycoprotein, NA, is encoded by strand 6. NA functions as a receptor destroying enzyme by cleavage of terminal sialic acid residues from the receptor and thereby facilitates the release and spread of progeny viruses away from the infected cell[24]. RNA strand 7 encodes two matrix proteins, M1 and M2, the latter arising by splicing of the M1 transcript[63,67,83,159]. M1 forms a shell surrounding the virus RNPs underneath the virus envelope which provides structure to the virus and interacts with the cytoplasmic tails of HA, NA and M2, and with the RNPs. M2 is anchored in the membranes as tetramers forming an ion channel allowing flow of ions from endosomes to the virus interior which is of importance in the process of replication[20,63,115,118,159,165] (see below). Finally, strand 8 encodes two nonstructural proteins designated NS1 and NS2. Like M2, NS2 is a splicing product of NS1. Both proteins are abundant in the infected cell but only the NS2 protein is found in progeny viruses where it associates with the M1 protein[128,189]. NS1 regulates nuclear export of mRNA and inhibits pre-mRNA splicing[3,9,35,93,124,125]. The function of NS2 is at present not known.

Replication cycle

An influenza virus particle binds to the cells of the respiratory tract via interaction between the receptor-binding site of HA1 and a terminal sialic acid residue of a cell surface receptor followed by endocytosis (receptor-mediated endocytosis) (Figure 2). The low pH in endosomes permits the flow of ions from the endosomes to the virus interior through the M2 proteins to disrupt protein-protein interactions and to free the RNPs from the M1 protein[56,95]. In addition, it triggers a conformational change in the HA which facilitates insertion of the hydrophobic N-terminus of HA2 into the

membrane of the endosome leading to fusion between this membrane and the viral membrane and subsequent release of the RNPs in the cytoplasm of the infected cell[153,184].

RNA segment (Nucleotides)	Gene product (Amino acids)	Molecules per virus
1. (2341)	polymerase PB2 (759)	30-60
2. (2341)	polymerase PB1 (757)	30-60
3. (2233)	polymerase PA (716)	30-60
4. (1778)	haemagglutinin HA (566)	500
5. (1565)	nucleocapsid protein NP (498)	1000
6. (1413)	neuraminidase NA (454)	100
7. (1027)	matrix protein M1 (252)	3000
	matrix protein M2 (97)	20-60
8. (890)	non structural protein NS1 (230)	-
	non structural protein NS2 (121)	130-200

TABLE 1. Influenza virus A gene segments and encoded proteins.

During influenza virus infection, a dramatic switch from cellular to viral protein synthesis occurs[82,87,151,152]. The RNPs migrate to the host cell nucleus and their associated polymerase proteins begin primary transcription of mRNAs[52]. The synthesis of viral mRNA requires initiation by capped host cell primers which are generated from host cell mRNAs by a viral cap-dependent endonuclease[15,77,119,120]. After primary transcription, complementary RNAs (cRNAs) are transcribed from all the viral RNAs (vRNAs) at equimolar amounts followed by selective transcription of cRNAs to vRNAs with the NP and NS vRNAs being the most abundant. Newly synthesized vRNAs then function as templates for secondary transcription of viral mRNAs. Since synthesis of vRNAs, mRNAs and viral proteins are coupled in the early phase, the NP and NS mRNAs and proteins are synthesized early[52,147,154]. NP is synthesized early because it is needed for cRNA and vRNA synthesis while NS1 is synthesized early because it regulates nuclear export of mRNA and inhibits pre-mRNA splicing[3,9,35,93,124,125,148]. During the late phase, synthesis of vRNAs, mRNAs and proteins is no longer coupled. Viral proteins are synthesized at high rates during the late phase, especially M1 and HA which are poorly synthesized in the early phase. Synthesis of M1 is delayed since it stops

transcription of vRNA into mRNA and mediates transport of vRNA (enclosed in RNPs) from the nucleus to the cytoplasm[96,194].

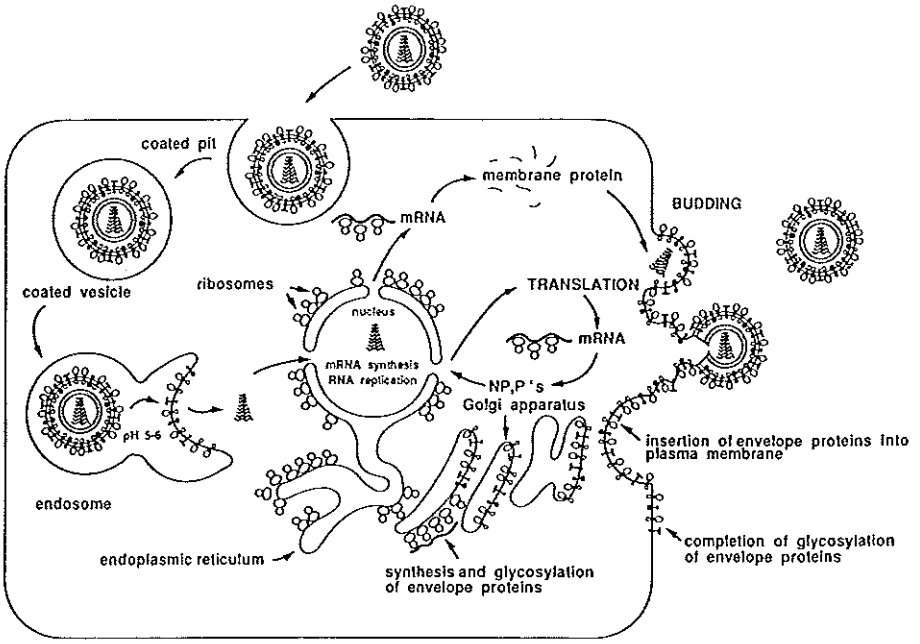

Figure 2. Schematic representation of replication cycle of influenza viruses. (Reproduced with permission from *Fields Virology*, Third Edition, 1996, Lippincott Williams and Wilkins, Philadelphia)

Newly synthesized NP, PB2, PB1 and PA, which contain karyophilic signals, migrate to the nucleus where new RNPs are assembled which associate with M1 protein to facilitate transport out of the nucleus to the cytoplasm[96]. Here, they become encased in a shell of M1 proteins. The surface glycoproteins HA and NA and the M2 protein, after passaging the ER and Golgi apparatus for modification and folding, are transported to the apical surface of cells where they are expressed at the cell surface. Progeny viruses are assembled at the apical surface and bud outward through the cell membrane[132]. Release of progeny viruses away from the infected cell is facilitated by NA[24]. New viruses then can infect other cells or being transmitted to another individual.

Host range and interspecies transmission

Influenza virus B and C are almost exclusively isolated from humans. Influenza virus C has also been isolated from pigs and, recently, influenza virus B has been isolated from seals[48,74,113]. In contrast, influenza A viruses infect a wide range of mammalian species including humans, pigs, horses and aquatic mammals[29]. Of the HA subtypes, H1, H2 and H3 (and incidently H5 and H9, see below) viruses are found in humans, H1 and H3 in pigs and H3 and H7 in horses. In contrast, all known HA subtypes are found in aquatic birds, most notably ducks, which are considered a natural reservoir of influenza A viruses from which all influenza A viruses originate[58-60,177]. This means that influenza A viruses can cross species barriers and can be transmitted from one species to another, either directly, or indirectly via an intermediate host.

Examples of direct transmission from the aquatic reservoir to pigs, horses, mink, domestic poultry and aquatic mammals which caused infections varying in severity from asymptomatic to major disease outbreaks have been described[49,50,61,75,141,176,177].

Transmission of influenza A viruses from pigs to humans occurs relatively frequently. While most of these transmissions do not cause disease in humans, exceptions have been reported[28,137,163,179,180]. In addition, it is not unlikely that pigs played a role in the emergence of viruses causing the pandemics of the 20th century (see below). Pigs are supposed to play an important role in interspecies transmission as they can act as an intermediate host between the avian virus reservoir and humans[72,144,178]. Since they can be infected with influenza A viruses of avian and human origin they also may create avian-human reassortant viruses with pandemic potential (see below).

Occasionally, influenza A viruses of avian origin are directly transmitted to humans[78,193]. A recent example of direct transmission from chickens to humans is the H5N1 virus that was isolated in 1997 in Hong Kong from humans[21,69,158]. The virus that was isolated closely resembled a H5N1 virus that had been shown responsible for an outbreak in chicken farms where it caused high mortality. Eighteen humans were shown to be infected of which 6 died. In addition, two cases have been reported of infection of humans with a H9N2 virus of avian origin[116].

The receptor specificity of HA, which differs between influenza A viruses, is considered to be an important determinant in host range restriction although a putative role of the NP has also been suggested[40,133,134,142].

However, also other influenza virus proteins and/or other yet unknown factors may play a role in host range restriction.

Epidemics and pandemics

Both influenza A and B viruses continuous undergo gradual changes of their surface glycoproteins HA and NA which is caused by the accumulation of point mutations, a process referred to as antigenic drift. In addition, influenza A viruses occasionally undergo more dramatic changes of their surface glycoproteins by replacing one or both for completely new ones, a process referred to as antigenic shift. Both processes, antigenic shift being the most dramatic, allow influenza viruses to evade pre-existing immunity in the human population. While antigenic drift is the mechanism behind recurrent influenza epidemics, antigenic shift has been the cause of three pandemics recorded the last century.

In 1918, the 'Spanish flu', estimated to cause 40-50 million deaths worldwide, resulted from the introduction of a novel influenza virus, H1N1, in the human population. This virus most probably was transmitted from pigs to humans. Sequencing of parts of four gene segments of an influenza virus isolated from a lung sample of a soldier who died in 1918 revealed that this virus was genetically similar to swine influenza viruses[160]. In 1957, the H1N1 virus was replaced by a H2N2 virus ('Asian flu') which in turn was replaced by a H3N2 virus ('Hong Kong flu') in 1968. The viruses causing these pandemics, like the one of 1918, most likely originated from pigs.

Since pigs are susceptible to both avian and human influenza A viruses and because the genome of influenza viruses is segmented, influenza virus gene segments may be exchanged upon dual infection of pigs with viruses of avian and human origin, a process referred to as genetic reassortment. If pigs become infected with avian and human influenza A viruses of different subtypes, then genetic reassortment may create a new (reassortant) influenza virus that has surface glycoproteins of avian origin against which limited or no immunity exists in the human population (antigenic shift). If such a virus is subsequently transmitted from pigs to humans and spreads in the human population it may initiate a pandemic. Thus, pigs have been implicated as 'mixing vessels' for the generation of pandemic influenza A viruses[143,144]. It is now generally believed that the pandemics of 1957 and 1968 resulted from avian-human reassortant viruses transmitted from pigs[71,140,174]. The

H2N2 influenza virus of 1957 acquired the HA, NA and PB1 gene segment from an avian virus and kept the other five gene segments from the strain circulating in humans at that time, while in the H3N2 virus of 1968 the HA and PB1 gene segments were of avian origin and the remaining six gene segments derived from the virus circulating in humans[71] (Figure 3).

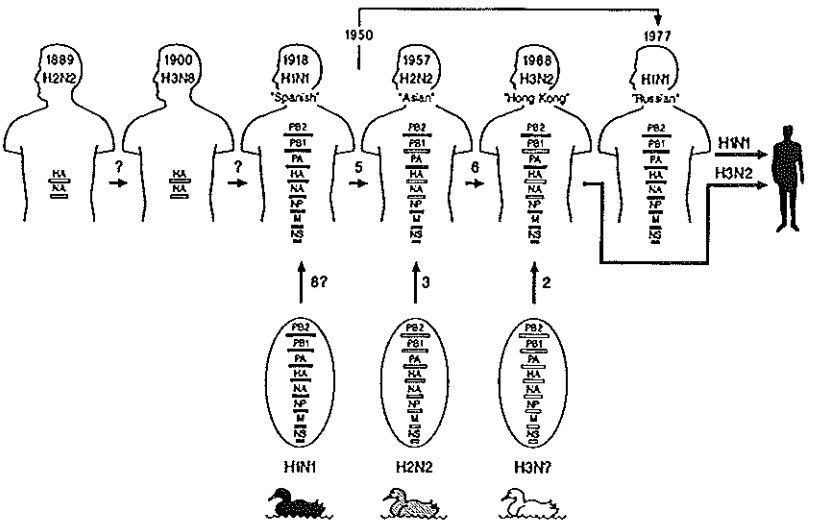

Figure 3. Origin of pandemic influenza A viruses.
(Reproduced with permission from *Fields Virology*, Third Edition, 1996, Lippincott Williams and Wilkins, Philadelphia)

In 1977, H1N1 viruses were re-introduced in the human population. The origin of this virus remains a mystery. Since this virus did not differ significantly from influenza A H1N1 viruses isolated in 1950, it most likely represents an escape from a laboratory[109]. Currently, both H1N1 and H3N2 influenza A viruses and influenza virus B circulate in the human population. In contrast to pandemics, which are unpredictable, epidemics occur virtually every year in the winter months (October to April in the northern hemisphere and in May to September in the southern hemisphere). Epidemics are less common in tropical countries.

Pathogenesis

Influenza virus is transmitted from one individual to another by aerosols or direct contact. Infection starts in the tracheobronchial epithelium and then spreads; virus replication may occur throughout the entire respiratory tract but is usually confined to the upper respiratory tract. Infection can be asymptomatic or accompanied with clinical symptoms typical of influenza like malaise, fever, chills, headache, dizziness, myalgia, arthralgia, lassitude, anorexia, rhinorrhea, sneezing, cough and sore throat. Symptoms appear within few days and usually persist for 3-4 days, but cough, lassitude and malaise may persist for 1-2 weeks. The clinical outcome may vary and is influenced by factors including prior influenza virus infections, intrinsic properties of the virus and health status. With regard to the latter, influenza risk groups are recognized which include people aged 65 or older, diabetics, people with chronic heart or lung diseases (e.g. asthma, cystic fibrosis) and people suffering from renal failure, infection with staphylococcus or a malfunction of the immune system (e.g. transplant recipients, HIV-infected patients). Influenza may be accompanied with complications which usually are respiratory (e.g. primary viral or secondary bacterial pneumonia) but may also involve other organ systems. Complications are more common in risk groups and may result in death. Because influenza-associated morbidity and mortality are highest among people of risk groups, they are recommended to receive annual vaccination against influenza. In general, influenza virus A and B infection cannot be distinguished from each other on the basis of clinical symptoms while influenza virus C infection usually causes less severe symptomatic illness.

In other mammals, influenza viruses also replicate in cells lining the respiratory tract, causing localized infection, and symptoms of infection can be compared to those in humans. In contrast, most avian influenza viruses are non-pathogenic to birds. Exceptions are H5 and H7 viruses which can be highly pathogenic to domestic poultry (chickens and turkeys). Infection with these viruses may be systemic, involving all organ systems rather than the respiratory tract only, and often has a lethal outcome. In ducks, in which influenza viruses preferentially replicate in cells lining the intestinal tract, infection is usually asymptomatic[175].

Immune responses

Both non-specific and specific humoral and cellular immune responses are induced following influenza virus infection[1]. Usually these responses are sufficient to clear infection and establish long-lived immunity against the infecting virus[36,38,41,111,122]. However, as discussed above, influenza viruses continuously change in the process of antigenic drift and, concomittantly, new virus variants emerge. While immunity against a particular influenza virus usually will protect against re-infection with the same or a closely related virus, it will be less protective or absent when a more distant virus is encountered. In other words, immunity to influenza wanes over time and largely depends on the extent of antigenic drift of the virus. In the case of antigenic shift, a new influenza virus A subtype is introduced in the human population to which little, if any, immunity exist. This has become clear during influenza pandemics of the last century. The fact that hetero-subtypic protective immunity appears weak in humans indicates that immunity against influenza is primarily mediated by responses to the surface glycoproteins and it is generally believed that protection against influenza is mediated by antibodies directed against these surface glycoproteins. In contrast, clearance of infection is supposed to be largely mediated by cellular responses[1,7,26,149,156].

During influenza virus infection, antibodies are most prominently raised against the HA, NA, NP and M proteins[27,122]. Neutralizing antibodies against the HA and NA of influenza virus are associated with resistance to infection and/or illness, whereas antibodies to NP and M proteins are not[4,6,23,32,103,157]. Functionally, antibodies against HA are believed to prevent virus from attaching to cells and thus inhibit initiation of infection. Antibodies against NA are believed to inhibit NA enzymatic activity and to cross-link viruses budding from cells thereby inhibiting virus release. Following primary infection, local (mucosal) antibodies of the IgA, IgG and IgM isotype directed against HA can be detected in nasal washes[5,26,105,106,136]. Since secretory IgA antibodies react with the virus at the site of entry, these antibodies are believed to be an important first line of defence against infection and have been shown to confer protection against infection[22,23,91,126,127]. Serum antibodies of the same isotypes are also detected in primary infections, while IgA en IgG antibodies predominate in subsequent infections[17,19,106,188]. Levels of serum antibodies correlate with resistance to illness[23,26,103].

Influenza virus-specific T helper (Th) cells are induced upon influenza virus infection. Th cells contribute to clear virus infection primarily by stimulating antibody production and proliferation of cytotoxic T lymphocytes (CTL) and by production of cytokines[79,81,191]. It has been reported that Th cells specific for NP or M proteins can stimulate B-cells specific for HA[79,80,138]. Although a subset of CD4+ cells has been shown to have a cytotoxic effect on cells infected with influenza virus, adoptive transfer experiments in mice demonstrated that Th cells by their own are unable to clear virus infection[139,164,191].

CTL directed against influenza virus antigens can be isolated from previously infected individuals[30,68]. CTL contribute to clearance of virus from the respiratory tract and accelerate recovery from infection[10,97,100]. CTL epitopes are less abundant when compared to B and Th cell epitopes and are mainly concentrated in the more conserved influenza virus proteins, like the NP[11,44,99,101,166,190]. CTL directed against conserved influenza virus A antigens usually are cross-reactive and will lyse cells infected with any subtype while CTL directed against the surface glycoproteins are largely subtype-specific[34,166,190,191,195]. The former have been shown to confer protection against heterosubtypic influenza A viruses[90,145,166,190]. In addition, numerous experiments with vaccinated or infected mice, including adoptive transfer experiments, have demonstrated that CTL are capable of clearing virus infection and do protect against challenge infection in the absence of B and Th cells[2,13,33,45,92,102,161,167,186,187]. However, despite the fact that heterosubtypic CTL are induced following infection, they do not confer long-lived immunity against influenza virus A infections in humans[98]. It has been suggested that this may be due to the limited life-span of memory CTL. The discrepancy with the animal studies may reflect the relatively short duration of these studies, i.e. the time span between vaccination and challenge infection.

Non-specific immune responses induced following influenza virus infection include the production of cytokines, most notably interferons, and the activation of natural killer (NK) cells[31,46,57,89,104,129]. NK cells may limit replication and spread of the virus, but NK cells on their own are not sufficient for viral clearance. Interferons are produced early in infection and may reduce viral spread by inducing an antiviral state in host cells and by activation of CTL and NK cells, thereby contributing to recovery from infection.

Vaccines and antivirals

Influenza vaccines currently in use consist of inactivated whole or disrupted (split) virus or purified preparations containing the surface glycoproteins HA and NA (Table 2). These vaccines are trivalent, i.e. they contain representatives of both influenza A viruses (H1N1 and H3N2) and influenza virus B. The composition of the vaccine is reconsidered each year by the WHO which is advised by influenza centers all over the world which monitor circulating viruses. Representative viruses are selected as vaccine strains and propagated in the allantoic cavity of embryonated chicken eggs. Usually, 'high-growth' reassortant viruses are used as vaccine strains, unless the selected virus itself has a high-growth phenotype. High-growth reassortant viruses are obtained after dual infection of embryonated chicken eggs with a vaccine strain and a high growth virus (a virus adapted to replicate to high titers in eggs). Reassortant viruses containing the surface glycoproteins HA and NA of the vaccine strain which inherited the high-growth phenotype (by exchanging some or all of the remaining gene segments) of the high-growth virus are selected for vaccine production. The currently used vaccines usually confer protection against infection and/or illness and are recommended for people at risk (see above).

Antivirals can be used prophylactically or after infection has been established and reduce influenza-associated illness. The antiviral amantadine and its analogue rimantadine are effective against all subtypes of influenza A viruses, but not against influenza B viruses[170]. Both agents inhibit virus replication by blocking the M2 ion channel, thereby preventing transport of RNPs to the nucleus and, consequently, RNA transcription and replication[20,53,173]. Analogues of the neuraminidase substrate N-acetylneuraminic acid (sialic acid) constitute a second group of antivirals. Two neuraminidase inhibitors, zanamivir and oseltamivir have been approved for human use and both drugs have been shown effective against both influenza virus A and B[8,18,47,51,54,73,88,172]. Although antivirals may be used prophylactically or therapeutically for individuals that have not been vaccinated or in case vaccination was inadequate, vaccination will remain the major means to control influenza[112].

Vaccine	Mode of action	Antiviral	Mode of action
Whole virus (in-activated)	Primarily induction of serum antibody responses	Amantadine	Inhibiton of M2 ion channel
Split virus	Same as above	Rimantadine	Same as above
Purified proteins	Same as above	Zanamivir	Neuraminidase inhibitor
MF59[a]	Same as above	Oseltamivir	Same as above
Live attenuated[b]	Induction of serum and secretory antibody and cellular responses		
Liposomes/ Virosomes[a]	Induction of serum antibody and cellular responses		
ISCOM[c]	Same as above		
Recombinant vaccinia[d]	Same as above		
DNA[d]	Same as above		

TABLE 2. Vaccines and antivirals.
[a]Licensed in some countries; [b]Not (yet) licensed; [c]Phase III trials, licensed for use in horses; [d]Not expected to become licensed soon.

New developments

An alternative to the currently used vaccines are the live-attenuated virus vaccines, e.g. cold-adapted virus vaccines. One advantage of these vaccines is that, when administered intranasally, they mimic natural infection and may thus induce local immune responses (involving secretory IgA) as well as CTL responses in addition to serum antibody responses. Thus, in contrast to the current vaccines which mainly aim at the induction of virus-neutralizing serum antibodies, live-attenuated vaccines challenge the entire immune system. Another (economical) advantage would be that more doses of vaccine can be obtained per embryonated chicken egg. Live-attenuated virus vaccines have already been extensively tested in humans but are still not licensed. A disadvantage of these vaccines is that it can not be excluded that they lose their attenuated phenotype and reverse to wild type virus. Other vaccines that have been, and still are, evaluated in humans include liposome/virosome and ISCOM (Immune Stimulating COMplex)-based vaccines[42,43,117,130,131,169]. In liposome/virosome based vaccines, the surface glycoproteins HA and NA are associated with phospholipids, e.g.

phosphatidylcholine, to form vesicles, while in ISCOMs the surface glycoproteins are incorporated into a structure composed of phospholipids, cholesterol and the adjuvant Quil A. Like live-attenuated virus vaccines, these vaccines induce cellular immune responses in addition to humoral responses and have been shown to confer protective immunity in animal models. Virosome-based influenza vaccines have been licensed for use in humans in some countries and ISCOM-based vaccines are currently being tested in clinical phase III trials. One other adjuvant that has been tested and is already licensed in some countries is MF59, consisting of squaleen, polysorbate 80 and sorbitan trioleaat. Vaccines which thus far have been tested experimentally in animal models only, include recombinant vaccina virus-based vaccines and DNA vaccines. Although promising results have been obtained with these vaccines they are not expected to become available for use in humans within the near future. In addition to the development and analysis of novel influenza vaccines using different adjuvant formulations and antigen presentation forms, also attempts are made to improve the current influenza vaccine production procedure. Since the use of embryonated chicken eggs has major disadvantages, the lack of flexibility (and hence the necessity of long-term planning) being the most important one, the use of continuous cell lines as a source to propagate influenza virus is being evaluated.

Laboratory diagnosis of influenza

There are numerous tests available for the diagnosis of influenza virus infection. These tests either detect viral antigens, viral nucleic acids or influenza virus induced antibodies. For rapid diagnosis of influenza virus infection, direct immunofluorescense (DIF) on patient material (nasal wash samples) is usually performed. DIF is usually based on the detection of influenza virus NP in infected cells. Another rapid method is the detection of influenza virus RNA by RT-PCR of a selected gene segment. While rapid diagnosis is an advantage of these tests, which in a hospital setting is of major importance, they usually only provide information about the type of influenza virus (A or B) but not about influenza virus A subtypes and antigenic properties of the virus, which is of importance for influenza surveillance. For this purpose, influenza virus is isolated after passaging patient material in embryonated chicken eggs or susceptible cells (e.g. MK or MDCK cells). The isolated virus then is antigenically characterized in

haemagglutination inhibition (HI) assays using a panel of sera raised against a series of antigenically distinct influenza viruses. The HI assay can be used to discriminate between different subtypes of influenza A viruses and antigenic variants of influenza virus A subtypes and influenza B viruses. In addition to the HI assay, also virus neutralization (VN) assays can be used to discriminate between antigenically distinct viruses. Serological assays for detection of antibodies or antibody titer rises induced after influenza virus infection include the complement fixation (CF) assay, the HI assay and enzyme-linked immunosorbent assays (ELISA). Since most influenza virus-infected patients have encountered influenza virus in the past, the HI assay requires paired serum samples to be able to measure antibody responses, i.e. titer rises, which may be difficult to obtain. However, the HI assay is particularly important in clinical trials for the evaluation of antibody responses induced upon vaccination where, in contrast to infection, paired serum samples can be obtained easily.

Aims and outline of this thesis

The studies described in this thesis have multiple objectives: development of ELISAs for measuring NP-specific antibody responses; evaluation of the usefulness of a continuous cell line for generation and propagation of reassortant influenza A viruses; evaluation of an ISCOM-based vaccine with respect to activation of CTL; and studying MHC class I processing and presentation of influenza virus antigen.

After summarizing the most important aspects of influenza viruses in this chapter, chapter 2 discusses the advantages and disadvantages of the use of a continuous cell line for the generation of reassortant influenza A viruses for vaccine production as a possible alternative to the current use of embryonated chicken eggs. Reassortant influenza A H1N1 and H3N2 viruses were generated in MDCK-SF1 cells, a continuous cell line adapted to grow without serum, and their genetic make-up as well as their growth properties in this cell line were studied. While chapter 2 focusses on improvement of the current vaccine production procedure, chapter 3 deals with some aspects of ISCOM, an antigen presentation form that may be the basis for future influenza vaccines. The potential of ISCOM to facilitate MHC class I processing and presentation of the influenza virus A matrix M1 protein to specific CTL was evaluated *in vitro*. The necessity of a membrane-spanning region and incorporation of the M1 protein into the

ISCOM structure was studied. The study of MHC class I processing of influenza virus antigen for presentation to CTL is continued in chapters 4 and 5. Chapter 4 describes MHC class I presentation of recombinant NP-derived peptides by B-lymphoblastoid cell lines (B-LCL). Possible pathways for processing of this exogenous antigen are discussed. Chapter 5 describes the consequences of mutations in CTL epitopes with respect to MHC class I presentation and recognition of influenza virus-infected cells by specific CTL. The introduction of mutations in CTL epitopes as a possible mechanism to escape CTL-mediated immunity is discussed. In chapter 6, the development and evaluation of ELISAs for measuring influenza virus A or B-induced NP-specific antibodies of the IgA and IgG isotypes in influenza virus-infected patients is described. Finally, chapter 7 summarizes and discusses the results of the studies described in this thesis.

REFERENCES

[01] Ada, G.L. and Jones, P.D. 1986. The immune response to influenza infection. *Curr.Top.Microbiol.Immunol.* 128:1-54.

[02] Allen, W., Tabi, Z., Cleary, A. and Doherty, P.C. 1990. Cellular events in the lymph node and lung of mice with influenza: consequences of depleting $CD4^+$ cells. *J.Immunol.* 144:3980-3986.

[03] Alonso-Caplen, F.V., Nemeroff, M.E., Qiu, Y. and Krug, R.M. 1992. Nucleocytoplasmic transport: the influenza virus NS1 protein regulates the transport of spliced NS2 mRNA and its precursor NS1 mRNA. *Genes Dev.* 6:255-267.

[04] Andrew, M.E. and Coupar, B.E.H. 1988. Efficacy of influenza haemagglutinin and nucleoprotein in protection: analysis using vaccinia virus recombinants. *Scand.J.Immunol.* 28:81-85.

[05] Artenstein, M.S., Bellanti, J.A. and Buescher, E.L. 1964. Identification of the antiviral substances in nasal secretions. *Proc.Soc.Exp.Biol.Med.* 117:558-564.

[06] Askonas, B.A., McMichael, A.J. and Webster, R.G. 1982. The immune response to influenza viruses and the problem of protection against infection. In: Beare, A.S. (ed.) *Basic and applied influenza research*, pp. 159-188. CRC Press, Boca Raton, Florida.

[07] Askonas, B.A. 1989. Influenza virus, a challenge to immunologists. *Res.Immunol.* 140:627-634.

[08] Bardsley-Elliott, A. and Noble, S. 1999. Oseltamivir. *Drugs* 58:851-860.

[09] Beaton, A.R. and Krug, R.M. 1986. Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5' capped end. *Proc.Natl.Acad.Sci.USA* 83:6282-6286.

[10] Bender, B.S., Croghan, T., Zhang, L. and Small Jr., P.A. 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. *J.Exp.Med.* 175:1143-1145.

[11] Bennink, J.R. and Yewdell, J.W. 1990. Recombinant vaccinia viruses as vectors for studying T lymphocyte specificity and function. *Curr.Top.Microbiol.Immunol.* 163:153-183.

[12] Blaas, D., Patzelt, E. and Kuechler, E. 1982. Cap-recognizing protein of influenza virus. *Virology* 116:339-348.

[13] Bodmer, H., Obert, G., Chan, S., Benoist, C. and Mathis, D. 1993. Environmental modulation of the autonomy of cytotoxic T lymphocytes. *Eur.J.Immunol.* 23:1649-1654.

[14] Booy, F.P., Ruigrok, R.W.H. and van Bruggen, E.F.J. 1985. Electron microscopy of influenza virus. A comparison of negatively stained and ice-embedded particles. *J.Mol.Biol.* 184:667-676.

[15] Bouloy, M., Plotch, S.J. and Krug, R.M. 1978. Globin mRNAs are primers for the transcription of influenza viral RNA in vitro. *Proc.Natl.Acad.Sci.USA* 75:4886-4890.

[16] Braam, J., Ulmanen, I. and Krug, R.M. 1983. Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. *Cell* 34:609-618.

[17] Brown, T.A., Murphy, b.R., Radl, J., Haaijman, J.J. and Mestecky, J. 1985. Subclass distribution and molecular from of immunoglobulin A hemagglutinin antibodies in sera and nasal secretions after experimental secondary infection with influenza A virus in humans. *J.Clin.Microbiol.* 22:259-264.

[18] Calfee, D.P. and Hayden, F.G. 1998. New approaches to influenza chemotherapy. Neuraminidase inhibitors. *Drugs* 56:537-553

[19] Callard, R.E. 1979. Specific *in vitro* antibody response to influenza virus by human blood lymphocytes. *Nature* 282:734-736.

[20] Chizhmakov, I.V., Geraghty, F.M., Ogden, D.C., Hayhurst, A., Antoniou, M. and Hay, A.J. 1996. Selective proton permeability and pH regulation of the influenza virus M2 channel expressed in mouse erythroleukaemia cells. *J.Physiol.* 494:329-336.

[21] Claas, E.C.J., Osterhaus, A.D.M.E., van Beek, R., de Jong, J.C., Rimmelzwaan, G.F., Senne, D.A., Krauss, S., Shortridge, K.F. and Webster, R.G. 1998. Human influenza A (H5N1) virus related to a highly pathogenic avian influenza virus. *Lancet* 351:472-477.

[22] Clements, M.L., O'Donnell, S., Levine, M.M., Chanock, R.M. and Murphy, B.R. 1983. Dose response of A/Alaska/6/77 (H3N2) cold-adapted reassortant vaccine virus in adult volunteers: role of local antibody in resistance to infection with vaccine virus. *Infect.Immun.* 40:1044-1051.

[23] Clements, M.L., Betts, R.F., Tierney, E.L. and Murphy, B.R. 1986. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. *J.Clin.Microbiol.* 24:157-160.

[24] Colman, P.M. 1989. Neuraminidase: Enzyme and antigen. In: Krug, R.M. (ed.) *The influenza viruses*, pp. 175-218. Plenum Press, New York.

[25] Compans, R.W., Content, J. and Duesberg, P. 1972. Structure of the ribonucleoprotein of influenza virus. *Virology* 10:795-800.

[26] Couch, R.B. and Kasel, J.A. 1983. Immunity to influenza in man. *Annu.Rev.Microbiol.* 37:529-549.

[27] Cretescu, L., Beare, A.S. and Schild, G.C. 1978. Formation of antibody to matrix protein in experimental human influenza A virus infections. *Infect.Immun.* 22:322-327.

[28] Dasco, C.C., Couch, R.B., Six, H.R., Young, J.F., Quarles, J.M. and Kasel, J.A. 1984. Sporadic occurrence of zoonotic swine influenza virus infections. *J.Clin.Microbiol.* 20:833-835.

[29] Easterday, B.C. 1975. Animal influenza. In: Kilbourne, E.D. (ed.) *The influenza viruses and influenza*, pp.449-481. Academic Press, Orlando.

[30] Ennis, F.A., Rook, A.H., Qi, Y.H, Schild, G.C., Riley, D., Pratt, R. and Potter, C.W. 1981. HLA-restricted virus-specific cytotoxic T-lymphocyte responses to live and inactivated influenza vaccines. *Lancet* 2:887-891.

[31] Ennis, F.A., Meagar, A., Beare, A.S., Qi, Y.H.K., Riley, D., Schwarz, G., Schild, G.C. and Rook, A.H. 1981. Interferon production and increased natural killer-cell activity in influenza infections in man. *Lancet* 2:891-893.

[32] Epstein, S.L., Misplon, J.A., Laswon, C.M., Subbarao., E.K., Connors, M. and Murphy, B.R. 1993. ß2-microglobulin-deficient mice can be protected against influenza A infection by vaccination with vaccinia-influenza recombinants expressing hemagglutinin and neuraminidase. *J.Immunol.* 150:5484-5493.

[33] Epstein, S.L., Lo, C.Y., Misplon, J.A. and Bennink, J.R. 1998. Mechanisms of protective immunity against influenza virus infection in mice without antibodies. *J.Immunol.* 160:322-327.

[34] Fleischer, B., Becht, H. and Rott, R. 1985. Recognition of viral antigens by human influenza A virus-specific T lymphocyte clones. *J.Immunol.* 135:2800-2804.

[35] Fortes, P., Beloso, A. and Ortin, J. 1994. Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. *EMBO J.* 13:704-712.

[36] Fox, J.P., Cooney, M.K., Hall, C.E. and Foy, H.M. 1982. Influenza virus infections in Seattle families, 1975-1979. II. Pattern of infection in invaded households and relation of age and prior antibody to occurrence of infection and related illness. *Am.J.Epidemiol.* 116:228-242.

[37] Francis, T. 1940. A new type of virus from epidemic influenza. *Science* 92:405-406.

[38] Frank, A.L., Taber, L.H. and Wells, J.M. 1983. Individuals infected with two subtypes of influenza A virus in the same season. *J.Infect.Dis.* 147:120-124.

[39] Fujiyoshi, Y., Kume, N.P., Sakata, K. and Sato, S.B. 1994. Fine structure of influenza A virus observed by electron cryo-microscopy. *EMBO J.* 13:318-326.

[40] Gammelin, M., Altmuller, A., Reinhardt, U., Mandler, J., Harley, V.R., Hudson, P.J., Fitch, W.M. and Scholtissek, C. 1990. Phylogenetic analysis of nucleoprotein suggests that human influenza A viruses emerged from a 19th century avian ancestor. *Mol.Biol.Evol.* 7:194-200.

[41] Gill, P.W. and Murphy, A.M. 1985. Naturally aquired immunity to influenza type A. Lessons from two coexisting subtypes. *Med.J.Aust.* 142:94-98.

[42] Glück, R. 1995. Liposomal presentation of antigens for human vaccines. In: Powell, M.F. and Newman, M.J. (eds.) *Vaccine Design: The Subunit and Adjuvant Approach*, pp.543-558. Plenum Press, New York.

[43] Glück, R. and Wegmann, A. 1997. Virosomes, a new liposome-like vaccine delivery system. In: Gander, B., Merkle, H.P. and Corradin, G. (eds.) *Antigen Delivery Systems; Immunological and Technological Issues*, pp. 101-122. Harwood Academic Publishers, Amsterdam.

[44] Gotch, F., McMichael, A., Smith, G. and Moss, B. 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. *J.Exp.Med.* 165:408-416.

[45] Graham, M.B. and Braciale, T.J. 1997. Resistance and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *J.Exp.Med.* 186:2063-2068.

[46] Green, J.A., Charette, R.P., Yeh, T.J. and Smith, C.B.. 1982. Presence of interferon in acute- and convalescent-phase sera of humans with influenza or an influenza-like illness of undetermined etiology. *J.Infect.Dis.* 145:837-841.

[47] Gubareva, L.V., Kaiser, L. and Hayden, F.G. 2000. Influenza virus neuraminidase inhibitors. *Lancet* 355:827-835.

[48] Guo, Y.J., Jin, F.G., Wang, P., Wang, M. and Zhu, J.M. 1983. Isolation of influenza C virus from pigs and experimental infection of pigs with influenza C virus. *J.Gen.Virol.* 64:177-182.

[49] Guo, Y.J, Wang, M., Kawaoka, Y., Gorman, O., Ito, T., Saito, T. and Webster, R.G. 1992. Characterization of a new avian-like influenza A virus from horses in china. *Virology* 188:245-255.

[50] Halvorson, D., Karunakaran, D., Senne, D., Kelleher, C., Bailey, C., Abraham, A., Hinshaw, V. and Newman, J. 1983. Epizootiology of avian influenza-simultaneous monitoring of sentinel ducks and turkeys in Minnesota. *Avian Dis.* 7:77-85.

[51] Hart, G. and Bethell, R. 1995. 2,3-Didehydro-2,4-dideoxy-4-guanidino-N-acetyl-D-neuraminic acid (4-guanidino-Neu5Ac2en) is a slow-binding inhibitor of sialidase from both influenza A virus and influenza B virus. *Biochem.Mol.Biol.Int.* 36:695-703.

[52] Hay, A.J., Lomniczi, B., Bellamy, A.R. and Skehel, J.J. 1977. Transcription of the influenza virus genome. *Virology* 83:337-355.

[53] Hay, A.J. 1992. The action of adamantanamines against influenza A viruses: inhibition of the M2 ion channel protein. *Semin.Virol.* 3:21-30.

[54] Hayden, F.G., Osterhaus, A.D.M.E., Treanor, J.J., Fleming, D.M., Aoiki, F.Y., Nicholson, K.G., Bohnen, A.M., Hisrt, H.M., Keene, O. and Wightman, K. 1997. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenzavirus infections. GG167 influenza study group. *N.Engl.J.Med.* 13:874-880.

[55] Heggeness, M.H., Smith, P.R., Ulmanen, I., Krug, R.M. and Choppin, P.W. 1982. Sudies on the helical nucleocapsid of influenza virus. *Virology* 118:466-470.

[56] Helenius, A. 1992. Unpacking the incoming influenza virus. *Cell* 69:577-578.

[57] Hill, D.A., Baron, S., Perkins, J.C., Worthington, M., Van Kirk, J.E., Mills, J., Kapikian, A.Z. and Chanock, R.M. 1972. Evaluation of an interferon inducer in viral respiratory disease. *JAMA* 219:1179-1184.

[58] Hinshaw, V.S., Webster, R.G., Bean, W.J. and Sriram, G. 1981. The ecology of influenza viruses in ducks and analysis of influenza viruses with monoclonal antibodies. *Comp.Immunol.Microbiol.Infect.Dis.* 3:155-164.

[59] Hinshaw, V.S., Webster, R.G. and Rodriguez, R.J. 1981. Influenza A viruses: combinations of hemagglutinin and neuraminidase subtypes isolated from animals and other sources. *Arch.Virol.* 67:191-201.

[60] Hinshaw, V.S. and Webster, R.G. 1982. The natural history of influenza A viruses. In: Beard, A.S. (ed.) *Basic and Applied Influenza Research*, pp.79-104. CRC press, Boca Raton, Florida.

[61] Hinshaw, V.S., Bean, W.J., Geraci, J., Fiorelli, P., Early, G. and Webster, R.G. 1986. Characterization of two influenza A viruses from a pilot whale. *J.Virol.* 58:655-656.

[62] Hirsch, A. 1883. In: *Handbook of Geographical and Historical Pathology.* New Sydenham Society, London.

[63] Holsinger, L.J. and Lamb, R.A. 1991. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. *Virology* 183:32-43.

[64] Horne, R.W., Waterson, A.P., Wildy, P. and Franham, A.E. 1960. The structure and composition of the myxoviruses. I. Electron microscope studies of the structure of myxovirus particles by negative staining techniques. *Virology* 11:79-98.

[65] Hoyle, L., Horne, R.W. and Waterson, A.P. 1961. The structure and composition of the myxoviruses. II. Components released from the influenza virus particle by ether. *Virology* 13:448-459.

[66] Inglis, S.C., Carroll, A.R., Lamb, R.A. and Mahy, B.W. 1976. Polypeptides specified by the influenza virus genome. I. Evidence for eight distinct gene products specified by fowl plague virus. *Virology* 74:489-503.

[67] Inglis, S.C. and Brown, C.M. 1981. Spliced and unspliced RNAs encoded by virus RNA segment 7 of influenza virus. *Nucleic Acids Res.* 9:2727-2740.

[68] Jameson, J., Cruz, J. and Ennis, F.A. 1998. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. *J.Virol.* 72:8682-8689.

[69] de Jong, J.C., Claas, E.C.J., Osterhaus, A.D.M.E., Webster, R.G. and Lim, W.L. 1997. A pandemic warning. *Nature* 389:554.

[70] Kates, M., Allsion, A.C., Tyrell, D.A. and James, A.T. 1962. Origin of lipids in influenza virus. *Cold Spring Harbor Symp.Quant.Biol.* 27:293-301.

[71] Kawaoka, Y., Krauss, S. and Webster, R.G. 1989. Avian-to-human transmission of the PB1 gene of influenza A virus in the 1957 and 1968 pandemics. *J.Virol.* 63:4603-4608.

[72] Kawaoka, Y., Bean, W.J., Gorman, O.T. 1993. The roles of birds and pigs the generation of pandemic strains of human influenza. In: Hannoun, C., Kendal, A.P., Klenk, H.D. and Ruben, F.L. (eds.) *Options for the control of influenza II*, pp. 187-191. Elsevier Science, Amsterdam.

[73] Kim, C.U., Chen, X. and Mendel, D.B. 1999. Neuraminidase inhibitors as anti-influenza virus agents. *Antivir.Chem.Chemother.* 10:141-154.

[74] Kimura, H., Abiko, C., Peng, G., Muraki, Y., Sugawara, K., Hongo, S., Kitame, F., Mizuta, K., Numazaki, Y., Suzuki, H. and Nakamura. 1997. Interspecies transmission of influenza C virus between humans and pigs. *Virus.res.* 48:71-79.

[75] Klingeborn, B., Englund, L., Rott, R., Juntti, N. and Rockborn, G. 1985. An avian influenza A virus killing a mammalian species-the mink. *Arch.Virol.* 86:347-351.

[76] Klumpp, K., Ruigrok, R.W.H. and Baudin, F. 1997. Roles of the influenza virus polymerase and nucleoprotein in forming a functional RNP structure. *EMBO J.* 16:1248-1257.

[77] Krug, R.M. 1981. Priming of influenza viral RNA transcription by capped heterologous RNAs. *Curr.Top.Microbiol.Immunol.* 93:125-149.

[78] Kurtz, J., Manvell, R.J. and Blanks, J. 1996. Avian influenza virus isolated from a woman with conjunctivitis. *Lancet* 348:901-902.

[79] Lamb, J.R., Wood, J.N., Hartzman, R.J. and Eckels, D.D. 1982. *In vitro* influenza virus-specific antibody production in man: Antigen-specific and HLA-restricted induction of helper activity mediated by cloned human T lymphocytes. *J.Immunol.* 129:1465-1470.

[80] Lamb, J.R., Eckels, D.D., Lake, P., Johnson, A.H., Hartzman, R.J. and Woody, J.N. 1982. Antigen-specific human T lymphocyte clones: Induction, antigen-specificity, and MHC restriction of influenza virus-immune clones. *J.Immunol.* 128:233-238.

[81] Lamb, J.R., McMichael, A.J. and Rothbarth, J.B. 1987. T-cell recognition of influenza viral antigens. *Human Immunol.* 19:79-89.

[82] Lamb, R.A. and Choppin, P.W. 1976. Synthesis of influenza virus proteins in infected cells: translation of viral polypeptides, including three P polypeptides, from RNA produced by primary transcription. *Virology* 74:504-519.

[83] Lamb, R.A., Lai, C.J. and Choppin, P.W. 1981. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: Colinear and interrupted mRNAs code for overlapping proteins. *Proc.Natl.Acad.Sci.USA* 78:4170-4174.

[84] Lamb, R.A., Zebedee, S.L. and Richardson, C.D. 1985. Influenza virus M2 protein is an integral membrane protein expressed on the infected cell-surface. *Cell* 40:627-633.

[85] Lamb, R.A. 1989. Genes and proteins of the influenza viruses. In: Krug, R.M. (ed.) *The influenza viruses*, pp. 1-87. Plenum Press, New York.

[86] Laver, W.G. and Valentine, R.C. 1969. Morphology of the isolated hemagglutinin and neuraminidase subunits of influenza virus. *Virology* 38:105-119.

[87] Lazarowitz, S.G., Compans, R.W. and Choppin, P.W. 1971. Influenza virus structural and non structural proteins in infected cells and their plasma membranes. *Virology* 46:830-843.

[88] Lew, W., Chen, X. and Kim, C.U. 2000. Discovery and development of GS4104 (oseltamivir): an orally active influenza neuraminidase inhibitor. *Curr.Med.Chem.* 7:663-672.

[89] Lewis, D.E., Gilbert, B.E. and Knight, V. 1986. Influenza virus infection induces functional alterations in peripheral blood lymphocytes. *J.Immunol.* 137:3777-3781.

[90] Liang, S., Mozdzanowska, Palladino, G. and Gerhard, W. 1994. Heterosubtypic immunity to influenza type A virus in mice. *J.Immunol.* 152:1653-1661.

[91] Liew, F.Y., Russell, S.M., Appleyard, G., Brand, C.M. and Beale, J. 1984. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. *Eur.J.Immunol.* 14:350-356.

[92] Lightman, S., Cobbold, S., Waldmann, H. and Askonas, B.A. 1987. Do L3T4$^+$ cells act as effector cells in protection against influenza virus infection. *Immunology* 62:139-144.

[93] Lu, Y., Qian, X.Y. and Krug, R.M. 1994. The influenza virus NS1 protein: A novel inhibitor of pre-mRNA splicing. *Genes Dev.* 8:1817-1828.

[94] Magill, T.P. 1940. A virus from cases of influenza-like upper respiratory infection. *Proc.Soc.Exp.Biol.* 45:162-164.

[95] Marsh, M. 1992. Keeping the viral coat on. 1992. *Curr.Biol.* 2:379-381.

[96] Martin, K. and Helenius, A. 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell* 67:117-130.

[97] McMichael, A.J., Gotch, F.M., Noble, G.R. and Beare, P.A.S. 1983. Cytotoxic T-cell immunity to influenza. *N.Engl.J.Med.* 309:13-17.

[98] McMichael, A.J., Gotch, F.M., Dongworth, D.W., Clark, A. and Potter, C.W. 1983. Declining T-cell immunity to influenza, 1977-1982. *Lancet* 2:762-764.

[99] McMichael, A.J., Mitchie, C.A, Gotch, F.M., Smith, G.L. and Moss, B. 1986. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. *J.Gen.Virol.* 67:719-726.

[100] McMichael, A.J. and Gotch, F.M. 1989. Recognition of influenza A virus by human cytotoxic T lymphocytes. *Adv.Exp.Med.Biol.* 257:109-114.

[101] McMichael, A. 1994. Cytotoxic T lymphocytes specific for influenza virus. *Curr.Top.Microbiol.Immunol.* 189:75-91.

[102] Moskophidis, D. and Kioussis, D. 1998. Contribution of virus-specific CD8+ cytotoxic T cells to virus clearance or pathological manifestations of influenza virus infection in a T cell receptor transgeneic mouse model. *J.Exp.Med.* 188:223-232.

[103] Murphy, B.R., Kasel, J.A. and Chanock, R.M. 1972. Association of serum antineuraminidase antibody with resistance to influenza in man. *N.Engl.J.Med.* 286:1329-1332.

[104] Murphy, B.R., Baron, S., Chalhub, E.G., Uhlendorf, C.P. and Chanock, R.M. 1973. Temperature-sensitive mutants of influenza virus. IV. Induction of interferon in the nasopharynx by wild-type and a temperature-sensitive recombinant virus. *J.Infect.Dis.* 128:488-493.

[105] Murphy, B.R., Nelson, D.L., Wright, P.F., Tierney, E.L., Phelan, M.A. and Chanock, R.M. 1982. Secretory and systemic immunological response in children infected with live attenuated influenza A virus vaccines. *Infect.Immun.* 36:1102-1108.

[106] Murphy, B.R. and Clements, M.L. 1989. The systemic and mucosal immune response of humans to influenza A virus. *Curr.Top.Microbiol.Immunol.* 146:107-116.

[107] Murti, K.G. and Webster, R.G. 1986. Distribution of haemagglutinin and neuraminidase on influenza virus as revealed by immunoelectron microscopy. *Virology* 149:36-43.

[108] Nakagawa, Y., Oda, K. and Nakada, S. 1996. The PB1 subunit alone can catalyse cRNA synthesis, and the PA subunit in addition to the PB1 subunit is required for viral RNA synthesis in replication of the influenza virus genome. *J.Virol.* 70:6390-6394.

[109] Nakajima, K., Desselberger, U. and Palese, P. 1978. Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950. *Nature* 274:334-339.

[110] Nermut, M.V. 1972. Further investigation on the fine structure of influenza virus. *J.Gen.Virol.* 67:4831-4841.

[111] Noble, G.R. 1982. Epidemiological and clinical aspects of influenza. In: Beare, A.S. (ed.) *Basic and applied influenza research*, pp. 11-50. CRC Press, Boca Raton, Florida.

[112] Osterhaus, A.D.M.E. and de Jong, J.C. 1999. The control of influenza: antivirals as an adjunct to vaccines. *Vaccine* 18:779-780.

[113] Osterhaus, A.D.M.E., Rimmelzwaan, G.F., Martina, B.E., Bestebroer, T.M. and Fouchier, R.A.M. 2000. Influenza B virus in seals. *Science* 288:1051-1053.

[114] Oxford, J.S. and Hockley, D.J. 1987. Orthomyxoviridae. In: Nermut, M.V. and Steven, A.C. (eds.) *Animal Virus Structure*, pp. 213-232. Elsevier, Amsterdam.

[115] Panayotov, P.P. and Schlesinger, R.W. 1992. Oligomeric organization and strain specific proteolytic modification of the virus M2 protein of influenza A H1N1 viruses. *Virology* 186:352-355.

[116] Peiris, M., Yuen, K.Y., Leung, C.W., Chan, K.H., Ip, P.L., Lai, R.W., Orr, W.K. and Shortridge, K.F. 1999. Human infection with influenza H9N2. *Lancet* 354:916-917.

[117] Pietrobon, P.J.F. 1995. Liposome design and vaccine development. In: Powell, M.F. and Newman, M.J. (eds.) *Vaccine Design: The Subunit and Adjuvant Approach*, pp.543-558. Plenum Press, New York.

[118] Pinto, L.H., Holsinger, L.J. and Lamb, R.A. 1992. Influenza virus M2 protein has ion channel activity. *Cell* 69:517-528.

[119] Plotch, S.J., Bouloy, M. and Krug, R.M. 1979. Transfer of 5'-terminal cap of globin mRNA to influenza viral complementary RNA during transcription in vitro. *Proc.Natl.Acad.Sci.USA* 76:1618-1622.

[120] Plotch, S.J., Bouloy, M., Ulmanen, I. and Krug, R.M. 1981. A unique cap (m^7Gpp-pXm)-dependent influenza virus endonuclease cleaves capped RNA to generate the primers that initiate viral RNA transcription. *Cell* 23:847-858.

[121] Pons, M.W., Schulze, I.T., Hirst, G.K. and Hauser, R. 1969. Isolation and characterization of the ribonucleoprotein of influenza virus. *Virology* 39:250-259.

[122] Potter, C.W. and Oxford, J.S. 1979. Determinants of immunity to influenza infection in man. *Br.Med.J.* 35:69-75.

[123] Pyle, G.F. 1986. In: *The Diffusion of Influenza: Patterns and Paradigms.* Rowan and Littlefield, New Jersey.

[124] Qian, X., Alonso-Caplen, F. and Krug, R.M. 1994. Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. *J.Virol.* 68: 2433-2441.

[125] Qiu, Y. and Krug, R.M. 1994. The influenza virus NS1 protein is a poly (A) - binding protein that inhibits nuclear export of mRNAs containing poly (A). *J.Virol.* 68:2425-2432.

[125] Renegar, K.B. and Small Jr., P.A. 1991. Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. *J.Virol.* 65:2146-2148.

[127] Renegar, K.B. and Small Jr., P.A. 1991. Passive transfer of local immunity to influenza virus infection by IgA antibody. *J.Immunol.* 146:1972-1978.

[128] Richardson, J.C. and Akkina, R.K. 1991. NS2 protein of influenza virus is found in purified virus and phosphorylated in infected cells. *Arch.Virol.* 116:69-80.

[129] Richmann, D.D., Murphy, B.R., Baron, S. and Uhlendorf, C. 1976. Three strains of influenza A virus (H3N2): Interferon sensitivity *in vitro* and interferon production in volunteers. *J.Clin.Microbiol.* 3:223-226.

[130] Rimmelzwaan, G.F. and Osterhaus, A.D.M.E. 1995. A novel generation of viral vaccines based on the ISCOM matrix. In: Powell, M.F. and Newman, M.J. (eds.) *Vaccine Design: The Subunit and Adjuvant Approach*, pp.543-558. Plenum Press, New York.

[131] Rimmelzwaan, G.F. and Osterhaus, A.D.M.E. 1997. ISCOMs: Materials, preparation, antigen delivery and immune response. In: Gander, B., Merkle, H.P. and Corradin, G. (eds.) *Antigen Delivery Systems; Immunological and Technological Issues*, pp. 123-138. Harwood Academic Publishers, Amsterdam.

[132] Rodriguez-Boulan, E. and Sabatini, D.D. 1978. Asymmetric budding of viruses in epithelial monolayers; a model system for study of epithelial polarity. *Proc.Natl.Acad.Sci.USA* 75:5071-5075.

[133] Rogers, G.N. and Paulson, J.C. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* 127:361-373.

[134] Rogers, G.N., Paulson, J.C., Daniels, R.S., Skehel, J.J., Wilson, I.A. and Wiley, D.C. 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature* 304:76-78.

[135] Romanos, M.A. and Hay, A.J. 1984. Identification of the influenza virus transcriptase by affinity-labeling with pyroxidal 5'-phosphate. *Virology* 132:110-117.

[136] Rossen, R.D., Butler, W.T., Waldman, R.H., Alfor, R.H., Hornick, R.B., Togo, Y. and Kasel, J.A. 1970. The proteins in nasal secretion. II. A longitudinal study of IgA and neutralizing antibody levels in nasal washings from men infected with influenza virus. *JAMA* 211:1157-1161.

[137] Rota., P.A., Rocha, E.P., Harmon, M.W., Hinshaw, V.S., Sheerar, M.G., Kawaoka, Y., Cox, N.J. and Smith, T.F. 1989. Laboratory characterization of a swine influenza virus isolated from a fatal case of human influenza. *J.Clin.Micorbiol.* 27:1413-1416.

[138] Scherle, P.A. and Gerhard, W. 1986. Functional analysis of influenza-specific helper T cell clones *in vivo*: T cell specific for internal proteins provide cognate help for B cell responses to hemagglutinin. *J.Exp.Med.* 164:1114-1128.

[139] Scherle, P.A., Palladino, G. and Gerhard, W. 1992. Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. *J.Immunol.* 148:212-217.

[140] Scholtissek, C., Rhode, W., von Hoyningen, V. and Rott, R. 1978. On the origins of the human influenza virus subtypes H2N2 and H3N2. *Virology* 87:13-20.

[141] Scholtissek, C., Burger, H., Bachmann, P.A. and Hannoun, C. 1983. Genetic relatedness of hemagglutinins of the H1 subtype of influenza A viruses isolated from swine and birds. *Virology* 129:521-523.

[142] Scholtissek, C., Burger, H., Kistner, O. and Shortridge, K.F. 1985. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. *Virology* 147:287-294.

[143] Scholtissek, C. 1990. Pigs as 'mixing vessels' for the creation of new pandemic influenza A viruses. *Med.Principles Prac.* 2:65-71.

[144] Scholtissek, C., Schultz, U., Ludwig, S. and Fitch, W.M. 1993. The role of swine in the origin of pandemic influenza. In: Hannoun, C., Kendal, A.P., Klenk, H.D. and Ruben, F.L. (eds.) *Options for the control of influenza II*, pp. 193-201. Elsevier Science, Amsterdam.

[145] Schulman, J.L. and Kilbourne, E.D. 1965. Induction of partial specific heterotypic immunity in mice by a single infection with influenza A virus. *J.Bacteriol.* 89:170-174.

[146] Schulze, I.T. 1972. The structure of influenza virus. II. A model based on the morphology and composition of subviral particles. *Virology* 47:181-196.

[147] Shapiro, G.I., Gutney, Jr T. and Krug, R.M. 1987. Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. *J.Virol.* 61:764-773.

[148] Shapiro, G.I. and Krug, R.M. 1988. Influenza virus RNA replication in vitro: synthesis of viral template RNAs and virus RNAs in the absence of an added primer. *J.Virol.* 62:2285-2290.

[149] Shaw, M.W., Arden, N.H. and Maassab, H.F. 1992. New aspects of influenza viruses. *Clin.Microbiol.Rev.* 5:74-92.

[150] Shi, L., Summers, D.F., Peng, Q. and Galarza, J.M. 1995. Influenza A virus RNA polymerase subunit PB2 is the endonuclease which cleaves host cell mRNA and functions only as the trimeric enzyme. *Virology* 208:38-47.

[151] Skehel, J.J. 1972. Polypeptide synthesis in influenza virus-infected cells. *Virology* 49:23-36.

[152] Skehel, J.J. 1973. Early polypeptide synthesis in influenza virus-infected cells. *Virology* 56:394-399.

[153] Skehel, J.J., Daniels, R.S., Hay, A.J., Ruigrok, R.W.H., Wharton, S.A., Wrigley, N.G., Weiss, W. and Wiley, D.C. 1986. Structure changes in influenza virus haemagglutinin at the pH of membrane fusion. *Biochem.Soc.Trans.* 14:252-253.

[154] Smith, G.L. and Hay, A.J. 1982. Replication of the influenza virus genome. *Virology* 118:96-108.

[155] Smith, W., Andrewes, C.H. and Laidlaw, P.P. 1933. A virus obtained from influenza patients. *Lancet* 1:66-68.

[156] Steinhoff, M.C., Fries, L.F., Karron, R.A., Clements, M.L. and Murphy, B.R. 1993. Effect of heterosubtypic immunity on infection with attenuated influenza A virus vaccines in young children. *J.Clin.Microbiol.* 31:836-838.

[157] Stitz, L., Schmitz, C., Binder, D., Zinkernagel, R., Paoletti, E. and Becht, H. 1990. Characterization and immunological properties of influenza A virus nucleoprotein (NP): cell-associated NP isolated from infected cells or viral NP expressed by vaccinia recombinant virus do not confer protection. *J.Gen.Virol.* 71:1169-1179.

[158] Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X.Y., Fukuda, K. and Cox, N. 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* 279:393-396.

[159] Sugrue, R.J. and Hay, A.J. 1991. Structural characteristics of the M2 protein of the influenza A viruses: evidence that it forms a tetrameric channel. *Virology* 180:617-624.

[160] Taubenberger, J.K., Reid, A.H., Krafft, A.E., Bijwaard, K.E. and Fanning, T.G. 1997. Initial genetic characterization of the 1918 'Spanish' influenza virus. *Science* 275:1793-1796.

[161] Taylor, P.M. and Askonas, B.A. 1986. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective *in vivo*. *Immunology* 58:417-420.

[162] Taylor, R.M. 1949. Studies on survival of influenza virus between epidemics and antigenic variants of the virus. *Am.J.Public Health* 39:171-178.

[163] Top, F.H. and Russell, P.K. 1977. Swine influenza A at Fort Dix, New Jersey (Januari-Februari 1976): IV. Summary and speculation. *J.Infect.Dis.* 136:S376-380.

[164] Topham, D.J. and Doherty, P.C. 1998. Clearance of an influenza A virus by CD4+ cells is inefficient in the absence of B cells. *J.Virol.* 72:882-885.

[165] Tosteson, M.T., Pinto, L.H., Holsinger, L.J. and Lamb, R.A. 1994. Reconstitution of the influenza virus M2 ion channel in lipid bilayers. *J.Membr.Biol.* 142:117-126.

[166] Townsend, A.R.M. and Skehel, J.J. 1984. The influenza A nucleoprotein gene controls the induction of both subtype-specific and crossreactive cytotoxic T cells. *J.Exp.Med.* 160:552-563.

[167] Tripp, R.A., Sarawar, S.R. and Doherty, P.C. 1995. Characteristics of the influenza virus-specific $CD8^+$ T-cell response in mice homozygous for disruption of the H-2IA^b gene. *J.Immunol.* 155:2955-2959.

[168] Ulmanen, I., Broni, B.A. and Krug, R.M. 1981. Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m^7GpppNm) on RNAs and in initiating viral RNA transcription. *Proc.Natl.Acad.Sci.USA* 78:7355-7359.

[169] Vadolas, J., Wijburg, O.L.C. and Strugnell, R.A. 1997. Liposomes as systemic and mucosal delivery vehicles. In: Gander, B., Merkle, H.P. and Corradin, G. (eds.) *Antigen Delivery Systems; Immunological and Technological Issues*, pp. 73-100. Harwood Academic Publishers, Amsterdam.

[170] Van Voris, L.P. and Newell, P.M. 1992. Antivirals for the chemoprophylaxis and treatment of influenza. *Semin.Respir.Infect.* 7:61-70.

[171] Varghese, J.N., Laver, W.G. and Colman, P.M. 1983. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9Å resolution. *Nature* 303:35-40.

[172] von Itzstein, I., Wu, W.Y., Kok, G.B., Pegg, M.S., Dyason, J.C., Jin, B., Van Phan, T., Smythe, M.L., White, H.F., Oliver, S.W., Colman, P.M., Varghese, J.N., Ryan, D.M., Woods, J.M., Bethell, R.C., Hotham, V.J., Cameron, J.M. and Penn, C.R. 1993. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* 363:418-423.

[173] Wang, C., Takeuchi, K., Pinto, L.H. and Lamb, R.A. 1993. Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. *J.Virol.* 67:5585-5594.

[174] Webster, R.G. and Laver, W.G. 1972. The origin of pandemic influenza. *Bull.WHO* 47:449-452.

[175] Webster, R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J. and Murti, K.G. 1978. Intestinal influenza: Replication and characterization of influenza viruses in ducks. *Virology* 84:268-278.

[176] Webster, R.G., Hinshaw, V.S., Bean, W.J., van Wyke, K.L., Geraci, J.R. and Petursson, G. 1981. Characterization of an influenza A virus from seals. *Virology* 113:712-724.

[177] Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. and Kawaoka, Y.1992. Evolution and ecology of influenza A viruses. *Microbiol.Rev.* 56:152-179.

[178] Webster, R.G., Sharp, G.B. and Claas, E.C. 1995. Interspecies transmission of influenza viruses. *Am.J.Respir.Crit.Care.Med.* 152:S25-S30.

[179] Wentworth, D.E., Thompson, B.L., Xu, X.Y., Regnery, H.L., Cooley, A.J., McGregor, M.W., Cox, N.J. and Hinshaw, V.S. 1994. An influenza A (H1N1) virus, closely related to swine influenza virus, responsible for a fatal case of human influenza. *J.Virol.* 68:2051-2058.

[180] Wentworth, D.E., McGregor, M.W., Macklin, M.D., Neumann, V. and Hinshaw, V.S. 1997. Transmission of swine influenza virus to humans after exposure to experimentally affected pigs. *J.Infect.Dis.* 175:7-15.

[181] Wharton, S.A., Weis, W., Skehel, J.J. and Wiley, D.C. 1989. Structure, function, and antigenicity of the haemagglutinin of influenza virus. In: Krug, R.M. (ed.) *The influenza viruses*, pp. 153-174. Plenum Press, New York.

[182] Wiley, D.C. and Skehel, J.J. 1977. Crystallization and x-ray diffraction studies on the haemagglutinin glycoprotein from the membrane of influenza virus. *J.Mol.Biol.* 112:343-347.

[183] Wiley, D.C., Skehel, J.J. and Waterfield, M.D. 1977. Evidence from studies with a cross-linking reagent that the hemagglutinin of influenza virus is a trimer. *Virology* 79:446-448.

[184] Wiley, D.C. and Skehel, J.J. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Ann.Rev.Biochem.* 56:365-394.

[185] Wilson, I.A., Skehel, J.J. and Wiley, D.C. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3Å resolution. *Nature* 289:366-373.

[186] Wraith, D.C., Vessey, A.E. and Askonas, B.A. 1987. Purified influenza virus nucleoprotein protects mice from lethal infection. *J.Gen.Virol.* 68:433-440.

[187] Yap, K.L., Ada, G.L. and McKenzie, I.F. 1978. Transfer of specific cytotoxic T cells protects mice inoculated with influenza viruses. *Nature* 273:230-237.

[188] Yarchoan, R., Murphy, B.R., Strober, W., Schneider, H.S. and Nelson, D.L. 1981. Specific anti-influenza virus antibody production *in vitro* by human peripheral blood mononuclear cells. *J.Immunol.* 127:2558-2594.

[189] Yasuda, J., Nakada, S., Kato, A., Toyoda, T. and Ishihama, A. 1993. Molecular assembly of influenza virus: association of the NS2 protein with virus matrix. *Virology* 196:249-255.

[190] Yewdell, J.W., Bennink, J.R., Smith, G.L. and Moss, B. 1985. Influenza A virus nucleoprotein is a major target antigen for cross reactive anti influenza A virus cytotoxic T lymphocytes. *Proc.Natl.Acad.Sci.USA* 82:1785-1789.

[191] Yewdell, J.W. and Hacket, C.J. 1989. The specificity and function of T lymphocytes induced by influenza A viruses. In: Krug, R. (ed.) *The influenza viruses*. Plenum Press, New York.

[192] Zebedee, S.L. and Lamb, R.A. 1988. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in viruss. *J.Virol.* 62:2762-2772.

[193] Zhou, N., He., S., Zhang, T., Zou, W., Shu, L., Sharp, G.B. and Webster, R.G. 1996. Influenza infection in humans and pigs in south-eastern China. *Arch.Virol.* 141:649-661.

[194] Zvonarjev, A.Y and Ghendon, Y.Z. 1980. Influence of membrane (M) protein on influenza A virus virus transcriptase activity in vitro and its susceptibility to rimantadine. *J.Virol.* 33:583-586.

[195] Zweerink, H.J., Courtneige, S.A., Skehel, J.J., Crumpton, M.J. and Askonas, B.A. 1977. Cytotoxic T cells kill influenza infected cells but do not distinguish between serologically distinct type A viruses. *Nature* 267:354-356.

CHAPTER 2

Characterization of high-growth reassortant influenza A viruses generated in MDCK cells cultured in serum-free medium

J.T.M. Voeten, R. Brands, A.M. Palache, G.J.M. van Scharrenburg, G.F. Rimmelzwaan, A.D.M.E. Osterhaus and E.C.J. Claas

Vaccine 17:1942-1950 (1999)

SUMMARY

In the present study reassortant influenza A viruses of both the H1N1 and H3N2 type were generated in Madin Darby Canine Kidney cells grown in the absence of fetal bovine serum (MDCK-SF1 cells). To this end, MDCK-SF1 cells were simultaneously infected with one of the high-growth laboratory strains A/Puerto Rico/8/34 (H1N1) or A/Hong Kong/2/68 (H3N2) and recent H3N2 and H1N1 vaccine strains, respectively. Reassortant viruses obtained from these mixed infections were genetically characterized by RT-PCR and restriction enzyme analysis and their growth properties were compared to those of the corresponding field strains. Reassortant H3N2 viruses inherited the matrix and polymerase pa gene whilst H1N1 reassortant viruses inherited the matrix and polymerase pb1 gene of the high-growth parent. Reassortant viruses generally gave higher viral yields, as measured by a haemagglutination assay, than their wild type counterparts. The procedure followed, results in the generation of high-growth reassortant viruses in weeks. The use of MDCK-SF1 cells together with these reassortants for generating influenza virus antigens can significantly speed up the vaccine production procedure.

INTRODUCTION

Influenza viruses, members of the family *Orthomyxoviridae*, are the causative agents of annual epidemics of acute respiratory disease. Influenza epidemics are associated with considerable morbidity and mortality, especially in people at risk, i.e. people suffering from heart or lung diseases, diabetics or a malfunction of the immune system. Vaccination is the most effective way to prevent the often fatal complications in these patients during influenza virus outbreaks.

Influenza vaccines contain the surface glycoproteins (haemagglutinin and neuraminidase) of the influenza viruses expected to circulate in the human population in the upcoming season. Currently, trivalent influenza vaccines are used that contain representative strains of influenza A (H1N1 and H3N2) and influenza B viruses. Due to antigenic drift, the antigenic epitopes of the surface glycoproteins continuously change which necessitates yearly adjustments of the vaccine. These changes are monitored by over a hundred National Influenza Centres worldwide. Based on the information collected, the World Health Organization (WHO) in Geneva yearly recommends the composition of the influenza vaccine, usually in

February. This leaves the vaccine manufacturers approximately six months to produce and register the vaccine for use in the Northern hemisphere. Vaccine production requires embryonated chicken eggs to propagate the influenza virus antigens. In case of an urgent worldwide demand for large quantities of vaccine, for example with the emergence of a novel subtype of influenza A virus (due to an antigenic shift), vaccine production may be hampered by the limited availability of eggs. Recently, an influenza A virus of the H5N1 subtype was isolated from humans[8]. Fortunately, human infections with this virus appeared to be restricted to 18 hospitalized cases. If, however, this virus would have managed to spread in the human population, it could have initiated a new influenza pandemic[4]. In that case it would have been difficult, if not impossible, to obtain sufficient embryonated eggs for rapid production of a new vaccine. The availability of embryonated eggs also can become a problem when flocks of chickens supplying the eggs become the victim of avian diseases or when the embryo's themselves are sensitive to the influenza virus used. The time limits on the one hand and the use of eggs on the other hand render influenza vaccine production unflexible and requires long-term planning by vaccine manufacturers.

The use of continuous cell lines like Madin Darby Canine Kidney (MDCK) cells for influenza virus antigen production can be considered an attractive alternative to embryonated chicken eggs for several reasons. Firstly, cell cultures are maintained easily and can be expanded in a relatively short time and therefore will allow initiaton and scaling up of antigen production at any time. Secondly, human viruses propagated in MDCK cells usually resemble the original human isolate more closely than do viruses propagated in eggs[14]. Also, egg-derived vaccine strains often constitute a heterogenous population of influenza viruses[6,15,20]. Due to adaptation to avian cells, these viruses undergo mutations in their surface glycoproteins which can render the ultimate vaccine less effective[11,13,18]. Thirdly, the use of MDCK cell derived influenza vaccines would overcome allergic reactions in vaccinated individuals sensitive to egg proteins and, finally, from an ethical point of view, it would reduce the amount of animals used for the benefit of human medicin.

A major draw-back in the use of MDCK cells has been the requirement to grow the cells in the presence of fetal bovine serum. The use of fetal bovine serum not only interferes with the activity of trypsin, necessary for propagation of most influenza A viruses, but its biological variation also complicates standardization of the culture conditions. Recently, a MDCK cell line (MDCK-SF1) that overcomes this draw-back has been developed as these cells grow in medium devoid of fetal bovine serum[1,2]. MDCK-SF1 cell

derived vaccines already have been extensively tested and shown to be equally effective as egg-derived vaccines[12].

Important factors in the production of an influenza vaccine are the growth properties of the virus and the amount of viral antigens (haemagglutinin and neuraminidase) that can be produced per unit of volume. Ideally, viruses should replicate to high titre in a short period of time and yield high amounts of viral antigens at the end of the process. Influenza A virus field strains, selected to be included in egg-based antigen production, are usually being genetically modified to generate a virus containing the genes encoding haemagglutinin and neuraminidase of the field strain while a high-growth phenotype is derived from a laboratory strain. Dual infection of embryonated chicken eggs with repression of replication of the laboratory strain results in a selective advantage of so called high-growth reassortants with the appropriate surface glycoproteins. This reassortment procedure, made possible by the segmented genome of influenza viruses, is currently carried out routinely for the egg-derived influenza vaccine viruses.

Here we describe the application of this approach for the generation of high-growth reassortant influenza A viruses that can be used for viral antigen production in MDCK-SF1 cells. Also, methods to characterize reassortant viruses are described.

MATERIALS AND METHODS

Viruses, cells and sera

The H1N1 viruses A/Puerto Rico/8/34 (PR 34), A/Taiwan/1/86 (TW 86), A/Johannesburg/82/96 (JB 96) and A/Shenzhen/227/95 (SZ 95) as well as the H3N2 viruses A/Hong Kong/2/68 (HK 68), A/Wuhan/359/95 (WH 95), A/Nanchang/933/95 (NC 95) and A/Johannesburg/33/94 (JB 94) were obtained from the repository of the Dutch National Influenza Centre. Most of these vaccine strains were primarily obtained from the National Institute for Biological Standards and Control (NIBSC), Potters Bar, United Kingdom. All viruses were passaged several times on MDKC-SF1 cells before the start of the experiments.

MDCK-SF1 cells were obtained from Solvay Pharmaceuticals, Weesp, The Netherlands. The cells were cultured in EpiSerf medium (Gibco-BRL) supplemented with antibiotics (penicillin 10 IU/ml, streptamycin 50 µg/ml). For propagation of the viruses, trypsin (5 µg/ml) was added.

PR 34 and HK 68-specific antisera were raised in rabbits injected with sucrose gradient purified virus and TW 86, JB 96, SZ 95, NC 95 and JB 94-specific antisera were obtained from ferrets after intranasal infection.

Dual infections and isolation of viruses

MDCK-SF1 cells were grown in 96-wells plates until confluency and infected either with a single virus or with a mixture of a H1N1 and a H3N2 virus in 100 µl culture medium. Mixed infections were carried out with the H1N1 virus PR 34 and one of the H3N2 viruses WH 95, NC 95 or JB 94, or with the H3N2 virus HK 68 and one of the H1N1 viruses TW 86, JB 96 or SZ 95. Infections were performed at various multiplicities of infection (moi's) ranging from 0.001 to 1. After one hour of incubation at 37°C, the inoculum was removed, cells were washed with culture medium, 200 µl fresh medium was added and the cells were further cultured for three days. At day 3, 20 µl of the supernatant was transferred to new MDCK-SF1 cells which were further cultured for another three days in the presence of PR 34 or HK 68-specific polyclonal rabbit antiserum (1:1000) in 200 µl culture medium. One round of plaque purifications were performed in 6-wells plates in the presence of antiserum (1:1000) and agarose (1%) added to the culture medium.

Haemagglutination assay

To 50 µl of two-fold diluted virus solutions (in Phosphate Buffered Saline; PBS), 25 µl PBS and 25 µl of a 1% turkey erythrocyte suspension in PBS was added and incubated for one hour at 4°C. Then the haemagglutination pattern was examined and expressed in haemagglutinating units (HAU). The amount of HAU corresponded to the reciprocal value of the highest virus dilution that showed full haemagglutination.

Haemagglutination inhibition assay

One volume of virus-specific antiserum (raised in ferrets or rabbits) was incubated with five volumes of cholera filtrate at 37°C for approximately 16 hours, followed by one hour incubation at 56°C. To 50 µl of two-fold dilution series of serum (in PBS), 25 µl of a virus solution of 4 haemagglutinating units (HAU) was added and incubated for 30 minutes at 37°C. Then, 25 µl of a 1% turkey erythrocyte suspension in PBS was added followed by one hour incubation at 4°C. Subsequently the haemagglutination pattern was examined and expressed as the reciprocal value of the highest serum dilution inhibiting haemagglutination.

RNA isolation, RT-PCR and restriction enzyme analysis

Viral RNA was extracted as previously described[3]. RT-PCR was performed as follows. To 10 µl of viral RNA 2 µl forward primer (10 pmol/µl) was added and incubated at 80°C for 2 minutes followed by a short incubation on ice. Then, cDNA was synthesized from the viral RNA by adding dNTP's (0.5 mM each), DTT (10 mM), RNasin (40 units) and MMLV-RT (200 units) in a total volume of 25 µl 1x reverse transcriptase buffer and incubation at 42°C for 45 minutes. The reaction was stopped by heating the mixture to 95°C for three minutes. The PCR mixture contained cDNA, 2 µl forward and 2 µl reverse primer (both 10 pmol/µl), dNTP's (0.2 mM each), $MgCl_2$ (1.5 mM) and Taq polymerase (2.5 units) in a total volume of 100 µl 1x Taq buffer. The PCR cycles consisted of 1 minute 94°C, 2 minutes 40°C and 3 minutes 72°C for a total of 40 cycles. PCR products were ethanol precipitated and dissolved in 50 µl H_2O. 8 µl of a PCR product was incubated with a particular restriction enzyme for one hour at 37°C and subsequently run on a 1% agarose gel containing ethidium bromide.

Comparison of wild type and reassortant viruses

MDCK-SF1 cells were grown in 24 wells plates and infected with 100 µl reassortant virus or the corresponding field strain at a broad range of moi's (1.10^{-6} to 1.10^{-3} pfu/cell). After one hour incubation at 37°C, the inoculum was removed and 2 ml fresh culture medium was added. The amount of HAU in the culture supernatant was measured in a haemagglutination assay from day 1 to day 6 post infection. The experiment was performed twice in duplicate and the average HAU values were calculated from both experiments.

RESULTS

Dual infections

For generation of H3N2 and H1N1 reassortant influenza A viruses, PR 34 and HK 68 were chosen as high-growth parents, respectively, because on comparison with a series of other viruses, PR 34 and HK 68 yielded the highest amounts of haemagglutinating units (HAU) when propagated in MDCK-SF1 cells. Replication of these viruses was completely inhibited by their respective antisera since no haemagglutinating units (HAU) could be measured in supernatants of cells infected with PR 34 or HK 68 only. The supernatants of cells infected with H3N2 or H1N1 field strains and cultured in the presence of PR 34 or HK 68-specific antiserum, respectively, caused haemagglutination of turkey erythrocytes indicating that the antisera did not inhibit these field strains. Viruses derived from dual infections which escaped the antiserum pressure (positive supernatants) were subjected to plaque purification. Of each dual infection, three randomly selected plaque purified viruses were characterized to determine whether they represented wild type (field strain) or reassortant viruses.

Determination of the type of surface glycoproteins

To check the subtype of haemagglutinin of the plaque purified viruses grown in the presence of antiserum, haemagglutination inhibition assays using antisera raised against PR 34, HK 68 and the field strains were carried out. From table 1 it is clear that the plaque purified viruses obtained from dual infections with PR 34 contained H3 haemagglutinin while those obtained from dual infection with HK 68 contained H1 haemagglutinin. The neuraminidase subtype of the plaque purified viruses was determined by RT-PCR using neuraminidase N1 and N2-specific primer sets. RT-PCR's with RNA isolated from the plaque purified viruses obtained from dual infection with PR 34 were only positive using the N2 primer set, whereas the plaque purified viruses

obtained from dual infection with HK 68 were only positive when the N1 primer set was used. Thus, all plaque purified viruses were of the same subtype as their corresponding field strains.

Virus[a]	Antisera[b]			Virus	Antisera			
	NC95 (H3N2)	JB94 (H3N2)	PR34 (H1N1)		TW86 (H1N1)	JB96 (H1N1)	SZ95 (H1N1)	HK68 (H3N2)
WH 95	640[c]	nt	<10[d]	TW 86	2560	nt	nt	<10
WHxPR1	640	nt	<10	TWxHK1	2560	nt	nt	<10
WHxPR2	640	nt	<10	TWxHK2	2560	nt	nt	<10
WHxPR3	640	nt	<10	TWxHK3	2560	nt	nt	<10
NC 95	2560	nt	<10	JB 96	nt	2560	nt	<10
NCxPR1	2560	nt	<10	JBxHK1	nt	2560	nt	<10
NCxPR2	2560	nt	<10	JBxHK2	nt	2560	nt	<10
NCxPR3	2560	nt	<10	JBxHK3	nt	2560	nt	<10
JB 94	Nt	2560	<10	SZ 95	nt	nt	5120	<10
JBxPR1	Nt	2560	<10	SZxHK1	nt	nt	5120	<10
JBxPR2	Nt	2560	<10	SZxHK2	nt	nt	5120	<10
JBxPR3	Nt	2560	<10	SZxHK3	nt	nt	5120	<10
PR 34	<10	<10	>20480	HK 68	<10	<10	<10	>20480

TABLE 1. Haemagglutination inhibition titers for field strains and reassortant influenza viruses.
[a]Viruses are abbreviated as follows: WH 95 (A/Wuhan/359/95), NC 95 (A/Nanchang/933/95), JB 94 (A/Johannesburg/33/94), PR 34 (A/Puerto Rico/8/34), WHxPR1-3, NCxPR1-3 or JBxPR1-3 reassortant viruses obtained from mixed infections of PR 34 with WH 95, NC 95 or JB 94 respectively), TW 86 (A/Taiwan/1/86), JB 96 (A/Johannesburg/82/96), SZ 95 (A/Shenzhen/227/95), HK 68 (A/Hong Kong/2/68), TWxHK1-3, JBxHK1-3 or SZxHK1-3 (reassortant viruses obtained from mixed infections of HK 68 with TW 86, JB 96 or SZ 95 respectively); [b]PR 34 and HK 68-specific antisera were derived from immunized rabbits; other sera were derived from infected ferrets; [c]WH 95 and the reassortant viruses WHxPR1-3 were measured against NC 95 antiserum as WH 95 antiserum was not available (nt: not tested); [d]A titer <10 corresponded to no detectable inhibition of haemagglutination.

Determination of the origin of the other gene segments

The results of the haemagglutination inhibition assays and neuraminidase RT-PCR's do not discriminate between wild-type viruses (field strains) and reassortant viruses. To establish whether the plaque purified viruses derived from the dual infections were actually reassortant viruses, the origin of the

remaining six gene segments encoding the internal viral proteins (PB2, PB1, PA, NP, MA and NS) was determined. RT-PCR followed by restriction enzyme analysis has previously been found to be a suitable method[10,17]. Primer sequences were selected for amplification of the six gene segments in regions conserved within H1N1 and H3N2 viruses. However, different recognition sequences for restriction endonucleases were present within the amplified region to distinguish gene segments derived from H1N1 or H3N2 viruses (Table 2). An example of a plaque purified virus obtained from mixed infection with PR 34 and WH 95 is shown in figure 1. This figure clearly demonstrates that all gene segments encoding the internal viral proteins of the plaque purified virus originated from PR 34. Therefore, this virus is the result of genetic reassortment between PR 34 and WH 95. The origin of the gene segments of other plaque purified viruses was determined as well and all these viruses proved to be reassortant viruses. Table 3 shows the genomic composition of the reassortant viruses derived from three different mixed infections with PR 34 or HK 68. All reassortant viruses contained the matrix gene of the high-growth laboratory strain. In addition to the matrix gene, also the gene segment encoding polymerase PA originated from the PR 34 strain in all H3N2 reassortant viruses. Four out of nine reassortant H3N2 viruses contained a complete PR 34 background; in these reassortant viruses all gene segments, except the ones encoding the surface glycoproteins, were of PR 34 origin.

Gene	Amplified region	Restriction enzyme(s)[a]
PB2	1-764	BglII (H1N1) or PvuII (H3N2)
PB1	1-623	RsaI (H1N1)
PA	1132-1790	BamHI (H1N1) or BglII (H3N2)
NP	46-1542	BamHI (both)[b] or PvuII (H3N2)
MA	26-784	BglII (H3N2)
NS	1-574	HindIII (H1N1)

TABLE 2. Restriction enzymes used to determine the origin of gene segments encoding internal viral proteins.
[a]The type of virus whose gene segment contains the recognition sequence for a particular restriction enzyme is shown in brackets; [b]*Bam*HI cuts the NP PCR product of H1N1 viruses once and that of A/Hong Kong/2/68 (H3N2) twice.

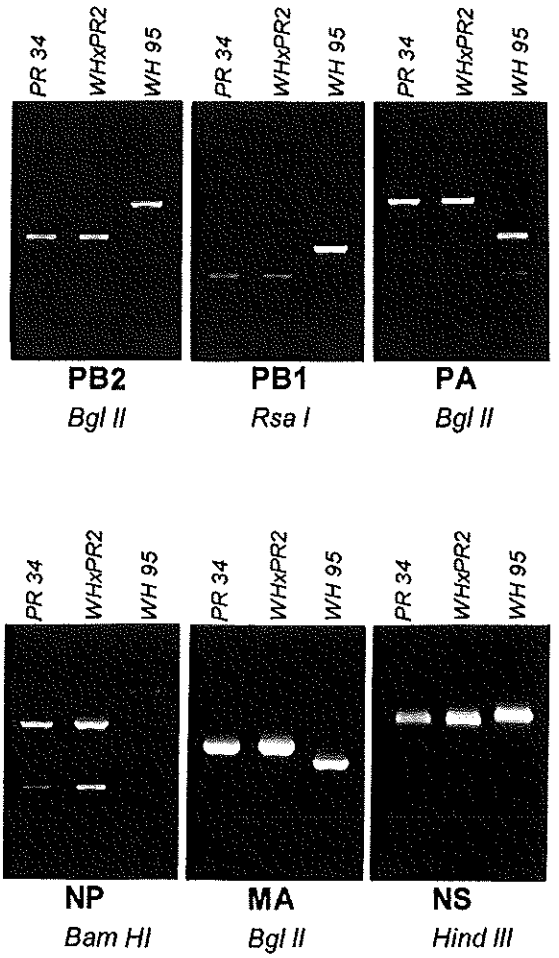

Figure 1. RT-PCR and restriction enzyme analysis of the gene segments encoding the internal viral proteins of A/Puerto Rico/8/34 (H1N1), a H3N2 reassortant (WHxPR) and A/Wuhan/359/95 (H3N2).

The H1N1 reassortant viruses shared the gene segments encoding polymerase PB1 and the matrix proteins of HK 68. The gene segments encoding the nucleoprotein and the nonstructural proteins were of H1N1 origin in all H1N1 reassortants and consequently none of the H1N1 reassortant viruses contained a complete HK 68 background.

Gene	WHxPR			NCxPR			JBxPR			TWxHK			JBxHK			SZxHK		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
PB1	P	P	P	P	P	N	P	P	P	H	H	T	H	H	H	S	S	H
PB2	P	P	P	P	N	N	J	P	J	H	H	H	H	H	H	H	H	H
PA	P	P	P	P	P	P	P	P	P	T	T	T	B	B	B	H	H	H
HA	W	W	W	N	N	N	J	J	J	T	T	T	B	B	B	S	S	S
NP	P	P	P	N	N	N	P	P	P	T	T	T	B	B	B	S	S	S
NA	W	W	W	N	N	N	J	J	J	T	T	T	B	B	B	S	S	S
MA	P	P	P	P	P	P	P	P	P	H	H	H	H	H	H	H	H	H
NS	P	P	P	N	N	N	P	P	P	T	T	T	B	B	B	S	S	S

TABLE 3. Genomic composition of influenza A H3N2 and H1N1 reassortant viruses.
[a]A one letter code is used to depict the origin of each of the gene segments: P (A/Puerto Rico/8/34), W (A/Wuhan/359/95), N (A/Nanchang/933/95), J (A/Johannesburg/33/94), H (A/Hong Kong/2/68), T (A/Taiwan/1/86), B (A/Johannesburg/82/96) and S (A/Shenzhen/227/95).

Comparison of growth characteristics of the reassortant viruses and field strains

The viral yield, expressed as haemagglutinating units (HAU) of reassortant viruses resulting from each dual infection was compared with that of the corresponding field strain for different m.o.i.'s. Most viruses, both wild type and reassortant viruses, reached a maximum yield of HAU within three to four days post infection (Figures 2 and 3). In general, the reassortant viruses showed higher yields than the wild type viruses. Of the H3N2 reassortant viruses, the yields produced by the WH 95 reassortant (WHxPR2) were at least twofold higher than the yields produced by wild type WH 95 at all moi's tested. The NC 95 reassortant (NCxPR1) showed higher yields than wild type NC 95 at moi= 0.001 and 0.0001, and the JB 94 reassortant (JBxPR1) showed higher yields than wild type JB 94 at moi= 0.001 and 0.000001. Of the H1N1 reassortants, the SZ 95 reassortant (SZxHK1) yielded at least two times the amount of HAU of wild type SZ 95 irrespective of the moi. The JB 96 reassortant (JBxHK2) yielded at least twice the amount of HAU of wild type JB 96 at three of the four moi' tested. The difference between the TW 86 reassortant (TWxHK1) and wild type TW 86 was most pronounced at very low moi's (0.00001 and 0.000001).

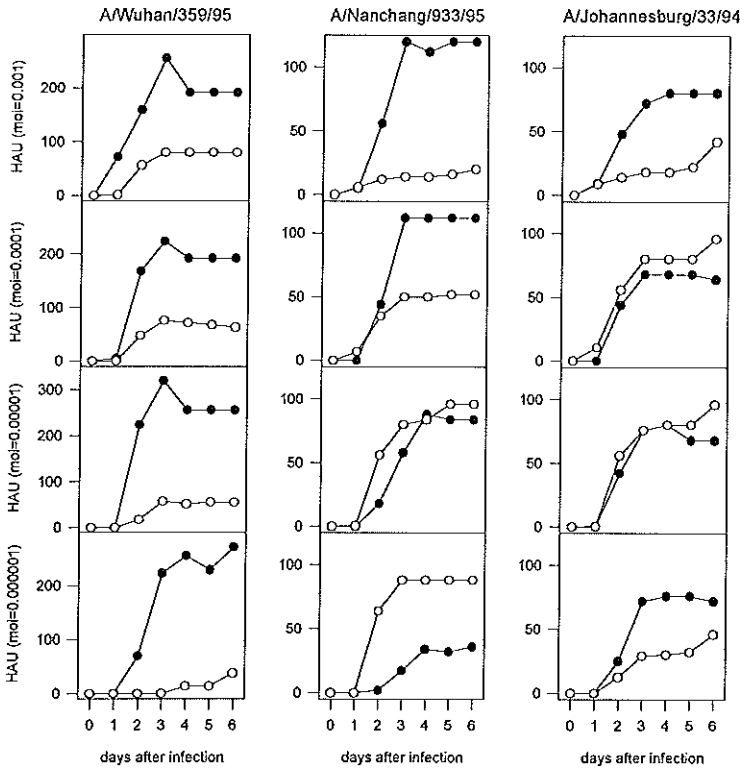

Figure 2. Kinetics of virus propagation measured in haemagglutinating units (HAU) from day 1 to 6 after infection of MDCK-SF1 cells at four different multiplicities of infection (moi). Average values of four similar experiments with H3N2 field strains A/Wuhan/359/-95, A/Nanchang/933/95 and A/Johannesburg/33/94 (open circles) and their corresponding reassortant viruses WHxPR2, NCxPR1 and JBxPR1 (solid circles) are shown.

DISCUSSION

MDCK-SF1 cells, a newly developed MDCK cell line, can be considered a serious candidate for production of tissue culture grown vaccines. In the present study it was shown that these cells allow genetic reassortment to take place after mixed infection resulting in potential vaccine seed strains with a high-growth phenotype.

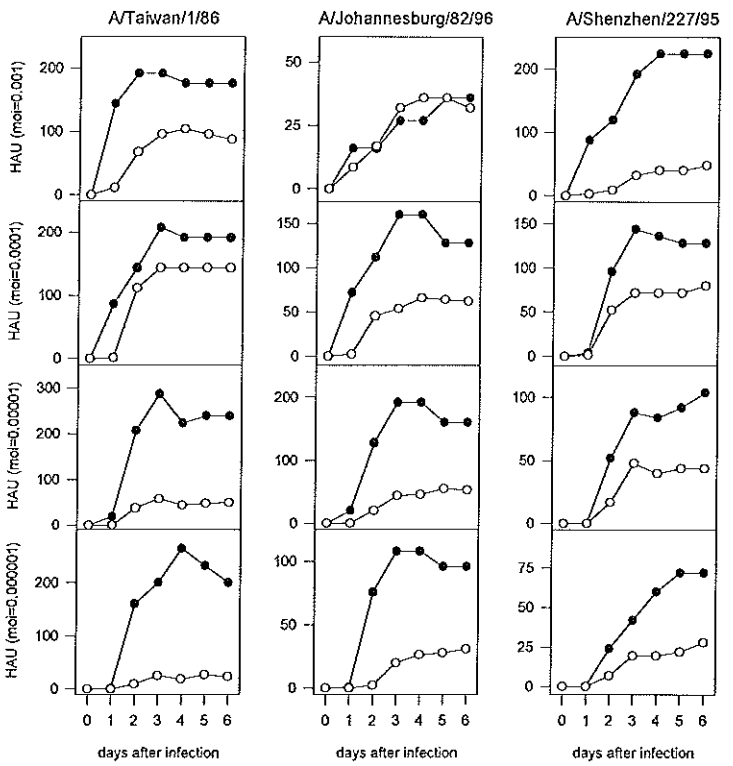

Figure 3. See legend to figure 2. Open circles represent the H1N1 field strains A/Taiwan/1/86, A/Johannesburg/82/96 and A/Shenzhen/227/95 and solid circles represent the corresponding reassortant viruses TWxHK1, JBxHK2 and SZxHK1.

The reassortant H3N2 and H1N1 influenza A viruses all contained the gene segment encoding the matrix proteins from the high-growth laboratory strains PR 34 or HK 68, respectively. In other studies it was shown that a high-growth phenotype of reassortant viruses correlates with the uptake of the matrix encoding gene of the high-growth parent virus[19,21-23]. Therefore, the improvement of the viral yield of the reassortant viruses (expressed in HAU) observed in the present study most likely can be attributed to the matrix protein of PR 34 or HK 68. However, the H3N2 reassortant viruses also shared the gene segment encoding polymerase PA of PR 34 while the H1N1 reassortant viruses shared the gene segment encoding polymerase

PB1 of HK 68, indicating that the matrix protein may be not the only important factor. The polymerase proteins PA and PB1 in the H3N2 and H1N1 reassortant viruses, respectively, could have contributed to the high-growth phenotype of these viruses as well.

The WH 95 reassortant (WHxPR2) contained a complete PR 34 background and this H3N2 reassortant virus consistently produced higher yields than wild type WH 95. The WH 95 strain was selected as the H3N2 vaccine strain in 1996/1997 but was replaced by the NC 95 strain that revealed better growth properties. For this strain, the reassortment procedure in MDCK-SF1 cells seems superior to embryonated chicken eggs as the WH 95 reassortant showed excellent growth properties. Although the WH 95 reassortant was found superior to the wild type strain, a complete PR 34 background does not necessarily result in the best growth characteristics. One reassortant virus (JBxPR2) contained the complete set of gene segments encoding the internal proteins of PR 34, but its yield did not differ from JBxPR1 (data not shown). Moreover, none of the H1N1 reassortant viruses contained a complete HK 68 background although a clear improvement in viral yield was measured. Taken together, the results show that the set of genes of a high-growth parent that confers a high-growth phenotype to a certain reassortant virus, do not necessarily benefit another type of reassortant virus and that it is not a prerequisite to inherit all gene segments of a high-growth parent to obtain a high yield. Apparently, the genomic constellation of reassortant viruses as a whole, rather than the inheritance of defined gene segments, determines their growth characteristics.

This statement is supported by the genetic characterization of the reassortant viruses. The genomic make-up of the three reassortant viruses isolated for each dual infection did not differ significantly from each other. The WH 95 reassortant viruses were identical to each other as were the JB 96 reassortant viruses. The reassortant viruses obtained from the other dual infections differed only from each other with respect to the origin of one or two gene segments encoding the polymerase proteins. Although we only characterized three reassortant viruses per dual infection, it seems that there is a restriction towards the random combination of gene segments. This is in agreement with other studies showing that the number of combinations of the six gene segments encoding the internal viral proteins (of which there are 64 possibilities) is limited[5,9,16,21].

The use of heterologous high-growth H1N1 and H3N2 virus for generation of H3N2 and H1N1 reassortant viruses, respectively, makes it easy to fully suppress reproduction of the high-growth parent virus, without affecting reproduction of desired reassortant viruses. In addition, it is very helpful to

determine the genetic make up of reassortant viruses. After a single passage of a mixed yield in the presence of the appropriate antiserum, the high-growth parent is neutralized and in subsequent plaque purifications the reassortant virus can be separated and cloned. This complete procedure, including the genetic characterization takes only 3-4 weeks.

The MDCK-SF1 cell line we used differs from the original MDCK cell line by its ability to be cultured in the absence of fetal bovine serum. Therefore, MDCK-SF1 cells do not need washing and replacement of culture medium so they can be directly infected with influenza viruses. This can be considered a great advantage in large-scale virus productions. Moreover, as shown in this study, high yields of virus in MDCK-SF1 cells can be obtained at low m.o.i. and a maximum amount of HAU usually is reached within three to four days. Obviously, in the scaling up of the procedure, the optimal m.o.i. will have to be reconsidered and determined for every new vaccine strain.

Although the use of MDCK-SF1 cells is likely to offer numerous advantages over embryonated chicken eggs in viral antigen production, two remarks must be made. First, influenza field strains may be contaminated with other viruses that also replicate in MDCK cells. Therefore, for safety reasons, an initial passage in embryonated chicken eggs will still be required to eliminate potential contamination of the ultimate influenza vaccine with adventitious human viruses. Second, the tumourigenic character of continous cell lines has withheld the use of these cells for viral antigen production so far as it was thought that residual cellular DNA present in the vaccine might be able to induce tumours. However, the purification procedures for the viral antigens will reduce the presence of residual cellular DNA to undetectable amounts and it has already become clear by safety studies that induction of tumours by residual cellular DNA is highly unlikely[1,7].

Within the next few years, influenza vaccines based on MDCK-grown antigens are likely to become available. By then, integration of the procedure described in this paper will be of interest. The flexibility of using cell lines, the ease of making reassortant viruses together with the increased yield that can be obtained using these reassortants, make MDCK-SF1 cells an attractive alternative to embryonated chicken eggs.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Economical Affairs (PBTS project): Senter BIO95004. Part of this work was supported by the SRVI:

Foundation for Respiratory Virus Infections. The authors wish to thank Ger van der Water for continuous support.

REFERENCES

[01] Brands, R., Palache, A.M. and van Scharrenburg, G.J.M. 1996. Madin Darby Canine Kidney (MDCK)-cells for the production of inactivated influenza subunit vaccine. Safety characteristics and clinical results in the elderly. In: Brown, L.E., Hampson, A.W. and Webster, R.G. (eds.) *Options for the Control of Influenza III*, pp. 683-693. Elsevier, Amsterdam.

[02] Brands, R., Palache, A.M. and van Scharrenburg, G.J.M. 1997. Development of influenza subunit vaccine produced using mammalian cell culture technology. In: Carrondo, M.J.T., Griffiths, B. and Moreira, J.L.P. (eds.) *Animal Cell Technology: From vaccines to genetic medicine*, pp. 165-167. Kluwer Academic Publishers, Dordrecht.

[03] Claas, E.C.J., Sprenger, M.J.W., Kleter, G.E.M., van Beek, R., Quint, W.G.W. and Masurel, N. 1992. Type-specific identification of influenza A, B and C by the polymerase chain reaction. *J. Virol. Methods* 39:1-13.

[04] Claas, E.C.J., Osterhaus, A.D.M.E., van Beek, R., De Jong, J.C., Rimmelzwaan, G.F., Senne, D.A., Krauss, S., Shortridge, K. and Webster, R.G. 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* 351:472-477.

[05] Cox, N.J., Maassab, H.F. and Kendal, A.P. 1979. Comparative study of wild-type and cold- mutant (temperature-sensitive) influenza viruses: nonrandom reassortment of genes during preparation of live virus vaccine candidates by recombination at 25°C between recent H3N2 and H1N1 epidemic strains and cold-adapted A/Ann Arbor/6/60. *Virology* 97:190-194.

[06] Gubareva, L.V., Wood, J.M., Meyer, W.J., Katz, J.M., Robertson, J.S., Major, D. and Webster, R.G. 1994. Codominant mixtures of viruses in reference strains of influenza virus due to host cell variation. *Virology* 199:89-97.

[07] Horaud, F. Viral vaccines and residual cellular DNA. 1995. *Biologicals* 23:225-228.

[08] de Jong, J.C., Claas, E.C.J., Osterhaus, A.D.M.E., Webster, R.G. and Lim, W.L. A pandemic Warning? 1997. *Nature* 389:554-554.

[09] Kaverin, N.V., Rudneva, I.A., Smirnov, A. and Finskaya, N.N. 1988. Human-avian influenza virus reassortants: effect of reassortment pattern on multi-cycle reproduction in MDCK cells. *Arch. Virol.* 103:117-126.

[10] Klimov, A.I. and Cox, N.J. 1995. PCR restriction analysis of genome composition and stability of cold-adapted reassortant live influenza vaccines. *J. Virol. Methods* 52:41-49.

[11] Kodihalli, S., Justewicz, D.M., Gubareva, L.V. and Webster, R.G. 1995. Selection of a single amino acid substitution in the hemagglutinin molecule by chicken eggs can render influenza A virus (H3) candidate vaccine ineffective. *J. Virol.* 69:4888-4897.

[12] Palache, A.M., Brands, R. and van Scharrenburg, G.J.M. 1997. Immunogenicity and reactogenicity of influenza subunit vaccines produced in MDCK cells or fertilized chicken eggs. *J. Infect. Dis.* 176:S20-S23.

[13] Robertson, J.S., Bootman, J.S., Newman, R., Oxford, J.S., Daniels, R.S., Webster, R.G. and Schild, G.C. 1987. Structural changes in the haemagglutinin which accompany egg adapatation of an influenza A(H1N1) virus. *Virology* 160:31-37.

[14] Robertson, J.S., Bootman, J.S., Nicolson, C., Major, D., Robertson, E.W. and Wood, J.M. 1990. The hemagglutinin of influenza B virus present in clinical material is a single species identical to that of mammalian cell-grown virus. *Virology* 179:35-40.

[15] Robertson, J.S., Cook, P., Nicolson, C., Newman, R. and Wood, J.M. 1994. Mixed populations in influenza virus vaccine strains. *Vaccine* 12:1317-1320.

[16] Rudneva, I.A., Kovaleva, V.P., Varich, N.L., Farashyan, V.R., Gubareva, L.V., Yamnikova, S.S., Popova, I.A., Presnova, V.P. and Kaverin, N.V. 1993. Influenza A virus reassortants with surface glycoprotein genes of the avian parent viruses: effects of HA and NA gene combinations on virus aggregation. *Arch. Virol.* 133:437-450.

[17] Sakamoto, S., Kino, Y., Oka, T., Herlocher, M.L. and Maassab, M.L. 1996. Gene analysis of reassortant influenza virus by RT-PCR followed by restriction enzyme digestion. *J. Virol. Methods* 56:161-171.

[18] Schild, G.C., Oxford, J.S., de Jong, J.C. and Webster, R.G. 1983. Evidence for host-cell selection of influenza virus antigenic variants. *Nature* 303:706-709.

[19] Smeenk, C.A. and Brown, E.G. 1994. The influenza virus variant A/FM/1/47-MA possesses single amino acid replacements in the hemagglutinin, controlling virulence, and in the matrix protein, controlling virulence as well as growth. *J. Virol.* 68:530-534.

[20] Wang, M., Katz, J.M. and Webster, R.G. 1989. Extensive heterogeneity in the hemagglutinin of egg-grown influenza viruses from different patients. *Virology* 171:275-279.

[21] Yasuda, J., Toyoda. T., Nakayama, M. and Ishihama, A. 1993. Regulatory effects of matrix protein variations on influenza virus growth. *Arch. Virol.* 133:283-294.

[22] Yasuda, J., Bucher, D. and Ishihama, A. 1994. Growth control of influenza A virus by M1 protein: analysis of transfectant viruses carrying the chimeric M gene. *J. Virol.* 68:8141-8146.

[23] Xu, X., Kilbourne, E.D., Hall, H.E. and Cox, N.J. 1994. Nonimmunoselected intrastrain genetic variation detected in pairs of high yielding influenza A (H3N2) vaccine and parental viruses. *J. Infect. Dis.* 170:1432-1438.

CHAPTER 3

Introduction of the haemagglutinin transmembrane region in the influenza virus matrix protein facilitates its incorporation into ISCOM and activation of specific $CD8^+$ cytotoxic T lymphocytes

J.T.M. Voeten, G.F. Rimmelzwaan, N.J. Nieuwkoop,
K. Lövgren-Bengtsson and A.D.M.E. Osterhaus

Vaccine 19:522-530 (2000)

SUMMARY

The gene encoding the influenza virus A matrix (MA) protein was cloned into the bacterial expression vector pMalC with and without the sequence encoding the transmembrane region of the haemagglutinin (HA). With the resulting recombinant proteins, immunestimulating complexes (ISCOM) were prepared. The MA protein with the hydrophobic anchor region (rMAHA) associated more efficiently with ISCOM than the unmodified MA protein (rMA). A B-lymphoblastoid cell line (B-LCL) was lysed by an autologous $CD8^+$ cytotoxic T lymphocyte (CTL) clone specific for the MA protein after incubation with rMAHA-ISCOM but not after incubation with rMA, rMAHA, rMA-ISCOM or empty ISCOM. The B-LCL was also lysed by the CTL clone after incubation with empty ISCOM mixed with the respective MA proteins. Incubation of ISCOM with the rMAHA protein proved to be the most efficient in this respect. Addition of the proteasome inhibitors lactacystin or clasto-lactacystin β-lactone to the B-LCL incubated with rMAHA-ISCOM or the MA proteins mixed with empty ISCOM dramatically decreased the lysis by the $CD8^+$ CTL clone. These results indicate that the addition of a hydrophobic anchor to hydrophilic proteins in combination with ISCOM facilitates their entry in the MHC class I processing and presentation pathway. This may be an attractive approach for the development of subunit vaccines aiming at the induction of CTL-mediated immunity.

INTRODUCTION

The immunestimulating complex (ISCOM), a hydrophilic structure held together by hydrophobic interactions and composed of amphipathic and hydrophobic constituents, the adjuvant Quil A, cholesterol and phospholipids, was originally developed as an antigen presentation form for viral membrane proteins and proved to be a potent inducer of humoral and cell-mediated immunity, including cytotoxic T lymphocyte (CTL) immunity[18,23]. In the process of ISCOM preparation, the hydrophobic nature of the membrane-spanning region of such proteins allows their spontaneous incorporation into ISCOM. In contrast, the incorporation of hydrophilic non-transmembrane proteins is usually less efficient. Increasing the efficiency of incorporation of these proteins can be achieved by coupling them to hydrophobic carrier molecules, like bacterial lipopolysaccharide or fatty acids[20,30,37]. However, these procedures are usually cumbersome to

perform, lack universal applicability and may result in conformational changes. A universal approach that would enable the incorporation of hydrophilic proteins into ISCOM would allow a wider application of the ISCOM technology in the field of vaccine development.

The use of ISCOM is especially of interest when vaccination aims at the induction of specific CTL responses. For influenza, currently used vaccines are mainly based on the use of the membrane glycoproteins of the virus, the haemagglutinin (HA) and neuraminidase (NA). The HA of influenza viruses shows a high degree of variation due to the accumulation of mutations in the antigenic determinants, which necessitates the regular adjustment of the vaccine. Although ISCOM-based vaccines prepared with the influenza HA and NA have been shown to induce strong humoral as well as cellular immune responses, conferring protective immunity to vaccinated animals, also ISCOM-based influenza vaccines would have to be adjusted on a regular basis[7,9,15,24-26,28]. The internal influenza virus proteins, like the nucleoprotein (NP) and matrix protein (MA), are well conserved amongst different influenza virus strains and are major targets for CTL[16,27]. However, the role of CTL directed against these proteins in protection or recovery from infection is still controversial[22]. Some studies have shown protection against infection conferred by (vaccine-induced) CTL, while others have shown that CTL only contribute marginally to vaccine-induced protective immunity[2,8,10,14,19,33-35]. Nevertheless, by killing virus-infected cells, CTL do contribute to viral clearance. The induction of CTL-mediated immunity by vaccination largely depends on the nature of the vaccine[17,22]. For a vaccine to induce CTL it is important that the antigen would be delivered into the cytosol of cells where it enters the endogenous route of antigen processing. This leads to the expression of antigenic peptides associated with MHC class I molecules on the surface of the cell. It has been shown that ISCOM-based vaccines are efficient in inducing CTL responses[3,12,13,32].

Here, we describe the preparation of ISCOM with recombinant influenza virus MA protein modified by extending the C-terminus with the transmembrane region of the influenza HA by recombinant DNA technology, a method used previously for incorporation of malaria antigen into ISCOM[1]. The antigen processing of several MA protein ISCOM preparations and the presentation to a MA protein-specific CTL clone were evaluated *in vitro*.

MATERIALS AND METHODS

Cloning, production and purification of recombinant MA proteins

The MA protein encoding gene (RNA segment 7) of influenza virus A/Netherlands/018/94 (H3N2) was cloned into pBluescript after introduction of an *Eco*RI restriction endonuclease recognition site at the 5' end of the MA gene and a *Sal*I site at the 3' end by RT-PCR using the primers 5'CAGGAATTCCTCGAGACGCGTATGAGCCTTCTAACCGAGGTCG and 5'GCGGTCGACTCGCGATCAGTTAACCTTGAATCGTTGCATCTGCACC (restriction sites underlined, start and stop codon in bold). A *Hpa*I site was introduced upstream of the stop codon of the MA gene. The transmembrane and cytoplasmic tail encoding region (aa 530-566) of the HA gene (RNA segment 4) of the same influenza virus was amplified by RT-PCR using the primers 5'GTCGTTAACTGGATC-CTGTGGATTTCCTTTGCC and 5'TCGGTCGACTCGCGATCAAATGCAAATGTT-GCACCTAATG, which introduced a *Hpa*I site at the 5' end and a *Sal*I site at the 3' end, and subsequently subcloned into the pBluescript construct as a *Hpa*I-*Sal*I fragment. To minimize the mutation error rate, Pfu polymerase (Stratagene, La Jolla, USA) was used in the PCR reactions. Both constructs (the MA gene and the MA gene extended with the transmembrane and cytoplasmic tail region of HA) were subcloned as *Eco*RI-*Sal*I fragments into the bacterial expression vector pMalC (New England Biolabs, Hertfordshire, UK) in frame with the maltose binding protein encoding gene (MBP) to yield recombinant fusion proteins consisting of MBP and the MA protein with or without the C- terminal region of HA, referred to as rMAHA and rMA, respectively. RNA isolation, RT-PCR, cloning, production and purification of the recombinant proteins were performed by methods described previously[36].

Analysis of recombinant MA proteins

Recombinant MA proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting according to standard procedures[29]. Nitrocellulose membranes were incubated with blocking buffer (2% nonfat milk powder, 0.05% Tween 20 in phosphate buffered saline (PBS)) for 1 h, followed by 1 h of incubation with 1:1000 diluted mouse monoclonal antibody directed against influenza virus MA protein (kindly provided by Dr. W. Gerhard of the Wistar Institute of Anatomy & Biology, Philadelphia). After washing the blot with PBS, the blot was incubated for 1 h with 1:1000 diluted horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG antibodies (Dako, Glostrup, Denmark). Then, the blot was washed with PBS followed by incubation in diaminobenzidine-H_2O_2 in PBS (250 µg of diaminobenzidine/ml, 0.002% H_2O_2). The reaction was stopped with H_2O after protein bands became visible.

Preparation of immunestimulating complexes (ISCOM)

ISCOM were prepared by adding 1 mg phosphatidylcholine, 1 mg cholesterol and 5 mg semi purified saponins of the tree *Quillaja saponaria* (Iscoprep 703, kindly provided by Dr. B. Sundquist of ISCOTEC, Uppsala, Sweden) to 1 mg recombinant protein in a total volume of 2 ml aqua bidest. containing 2% decanoyl N-methyl-glucamide (Mega-10, Sigma, St. Louis, USA). ISCOM without recombinant protein were also prepared and

referred to as empty ISCOM. The mixture was sonicated for 30 minutes and transferred to a dialysis cassette (Pierce, Rockford, USA). After 30 hrs dialysis against PBS, the ISCOM preparations were harvested from the cassette and stored at -20°C until use. In parallel, ISCOM were prepared in the presence of ^{3}H-labeled cholesterol (2.5 µCi/µl) for analytical purposes. These ISCOM were loaded on 10-60% discontinuous sucrose gradients and centrifuged at 35,000 rpm (Beckmann Ultracentrifuge, SW41 rotor) and 16°C for 16 hrs. Subsequently, 1 ml fractions were collected and analyzed for ^{3}H, as a measure for ISCOM formation, by measuring radioactivity in a scintillation counter (expressed as CPM). The protein content of these fractions was determined by measuring the optical density at a wavelength of 620nm (OD620) after the addition of Bradford reagent[4]. MA protein content was analyzed by an immuno dot blot assay. 20 µl of each fraction was blotted onto nitrocellulose and the blot was further processed as described for the Western Blot.

Isolation of MA protein-specific HLA-A2-restricted CTL clones

In round bottom microtiter plates, 1000 peripheral blood mononuclear cells (PBMCs) of a HLA-A2 positive donor were stimulated twice, with an interval of one week, with 2,5 x 10^4 gamma irradiated (30 minutes 3000 rd) autologous PHA-stimulated PBMCs pulsed with the peptide GILGFVFTL (a HLA-A2-restricted CTL epitope of the influenza virus A MA protein (aa 58-66)). The cells were cultured in RPMI 1640 medium containing L-glutamine (2 mM), penicillin (100 IU/ml), streptamycin (100 µg/ml), 2-mercaptoethanol (2 x 10^{-5} M), IL-2 (50 U/ml) and 10% human pool serum at 37°C and 5% CO_2. One week after the second stimulation, expanding cells were analyzed for peptide-specific CTL activity. Cells from wells showing CTL activity were cloned by limiting dilution (0,3 , 1 and 3 cells per well) and stimulated non-specifically by adding 3 x 10^4 APD B-LCL (B-lymphoblastoid cell line), 3 x 10^4 BSM B-LCL and 6 x 10^5 allogeneic PBMCs (which were all gamma irradiated), 1 µg PHA and 50 U IL-2 per ml[11]. After an incubation of two weeks, clones showing CTL activity were stimulated specifically with gamma irradiated peptide-pulsed autologous PBMCs. After incubating the clones for twelve days they were stimulated non-specifically as described above in 75 cm^2 flasks. After two weeks, cells were harvested, aliquoted and stored at -135°C until use. The $CD3^+$ $CD8^+$ $CD4^-$ phenotype was confirmed by FACS analysis, after staining for CD3, CD4 and CD8, and their MA protein-specificity and HLA-A2 restriction was confirmed in CTL assays. To this end, 1 x 10^6 autologous and mismatched EBV-transformed B-LCL were incubated with the peptide GILGFVFTL (10 µM) or infected with a recombinant vaccinia virus expressing the MA protein of influenza virus A/Netherlands/018/94 (generated essentially as previously described[21]), a control vaccinia virus (VSC65), both at a multiplicity of infection of 10, or infected with 5 x 10^4 $TCID_{50}$/ml influenza virus A/Nanchang/933/95 (kindly provided by Dr. J. Bates, CSL, Sydney, Australia) in 1 ml RPMI 1640 medium for one hour at 37°C and 5% CO_2. Cells were washed with complete RPMI 1640 medium (RPMI 1640 medium containing L-glutamine (2 mM), penicillin (100 IU/ml), streptamycin (100 µg/ml) and 10% fetal bovine serum) and either directly used as target cells in CTL assays (peptide-pulsed cells) or after a further incubation in this medium for 16 hrs (vaccinia and influenza virus-infected cells).

Preparation of target cells for studying processing of antigen preparations

1 x 10^6 autologous EBV-transformed B-LCL were incubated with different antigen preparations in 1 ml complete RPMI 1640 medium at 37°C and 5% CO_2. The antigen

preparations included rMA protein, rMAHA protein, rMA-ISCOM, rMAHA-ISCOM, rMA and rMAHA protein mixed with empty ISCOM and empty ISCOM only. B-LCL were also incubated with empty ISCOM for 1 h, washed with medium and subsequently incubated with rMA or rMAHA protein. The antigen preparations were added to the B-LCL to a final concentration of 7,5 µg protein per ml. All ISCOM were prepared with identical amounts of Quil A, cholesterol and phospholipids and gave similar profiles when analysed by sucrose gradient centrifugation. The amount of protein in the preparations containing empty ISCOM mixed with protein was adjusted to the amount of protein present in ISCOM prepared with protein. Untreated B-LCL served as a negative control. 24 hrs after the addition of the antigen preparations, cells were washed with medium and used as target cells in CTL assays. In order to study proteasome involvement in antigen processing, the highly specific proteasome inhibitors lactacystin or clasto-lactacystin β-lactone (Affinity Research Products, Mamhead Castle, UK) were added to a final concentration of 3 µM.

CTL assays

Target cells (B-LCL) were labeled for 1 h with 100 µCi $Na_2[^{51}Cr]O_4$ in RPMI 1640 medium. Cells were washed three times in complete RPMI 1640 medium and resuspended in this medium to a concentration of 10^4 cells / 50 µl. Effector cells (CTL) were suspended in this medium to a concentration of 10^5 cells / 100 µl. 50 µl Target cells were incubated either with 100 µl medium (spontaneous release), with 100 µl 10% Triton X-100 (maximum release) or with 100 µl effector cells (experimental release; effector to target ratio = 10) for 4 hrs at 37° C. For other effector to target ratios, the concentration of effector cells was adjusted. Supernatants were harvested and radioactivity was measured by gamma counting. The percentage specific lysis was calculated as: 100 x [experimental release - spontaneous release] / [maximum release - spontaneous release]. All CTL assays were performed with ten replicates per target per experiment. Experiments were performed twice for B-LCL incubated with rMA or rMAHA protein mixed with empty ISCOM and three times for control B-LCL and B-LCL incubated with rMA protein, rMAHA protein, rMA-ISCOM, rMAHA-ISCOM or empty ISCOM. Mean values +/- S.E.M. were calculated. Statistical analysis was performed using the Student T test.

RESULTS

Recombinant MA proteins rMA and rMAHA

Highly purified recombinant fusion proteins consisting of MBP and influenza virus MA protein were produced using the pMalC expression system with an estimated purity of 90% for both the rMA and rMAHA proteins (Figure 1A). The molecular mass of rMA protein was approximately 70 kD which correlates with the calculated molecular mass of the fusion protein (40 kD for MBP and 30 kD for the MA protein). The molecular mass of rMAHA protein proved to be slightly higher probably

due to the addition of the C terminal region of the HA. Besides a major band at 70 kD, minor bands of smaller molecular masses were observed. These bands are likely to be the result of some degradation by bacterial proteases or may represent incomplete translation products. The identity of both proteins was confirmed by Western blot analysis using mouse monoclonal antibodies, directed against the MA protein, which showed equal reactivity with both recombinant proteins (Figure 1B).

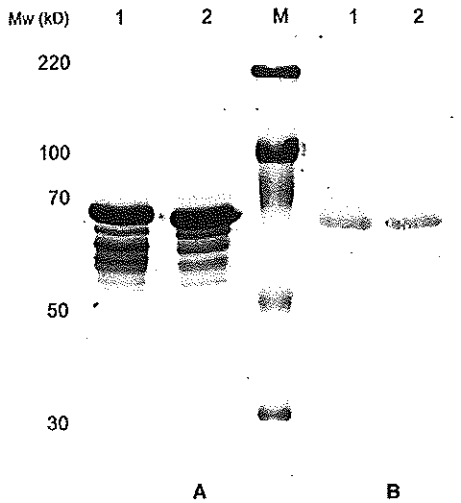

Figure 1. Analysis of rMA and rMAHA protein by SDS-PAGE and Western Blotting. rMAHA (lane 1) and rMA (lane 2) were separated on a SDS-10% polyacrylamide gel and stained with coomassie brilliant blue (A) or transferred to a nitrocellulose membrane which was incubated with mouse monoclonal antibodies specific for influenza virus A MA protein (B) as described in materials and methods.

Analysis of sucrose gradient fractionated ISCOM preparations

Sucrose gradient fractions of rMA-ISCOM and rMAHA-ISCOM were analyzed for ^{3}H-labeled cholesterol and protein content. As shown in figure 2, ISCOM were formed since they typically migrated at a density of 40-45% sucrose which coincides with the fraction containing the highest amount of

cholesterol (fraction 7). This was confirmed by analysis of these fractions by negative contrast electron microscopy which revealed the typical cage-

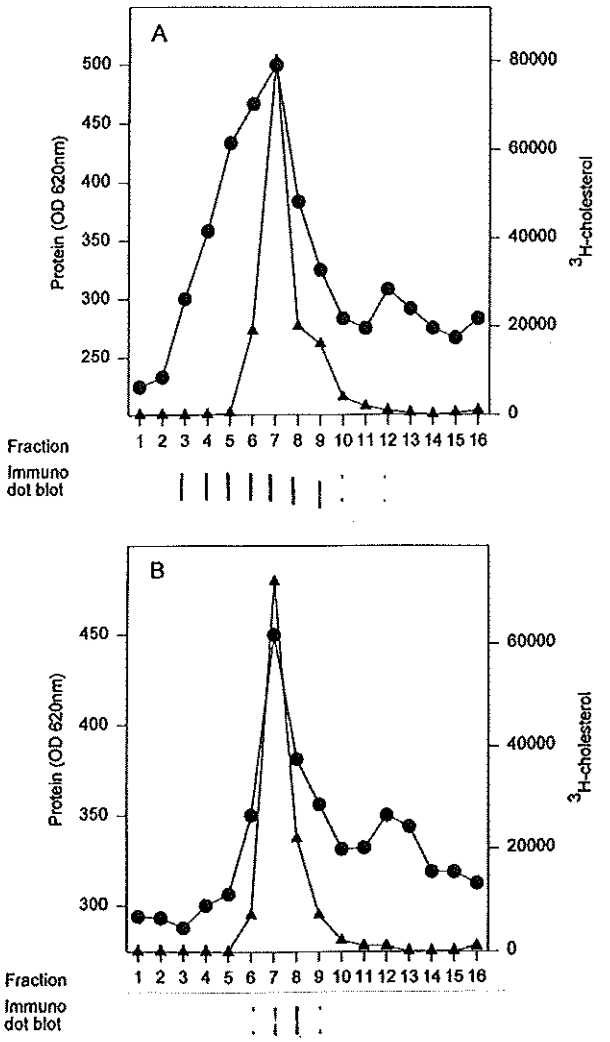

Figure 2. Analysis of rMA and rMAHA-ISCOM. ISCOM were prepared in the presence of rMA (A) or rMAHA (B) protein and loaded onto a 10-60 dicontinuous sucrose gradient. 16 Fractions (from bottom to top) were collected and analyzed for total protein content, expressed as the optical density at a wavelength of 620 nm (circles), and ^{3}H-labeled cholesterol, expressed as CPM (triangles). Fractions 3 to 14 were also analyzed for the presence of MA protein in an immuno dot blot assay using mouse monoclonal antibodies directed against influenza virus MA protein. Reactivities of the individual fractions are shown at the bottom of each graph.

like structure of the ISCOM (data not shown). The rMAHA protein comigrated with the ^{3}H-labeled cholesterol whereas the rMA protein showed a wider distribution. Although equal amounts of soluble rMA- and rMAHA protein were used in the preparation of ISCOM, the total amount of rMAHA protein retrieved after ISCOM formation, centrifugation and fractionation was lower compared to rMA protein. It is possible that part of the rMAHA has become insoluble during the preparation process and subsequently was not incorporated into ISCOM. Nevertheless, the fact that the distribution of rMAHA protein in the gradient exactly followed the distribution of ISCOM and that rMA protein was also detected in fractions devoid of any ISCOM material may indicate a more efficient incorporation of rMAHA protein into ISCOM. Immuno dot blot analysis confirmed the identity of the proteins present in the ISCOM fractions.

Analysis of MA protein-specific, HLA-A2-restricted, CTL clones

In order to study MHC class I-restricted recognition of MA protein, CTL clones were isolated after stimulating PBMCs of a HLA-A2 donor with the known A2 peptide GILGFVFTL. From one cloning event, five clones were isolated. The results with one representative clone are shown in figure 3. It was shown that the CTL clone lysed peptide-pulsed target cells but not control target cells. It lysed target cells infected with influenza virus or a recombinant vaccinia virus expressing the influenza virus MA protein but failed to lyse target cells infected with a control vaccinia virus or mismatched target cells. This confirmed that the CTL clone recognized naturally liberated peptides from the MA protein in a HLA-A2-restricted fashion. FACS analysis confirmed that the clones were all $CD3^{+}$ $CD8^{+}$ $CD4^{-}$ (not shown).

Processing of MA protein and MHC class I antigen presentation by ISCOM

To measure MHC class I presentation of the peptide derived from the influenza virus MA protein, the recognition of the MHC class I peptide complex by the CTL clone was analyzed in CTL assays using autologous B-LCL as target cells. As shown in figure 4A, control B-LCL or B-LCL incubated with empty ISCOM were not recognized by the MA protein-specific CTL clone. Also B-LCL incubated with the soluble recombinant proteins with or without the addition of the transmembrane region of HA were poorly recognized by the CTL clone. B-LCL incubated with ISCOM

prepared with the rMA protein did not significantly differ in their recognition by the CTL clone from B-LCL incubated with the soluble rMA protein. In contrast, the specific lysis observed with B-LCL incubated with rMAHA-ISCOM was significantly higher ($p<0.005$) than that observed after incubation with soluble rMAHA protein or rMA-ISCOM. Interestingly, target cells simultaneously incubated with empty ISCOM and recombinant proteins rMA or rMAHA were lysed significantly more efficient by the CTL clone ($p<0.0001$) than B-LCL incubated with rMA-ISCOM or rMAHA-ISCOM. The specific lysis of B-LCL incubated with empty ISCOM and rMAHA protein was significantly higher than that of B-LCL incubated with empty ISCOM and rMA protein ($p<0.0001$). To assess whether this observation was due to association of the proteins with empty ISCOM, or the result of protein entry into the B-LCL after being sensitized by ISCOM, B-LCL were incubated with empty ISCOM and, after washing, subsequently incubated with rMA or rMAHA protein. No lysis of these target cells was observed (data not shown). To confirm that the MA protein was processed in the endogenous route of antigen processing, B-LCL were incubated with the respective antigen preparations in the presence of the proteasome inhibitors lactacystin or clasto-lactacystin β-lactone[6]. The results obtained with B-LCL incubated with empty ISCOM and rMAHA protein are shown in figure 4B. Addition of 3 μM of the proteasome inhibitors dramatically decreased the specific lysis of these target cells by the CTL clone, demonstrating that presentation was dependend on proteasome activity. Similar results were obtained with target cells incubated with empty ISCOM and rMA protein or target cells incubated with rMAHA-ISCOM (data not shown). Full inhibition could not be demonstrated as the concentration of these agents necessary to completely block proteasome activity (>10 μM) is toxic for B-LCL[5].

DISCUSSION

Since ISCOM were first described as an antigen presentation form for viral membrane proteins, their potential as candidate vaccines against a variety of viruses has been demonstrated[18,23]. One of the limitations of the ISCOM technology is that only proteins with highly hydrophobic regions such as envelope glycoproteins, can efficiently be incorporated into ISCOM, whereas hydrophilic proteins, such as most of the internal proteins of enveloped viruses, are incorporated poorly or not at all. Incorporation of such proteins was only observed after chemical modification or conjugation

to hydrophobic carrier molecules[20,30,37]. Here, we describe an alternative approach taking advantage of the transmembrane region of influenza virus A HA which has been shown to facilitate efficient incorporation of this protein into ISCOM. The sequence encoding the transmembrane region of HA was cloned into the 3' end of the influenza virus MA protein encoding gene, which was subsequently expressed as a recombinant fusion protein. The transmembrane region of the HA indeed facilitated the incorporation of the MA protein into ISCOM. To investigate whether the MA protein containing ISCOM were processed by the endogenous antigen processing pathway resulting in MHC class I presentation of the MA protein-derived peptide GILGFVFTL, the recognition of antigen-pulsed target cells by a HLA-A2-restricted $CD8^+$ CTL clone specific for this peptide was studied in CTL assays. It was shown that target cells incubated with rMAHA-ISCOM were lysed by the MA protein-specific CTL clone, whereas target cells incubated with ISCOM prepared with the MA protein lacking the hydrophobic extension or soluble rMA or rMAHA protein were not recognized. From these data it was concluded that the addition of the transmembrane region of HA facilitated incorporation into the ISCOM structure and that this was necessary for entry into the endogenous route of antigen processing.

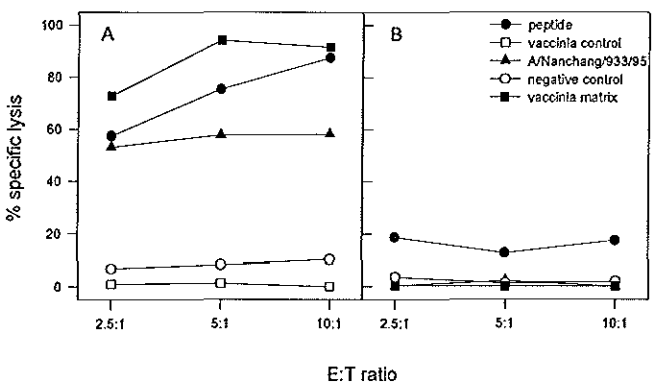

Figure 3. Confirmation of influenza virus MA protein-specificity and HLA-A2 restriction of one CTL clone. B-LCL of a HLA-A2 positive (A) and a HLA-A2 negative (B) donor were incubated with peptide GILGFVFTL (solid circles), infected with recombinant vaccinia virus expressing the influenza virus MA protein (solid squares), control vaccinia virus (open squares) or influenza virus (triangles) and used as target cells in CTL assays with the CTL clone as effector cells. Untreated B-LCL (open circles) served as a negative control.

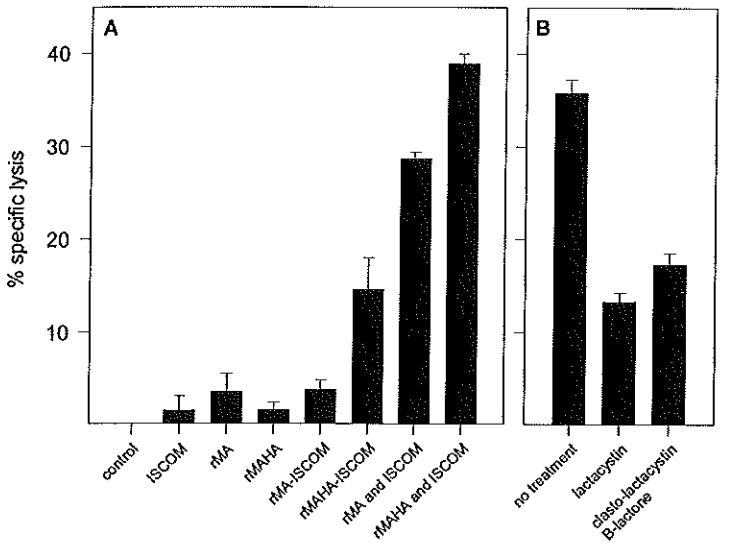

Figure 4. MHC class I presentation of influenza virus MA protein and influence of proteasome inhibitors. B-LCL were incubated with empty ISCOM, rMA protein, rMAHA protein, rMA-ISCOM, rMAHA-ISCOM, rMA protein and empty ISCOM, rMAHA protein and empty ISCOM or left untreated (A). In another experiment, B-LCL were incubated with rMAHA protein and empty ISCOM in normal medium or in medium containing 3 μM of the proteasome inhibitor lactacystin or clasto-lactacystin β-lactone (B). The B-LCL were used as target cells in CTL assays with a MA protein–specific CTL clone as effector cells. CTL assays were performed with ten replicates per target per experiment at an effector to target ratio of 10. Mean percentages specific lysis +/- S.E.M. are shown for one (B) or two similar experiments (A).

Interestingly, autologous B-LCL incubated with empty ISCOM together with the recombinant proteins rMA or rMAHA also resulted in the specific lysis of these cells by the MA protein-specific CTL clone. These target cells were lysed significantly more efficiently than the target cells incubated with rMA- or rMAHA-ISCOM. It can not be excluded, however, that possible differences in the composition of the respective ISCOM preparations had an influence on the level of antigen presentation. This finding conflicts with data obtained in previous studies showing that the measles virus fusion (F) protein was only processed for MHC class I presentation by B-LCL after

incorporation into ISCOM and not when it was administered in the presence of empty ISCOM[3]. The processing and presentation we observed after mixing empty ISCOM with proteins indicate that the proteins can associate with the ISCOM through hydrophobic interactions. Indeed, the MA protein contains hydrophobic domains which are most likely involved in interactions with the lipid membrane of the virus[31,38]. The finding that the addition of the hydrophobic transmembrane region of HA improves presentation of the MA peptide is in agreement with this hypothesis. Furthermore it was shown that the physical interaction between empty ISCOM and protein is required for MHC class I processing and presentation of the MA protein since pre-incubation of the target cells with empty ISCOM followed by an incubation with protein did not result in recognition of the target cells by the CTL clone. The bacterial fusion part, MBP, did not appear to play a direct role in incorporation into ISCOM or entry of recombinant proteins into the endogenous processing pathway since rMA-ISCOM and soluble rMA- or rMAHA failed to sensitize B-LCL for recognition by the CTL clone.

Recognition of the target cells incubated with rMAHA-ISCOM or empty ISCOM together with rMA or rMAHA protein by the CTL clone showed to be influenced by the proteasome inhibitors lactacystin and clasto-lactacystin β-lactone. This indicates that processing was dependend on proteasome activity, which is important for cytosolic protein degradation typical for endogenous processing, and that the antigenic peptides were liberated from ISCOM-delivered MA protein in the cytosolic compartment rather than in lysosomes from which they could have leaked into the cytosol.

In conclusion, we have demonstrated that ISCOM facilitate the entry of influenza virus MA protein into the endogenous processing pathway leading to MHC class I presentation of a HLA-A2-restricted CTL epitope. This was improved after its co-expression with the transmembrane region of influenza HA. The introduction of a hydrophobic region into hydrophilic proteins by way of recombinant DNA technology would in principle be universally applicable and could therefore be exploited for the development of ISCOM vaccines for a variety of virus infections against which CTL-mediated immunity plays a role.

ACKNOWLEDGEMENTS

This study was financially supported by ISCOTEC, Uppsala, Sweden. Part of this work was supported by the Foundation for Respiratory Virus

Infections, notably Influenza (SRVI). The authors wish to thank Ger van der Water for continuous support.

REFERENCES

[01] Andersson, C., Sandberg, L., Murby, M., Sjölander, A., Lövgren-Bengtsson, K. and Ståhl, S. 1999. General expression vectors for production of hydrophobically tagged immunogens for direct iscom incorporation. *J. Immunol. Meth.* 222:171-182.

[02] Bender, B.S., Bell, W.E., Taylor, S. and Small Jr, P.A. 1994. Class I major histocompatibility complex-restricted cytotoxic T lymphocytes are not necessary for heterotypic immunity to influenza. *J. Inf. Dis.* 170:1195-1200.

[03] van Binnendijk, R.S., van Baalen, C.A., Poelen, M.C.M., de Vries, P., Boes, J., Cerundolo, V., Osterhaus, A.D.M.E. And UytdeHaag, F.G.C.M. 1992. Measles virus transmembrane fusion protein synthesized de novo or presented in immunostimulating complexes is endogenously processed for HLA class I- and classII-restricted cytotoxic t cell recognition. *J. Exp. Med.* 176:119-128.

[04] Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

[05] Cerundolo, V., Benham, A., Braud, V., Mukherjee, S., Gould, K., Macino, B., Neefjes, J. and Townsend, A. 1997. The proteasome-specific inhibitor lactacystin blocks presentation of cytotoxic T lymphocyte epitopes in human and murine cells. *Eur. J. Immunol.* 27:336-341.

[06] Craiu, A., Gaczynska, M., Akopian, T., Gramm, C.F., Fenteany, G., Goldberg, A.L. and Rock, K.L. 1997. Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. *J. Biol. Chem.* 272:13437-13445.

[07] Deliyannis, G., Jackson, D.C., Dyer, W., Bates, J., Coulter, A., Harling-McNabb, L. and Brown, L.E. 1998. Immunopotentiation of humoral and cellular responses to inactivated influenza vaccines by two different adjuvants with potential for human use. *Vaccine* 16:2058-2068.

[08] Fu, T.M., Friedman, A., Ulmer, J.B., Liu, M.A. and Donnelly, J.J. 1997. Protective cellular immunity: cytotoxic T-lymphocyte responses against dominant and recessive epitopes of influenza virus nucleoprotein induced by DNA immunization. *J.Virol.* 71:2715-2721.

[09] Ghazi, H.O., Potter, C.W., Smith, T.L. and Jennings, R. 1995. Comparative antibody responses and protection in mice immunised by oral or parenteral routes with influenza virus subunit antigens in aqueous form or incorporated into ISCOMs. *J. Med. Microbiol.* 42:53-61.

[10] Graham, M.B. and Braciale, T.J. 1997. Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *J. Exp. Med.* 186:2063-2068.

[11] van de Griend, R.J., van Krimpen, B.A., Bol, S.J.L., Thompson, A. and Bolhuis, R.L.H. 1984. Rapid expansion of human cytotoxic T cell clones: Growth promotion by a heat-labile serum component and various types of feeder cells. *J. Immunol. Meth.*

66:285-298.

[12] Heeg, K., Kuon, W. and Wagner, H. 1991. Vaccination of class I major histocompatibility complex (MHC)-restricted murine CD8+ cytotoxic T lymphocytes towards soluble antigens: immunostimulating-ovalbumin complexes enter the class I MHC-restricted antigen pathway and allow sensitization against the immunodominnant peptide. *Eur. J. Immunol.* 21: 1523-1527.

[13] Jones, P.D., Tha Hla, R., Morein, B., Lovgren, K. and Ada, G.L. 1988. Cellular immune responses in the murine lung to local immunization with influenza A virus glycoproteins in micelles and immunostimulatory complexes (iscoms). *Scand. J. Immunol.* 27:645-652.

[14] Lawson, C.M., Bennink, J.R., Restifo, N.P., Yewdell, J.W. and Murphy, B.R. 1994. Primary pulmonary cytotoxic T lymphocytes induced by immunization with a vaccinia virus recombinant expressing influenza A virus nucleoprotein peptide do not protect mice against challenge. *J. Virol.* 68:3505-3511.

[15] Lövgren, K., Kaberg, H. and Morein, B. 1990. An experimental influenza subunit vaccine (iscom): induction of protective immunity to challenge infection in mice after intranasal or subcutaneous administration. *Clin. exp. Immunol.* 82:435-439.

[16] McMichael, A.J., Mitchie, C.A., Gotch, F.M., Smith, G.L. and Moss, B. 1986. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. *J. Gen. Virol.* 67:719-726.

[17] McMichael, A. 1994. Cytotoxic T lymphocytes specific for influenza virus. *Curr. Top. Microbiol. Immunol.* 189:75-91.

[18] Morein, B., Sundquist, B., Hoglund, S., Dalsgaard, K. and Osterhaus, A.D.M.E. 1984. Iscom, a novel structure for antigenic presentation of membrane proteins of enveloped viruses. *Nature* 308:457-459.

[19] Moskophidis, D. and Kioussis, D. 1998. Contribution of virus-specific CD8+ cytotoxic T cells to virus clearance or pathological manifestations of influenza virus infection in a T cell receptor transgenic mouse model. *J. Exp. Med.* 188:223-232.

[20] Reid, G. 1992. Soluble proteins incorporate into iscoms after covalent attachment of fatty acid. *Vaccine* 10:597-602.

[21] Rimmelzwaan, G.F., Siebelink, K.H., Huisman, R.C., Moss, B., Francis, M.J. and Osterhaus, A.D.M.E. 1994. Removal of the cleavage site of recombinant feline immunodeficiency virus envelope protein facilitates incorporation of the surface glycoprotein in immune-stimulating complexes. *J. Gen. Virol.* 75:2097-2102.

[22] Rimmelzwaan, G.F. and Osterhaus, A.D.M.E. 1995. Cytotoxic T lymphocyte memory: role in cross-protective immunity against influenza? *Vaccine* 13:703-705.

[23] Rimmelzwaan, G.F. and Osterhaus, A.D.M.E. 1997. ISCOMs: Materials, Preparation, Antigen Delivery and Immune Response. In: Gander, B., Merkle, H.P. and Corradin, G (eds.) *Antigen delivery systems. Immunological and technological issues,* 8:123-128. Harwood academic publishers, Amsterdam.

[24] Rimmelzwaan, G.F., Baars, M., van Beek, R., van Amerongen, G., Lovgren-Bengtsson, K., Claas, E.C.J. and Osterhaus, A.D.M.E. 1997. Induction of protective immunity against influenza virus in a macaque model: comparison of conventional and iscom vaccines. *J. Gen. Virol.* 78: 757-765.

[25] Rimmelzwaan, G.F., Claas, E.C.J., van Amerongen, G., de Jong, J.C. and Osterhaus, A.D.M.E. 1999. ISCOM vaccine induced protection against a lethal challenge with a human H5N1 influenza virus. *Vaccine* 17:1355-1358.

[26] Rimmelzwaan, G.F., Baars, M., de Lijster, P., Claas, E.C.J. and Osterhaus, A.D.M.E. 1999. Influenza virus subtype cross-reactivities of haemagglutination inhibiting and virus neutralising serum antibodies induced by infection and vaccination with an ISCOM-based vaccine. *Vaccine* 17:2512-2516.

[27] Rouse, B.T., Norley, S. and Martin, S. 1988. Antiviral cytotoxic T lymphocyte induction and vaccination. *Rev. Infect. Dis.* 10:16-33.

[28] Sambhara, S., Woods, S., Arpino, R., Kurichh, A., Tamane, A., Underdown, B., Klein, M., Lövgren-Bengtsson, K., Morein, B. and Burt, D. 1998. Heterotypic protection against influenza by immunostimulating complexes is associated with the induction of cross-reactive cytotoxic T lymphocytes. *J. Infec. Dis.* 177:1266-1274.

[29] Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

[30] Scheepers, K. and Becht, H. 1994. Protection of mice against an influenza virus infection by oral vaccination with viral nucleoprotein incorporated into immunestimulating complexes. *Med. Mircobiol. Immunol.* 183:265-278.

[31] Sha, B. and Luo, M. 1997. Structure of a bifunctional membrane-RNA binding protein, influenza virus matrix protein M1. *Nature Struct. Biol.* 4:239-244.

[32] Takahashi, H., Takeshita, T., Morein, B., Putney, S., Germain, R.N. and Berzofsky, J.A. 1990. Induction of CD8+ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMs. *Nature* 344:873-875.

[33] Tite, J.P., Hughes-Jenkins, C., O'Callaghan, D., Dougan, G., Russell, S.M., Gao, X.M. and Liew, F.Y. 1990. Anti-viral immunity induced by recombinant nucleoprotein of influenza A virus. II. Protection from influenza infection and mechanism of protection. *Immunology* 71:202-207.

[34] Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., DeWitt, C.M., Friedman, A., Hawe, L.A., Leander, K.R., Martinez, D., Perry, H.C., Shiver, J.W., Montgomery, D.L. and Liu, M.A. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259:1745-1749.

[35] Ulmer, J.B., Fu, T.M., Deck, R.R., Friedman, A., Guan, L., DeWitt, C., Liu, X., Wang, S., Liu, M.A., Donnelly, J.J. and Caulfield M.J. 1998. Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. *J. Virol.* 72:5648-5653.

[36] Voeten, J.T.M., Groen, J., van Alphen, D., Claas, E.C.J., de Groot, R., Osterhaus, A.D.M.E. and Rimmelzwaan, G.F. 1998. Use of recombinant nucleoproteins in enzyme-linked immunosorbent assays for detection of virus-specific immunoglobulin A (IgA) and IgG antibodies in influenza virus A- or B-infected patient. *J.Clin. Microbiol.* 36:3527-3531.

[37] Weiss, H., Stitz, I. and Becht, H. 1990. Immunogenic properties of iscom prepared with influenza virus nucleoprotein. *Arch. Virol.* 114:109-120.

[38] Ye, Z., Pal, R., Fox, J.W. and Wagner, R.R. 1987. Functional and antigenic domains of the matrix (M1) protein of influenza A virus. *J. Virol.* 61:239-246.

CHAPTER 4

Multiple routes of antigen processing for HLA-B27-associated presentation of exogenous influenza A virus nucleoprotein by B-lymphoblastoid cells

J.T.M. Voeten, G.F. Rimmelzwaan, N.J. Nieuwkoop,
R.A.M. Fouchier and A.D.M.E. Osterhaus

Submitted for publication

SUMMARY

In general, exogenous proteins are processed by APC in the endosomes for MHC class II presentation to $CD4^+$ T cells, while proteins synthesized endogenously are processed in the cytoplasm for MHC class I presentation to $CD8^+$ T cells. However, it is recognized that exogenous proteins can be processed for MHC class I presentation also and evidence in favour of alternatives to the conventional MHC class I processing and presentation pathway is accumulating. Here, we show that exogenous recombinant influenza A virus nucleoprotein (rNP) is processed for MHC class I presentation to $CD8^+$ CTL by EBV-transformed B-lymphoblastoid cell lines (B-LCL). The presentation of the HLA-A3-restricted peptide ILRGSVAHK (aa 265-273) was found to be less efficient than that of the HLA-B27-restricted peptide SRYWAIRTR (aa 383-391), since approximately hundred times more rNP or peptide was required to sensitize B-LCL cells for recognition by HLA-A3-restricted CTL. Processing of rNP for HLA-B27-associated presentation seemed to follow the conventional MHC class I pathway predominantly, since presentation was diminished in the presence of lactacystin (inhibiting proteasome activity) and brefeldin A (inhibiting transport from the endoplasmic reticulum) but was less sensitive to chloroquine and NH_4Cl (both inhibiting proteolysis in endosomes). HLA-B27-associated presentation was also observed using cells lacking a functional TAP, suggesting that alternative pathways may be exploited for processing of rNP.

INTRODUCTION

In virus-infected cells, antigenic peptides are liberated in the cytoplasm from endogenously synthesized proteins and are presented to $CD8^+$ CTL by MHC class I molecules. These proteins are degraded by a multi-enzyme complex, the proteasome, which generates peptides 9-12 amino acids long that are subsequently transported to the endoplasmic reticulum (ER) by TAP. In the ER, the peptides associate with MHC class I molecules and the resulting complex traverses the Golgi apparatus to be presented at the surface of the cell for recognition by specific CTL[13,15,23,24,47].

Although proteins synthesized in the cytoplasm of infected cells are the main substrate for the conventional (endogenous) MHC class I processing

and presentation pathway, it has become evident that peptides liberated from exogenous proteins can be presented in a MHC class I-restricted fashion also and evidence in favour of alternatives to the conventional MHC class I pathway is accumulating[29,30,46]. Indeed, it has been shown that macrophages, as well as other APC, can process a vast diversity of exogenous antigens, including particulate antigens like bacteria, bead-coupled antigens, cell debris, inactivated viruses, and antigens associated with or incorporated into virosomes, liposomes or immune stimulating complexes (iscoms) for MHC class I presentation[46]. Recently, it was shown that MHC class I presentation of exogenous antigens may be necessary for the induction of CTL responses whereby APC function as messengers for infected non-lymphoid cells (by taking up viral proteins or virus-infected cells) which would otherwise be difficult to target by specific CTL[35,36].

Several mechanisms for processing of exogenous proteins and MHC class I presentation have been proposed. The results of several studies suggest a model in which endocytosed exogenous proteins gain access to the cytoplasm either by leakage from endosomal compartments or by a yet unknown transport mechanism, and subsequently follow the conventional MHC class I pathway[8,20,25,31,33]. Results of other studies suggest an alternative mechanism in which endocytosed exogenous proteins are degraded in an endosomal compartment and subsequently loaded onto MHC class I molecules[3,14,22,34,38]. This could take place either extracellularly following regurgitation, or in the endosomal compartment itself following internalization of MHC class I molecules from the cell surface.

Processing of exogenous proteins for MHC class I presentation has been demonstrated almost exclusively for macrophages and dendritic cells[46]. B-lymphocytes usually process exogenous antigen for MHC class II presentation by means of internalization of antigen bound to surface Ig (receptor-mediated endocytose). It has been demonstrated that B-lymphocytes can present exogenous antigen in association with MHC class I molecules also, but only if the antigen matches the surface Ig[6,17].

Here, we describe MHC class I presentation of exogenous recombinant influenza A virus nucleoprotein (rNP) by EBV-transformed B-lymphoblastoid cell lines (B-LCL). MHC class I processing and presentation of rNP-derived peptides were studied using CTL clones specific for the HLA-A3-restricted peptide ILRGSVAHK (aa 265-273) and the HLA-B27-restricted peptide SRYWAIRTR (aa 383-391).

MATERIALS AND METHODS

Production of rNP

rNP of influenza virus A/HongKong/2/68;H3N2 (A/HK/2/68), A/Netherlands/18/94;H3N2 (A/Neth/18/94), and B/Harbin/7/94 (B/Har/7/94) was produced as described previously[40]. In brief, the nucleoprotein (NP)-encoding genes were cloned at the 3' end of the maltose binding protein (MBP)-encoding gene using the bacterial expression vector pMalC (New England Biolabs) to yield fusion proteins consisting of MBP at the N-terminus and NP at the C-terminus. Recombinant MBP alone (rMBP) was generated from the empty vector as a control. The resulting recombinant proteins were purified by affinity chromatography using amylose resin columns (New England Biolabs).

Isolation and analysis of NP-specific CTL clones

A HLA-B27-restricted CTL clone, designated NP/B27, with specificity for the influenza A virus NP epitope SRYWAIRTR (aa 383-391) was isolated as previously described[41]. A HLA-A3-restricted CTL clone, designated NP/A3, specific for the influenza A virus NP epitope ILRGSVAHK (aa 265-273) was kindly provided by Dr. W. Biddison, NIH, Bethesda, MD. Phenotype, specificity and HLA restriction of both CTL clones were confirmed as described[41].

CTL assays

$1x10^6$ B-LCL cells of a HLA-A3 and -B27 positive donor were incubated with rNP derived from A/HK/2/68, A/Neth/18/94 or B/Har/7/94, or rMBP (0-50 µg/ml), or were incubated with the peptide ILRGSVAHK or SRYWAIRTR (0-10 µM) in 1 ml RPMI 1640 medium containing L-glutamin (2mM), penicillin (100 IU/ml), streptomycin (100 µg/ml) and 10% FBS (culture medium) at 37°C and 5% CO_2. In addition, B-LCL cells were infected with influenza virus (Resvir-9; a H3N2 reassortant influenza virus obtained from A/Nanchang/933/95;H3N2 and A/Puerto Rico/8/34;H1N1) at a multiplicity of infection (m.o.i.) of 0.1. Infections were performed for 1 h in 1 ml RPMI 1640 medium without FBS, washed with culture medium and further cultured in 1 ml of this medium. Also, $1x10^6$ BM28.7 and BM36.1 cells, both transfectant cell lines expressing HLA-B27 with the BM36.1 cell line differing from BM28.7 in a 2 bp deletion in TAP2 leading to a nonfunctional TAP1/TAP2 complex[18], were incubated with rNP derived from A/HK/2/68 (0-5 µg/ml), the peptide SRYWAIRTR (0-10 nM) or infected with influenza virus (m.o.i. = 0.1) as described above. The BM28.7 and BM36.1 cell lines were cultured in the presence of 300 µg/ml geneticin. In selected experiments, cells were cultured in the continuous presence of lactacystin (3 µM) or brefeldin A (50 µM). After an incubation of 1 h (peptide-pulsed cells) or 16 hrs (rNP-incubated or influenza virus-infected cells), target cells were washed with culture medium to remove excess peptide, antigen, lactacystin and brefeldin A and labeled for 1 h with 75 µCi $Na_2[^{51}Cr]O_4$. After washing three times with culture medium, 10^4 target cells were incubated with 10^5 cells of the NP/A3 or NP/B27 CTL clone (E:T ratio = 10) in a total volume of 150 µl for 4 hrs at 37°C. Since the BM28.7

and BM36.1 cell lines are HLA-A3 negative, only NP/B27 CTL were used as effector cells in experiments with these cell lines. Target cells were also incubated in culture medium (spontaneous release) or in 10% Triton X-100 (maximum release). Supernatants were harvested and radioactivity was measured by gamma counting. The percentage specific lysis was calculated as: 100 x [experimental release - spontaneous release] / [maximum release - spontaneous release]. CTL assays were performed at least in tenfold. Statistical analysis was performed using Student-T tests.

Lymphocyte Stimulation Test (LST)

$1x10^6$ B-LCL cells were incubated with rNP derived from A/HK/2/68 (50-100 µg/ml) or infected with influenza virus (m.o.i. = 0.1) as described above, and cultured in the presence or absence of lactacystin (3 µM), brefeldin A (50 µM), chloroquine (50 µM) or NH_4Cl (20 mM). Also, $1x10^6$ BM28.7 and BM36.1 cells were incubated with rNP derived from A/HK/2/68 (100 µg/ml) and cultured in the presence or absence of these inhibitors. Culture conditions were as described above. After an incubation of 16 hrs, cells were washed twice with PBS to remove antigen and inhibitors, fixed with paraformaldehyde (1.5%), and resuspended in RPMI 1640 medium containing L-glutamin (2mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), 2-mercapto-ethanol ($5x10^{-5}$ M) and 10% human pool serum. $5x10^4$ of these stimulator cells were incubated with $5x10^4$ cells of the NP/A3 or NP/B27 CTL clone in a total volume of 200 µl at 37°C and 5% CO_2. After 3 days, 10 µl ^{3}H-labeled thymidine (50 µCi/ml) was added and after an incubation of 16 hrs cells were harvested and thymidine incorporation was measured using a ß-plate reader (LKB Wallac). The stimulation index was calculated as: radioactivity sample/radioactivity negative control (medium). LST were performed in triplicate.

Radio Immuno Precipitation Assay (RIPA)

$6x10^6$ B-LCL cells were washed in PBS and resuspended in 3 ml RPMI 1640 medium without L-methionin and L-cystin, supplemented with L-glutamin (2mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), 5% dialysed FBS, and incubated at 37°C and 5% CO_2. After 30 minutes, 300 µCi of a mixture of ^{35}S-labeled L-methionin and L-cystin was added and incubated for 15 minutes. Cells were pelleted and resuspended in 2 ml ice cold RIPA buffer (10 mM Tris pH 7.8, 150 mM NaCl, 600 mM KCl, 4 mM EDTA, 2% Triton X-100, 2.5 mM iodocetamide and protease inhibitor (Complete, Boehringer Mannheim). After incubation on ice for 10 minutes, the lysate was centrifuged and the supernatant used for immuno-precipitation. 1% of the volume (20 µl), diluted in 300 µl PBS, was incubated with 50 µl of a 20% suspension of protein A beads and 10 µl control mouse ascites fluid for 1 h at 4°C. Beads were pelleted and the supernatant was incubated with 50 µl protein A beads and 5 µl normal mouse serum for 1 h at 4°C. Again, beads were pelleted and the pre-cleared supernatant was used for specific precipitation with monoclonal antibodies directed against HLA-A3 or -B27 (Biotest). 50% of the lysate was incubated with 100 µl protein A beads and 50 µl anti HLA-A3 antibody or anti HLA-B27 antibody and incubated for 1 h at 4°C. Beads were pelleted and resuspended in SDS-PAGE sample buffer to a final volume of 150 µl. The supernatant was subjected to a second round of precipitation with the respective monoclonal antibodies after which the beads were resuspended in SDS-PAGE

sample buffer to a final volume of 150 µl. Samples were boiled for 3 minutes and 50 µl was loaded onto a 15% polyacrylamide gel. After electrophoresis, gels were incubated in a solution of 40% methanol and 10% acetic acid for 1 h, followed by incubation in distilled H_2O for 30 minutes. Then, the gels were incubated with amplify solution (Pharmacia Amersham) for 30 minutes, dried under vacuum at 80°C for 2 hrs and exposed to X-ray film.

RESULTS

MHC class I presentation of rNP

B-LCL cells of a HLA-A3 and -B27 positive donor were incubated with various concentrations of rNP derived from A/HK/2/68 and used as target cells in CTL assays (Figure 1A). B-LCL cells incubated with as little as 0.2 µg rNP were recognized by NP/B27 CTL. The percentage of specific lysis by these CTL increased with increasing concentrations of rNP. The B-LCL cells were also recognized and lysed by NP/A3 CTL, but only after incubation with rNP at the highest concentration tested (50 µg/ml). The recognition of the rNP-derived peptides ILRGSVAHK (aa 265-273) and SRYWAIRTR (aa 383-391) by NP/A3 and NP/B27 CTL respectively, was also studied with B-LCL from other donors which were either HLA-A3, HLA-B27 or HLA-A3 and -B27 positive, but with mismatching other HLA-A and -B alleles. With these B-LCL, comparable results were obtained (data not shown). Using peptide-pulsed cells it was found that at saturating concentrations of peptide, the B-LCL cells were lysed equally well by NP/A3 and NP/B27 CTL whereas at low concentrations of peptide (below 0.01 µM) only NP/B27 CTL specifically lysed the B-LCL cells (Figure 1B). Thus, HLA-B27 requires approximately 100-fold less rNP or peptide for efficient lysis by NP/B27 CTL. Furthermore, in LST, proliferation of NP/B27 CTL was observed after stimulation with B-LCL cells incubated with rNP (Figure 4B) while NP/A3 CTL failed to respond (data not shown). In order to further address the observed differences in recognition by NP/A3 and NP/B27 CTL, the expression levels of HLA-A3 and HLA-B27 molecules in the B-LCL cells was studied. In a RIPA it was shown that the expression levels of HLA-A3 and HLA-B27 in the B-LCL cells used were comparable (Figure 2).

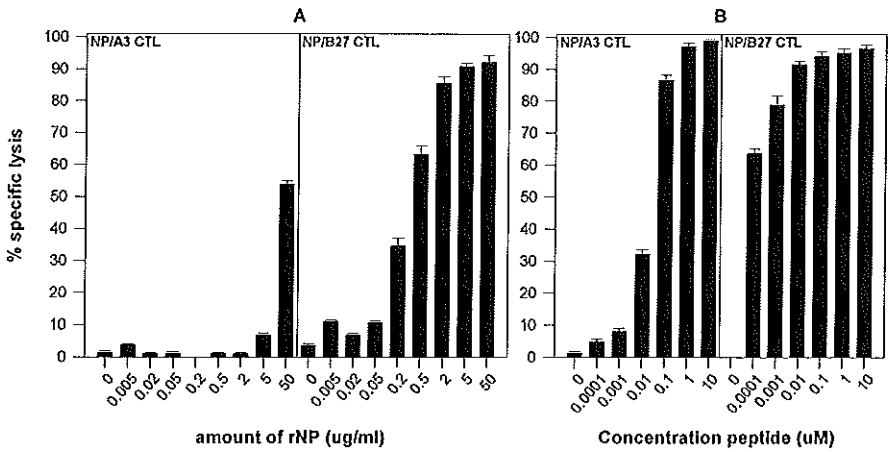

Figure 1. MHC class I presentation of rNP. B-LCL cells of a HLA-A3 and -B27 positive donor were incubated with various concentrations of rNP derived from A/HK/2/68 (A) or various concentrations of the HLA-A3-restricted peptide ILRGSVAHK (B, left panel) or the HLA-B27-restricted peptide SRYWAIRTR (B, right panel) and used as target cells in CTL assays with NP/A3 and NP/B27 CTL as effector cells. CTL assays were performed in tenfold. Mean percentages of specific lysis at an E:T ratio of 10:1 are shown.

To confirm that the recognition of the B-LCL cells incubated with rNP by NP/A3 and NP/B27 CTL was specific for the epitopes ILRGSVAHK and SRYWAIRTR, respectively, B-LCL cells were incubated with rNP derived from A/HK/2/68, A/Neth/18/94 (containing an amino acid mutation in the HLA-B27-restricted epitope), B/Har/7/94, or rMBP (both of which do not contain the HLA-A3 and HLA-B27-restricted epitopes and served as negative controls) (Figure 3A). B-LCL cells were lysed by NP/B27 CTL after incubation with 0.5 µg rNP derived from A/HK/2/68 but not after incubation with the same amount of rMBP or rNP derived from B/Har/7/94 or A/Neth/18/94 which contains a R384G mutation in the SRYWAIRTR epitope. This mutation has been associated with the loss of recognition by CTL specific for this epitope[41]. B-LCL cells incubated with 50 µg rNP (required to measure specific lysis by NP/A3 CTL; see figure 1A) derived from A/HK/2/68 or A/Neth/18/94 (which both contain the ILRGSVAHK

epitope) were recognized by NP/A3 CTL. Like NP/B27 CTL, NP/A3 CTL did not lyse B-LCL cells incubated with the same amount of rNP of B/Har/7/94 or rMBP.

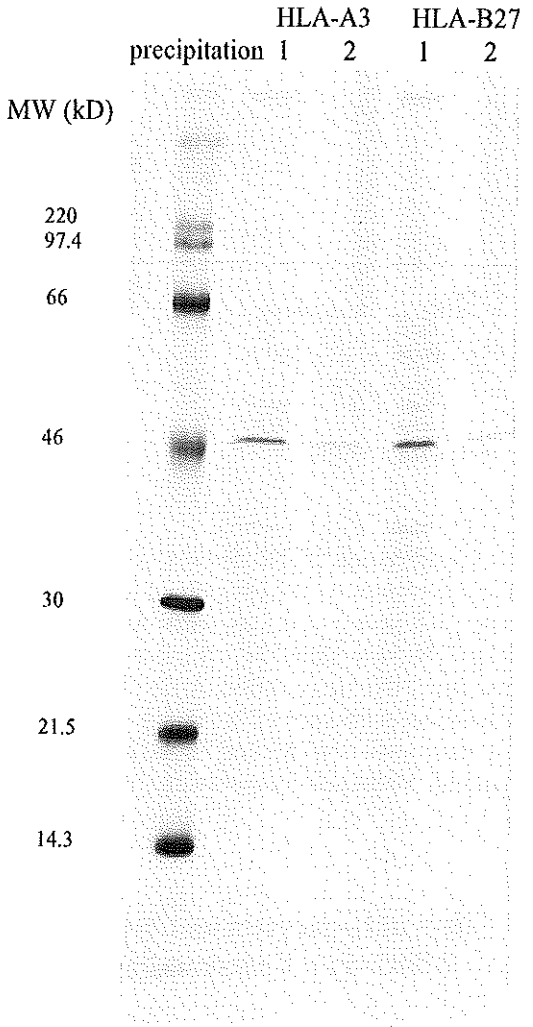

Figure 2. Analysis of expression levels of HLA-A3 and HLA-B27 in B-LCL cells. B-LCL cells incubated with ^{35}S-labeled cysteine and methionine were lysed and the pre-cleared lysate was subjected to two rounds of specific precipitation using HLA-A3 and HLA-B27-specific monoclonal antibodies. Lanes 1: first precipitation, lanes 2: second precipitation.

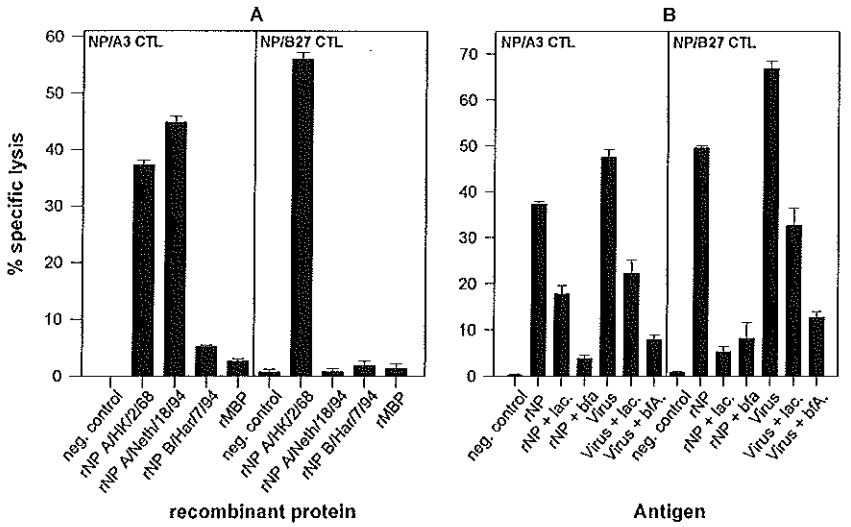

Figure 3. MHC class I presentation of rNP derived from several influenza viruses and the effect of lactacystin and brefeldin A. B-LCL cells of a HLA-A3 and -B27 positive donor were incubated with 50 (left panel) or 0.5 μg (right panel) rNP derived from A/HK/2/68, A/Neth/18/94 or B/Har/7/94, with rMBP or left untreated (A), or incubated with 50 (left panel) or 0.5 μg (right panel) rNP derived from A/HK/2/68 or infected with influenza virus (m.o.i. = 0.1) in the presence or absence of lactacystin or brefeldin A (B). Cells were used as target cells in CTL assays with NP/A3 and NP/B27 CTL as effector cells. CTL assays were performed in tenfold. Mean percentages of specific lysis at an E:T ratio of 10:1 are shown.

Effect of lactacystin and brefeldin A on MHC class I presentation of rNP

In order to study the route of processing of rNP, B-LCL cells were incubated with rNP derived from A/HK/2/68 in the continuous presence of the MHC class I pathway inhibitor lactacystin, which inhibits proteasome activity, or brefeldin A, which blocks transport from the ER (Figure 3B). As a control, B-LCL cells infected with influenza virus Resvir-9 (of which the NP contains both the HLA-A3 and HLA-B27-restricted epitopes) were

included. Both NP/A3 and NP/B27 CTL lysed virus-infected target cells. As expected, recognition of virus-infected cells by these CTL was sensitive to the action of lactacystin and brefeldin A ($p<0.001$). The recognition of B-LCL cells incubated with rNP in the presence of these inhibitors was also reduced ($p<0.001$) suggesting that the conventional MHC class I pathway is of importance for processing and presentation of rNP in these cells. The results obtained with the CTL assays were confirmed in a LST: proliferation of NP/A3 and NP/B27 CTL was reduced after stimulation with influenza virus-infected B-LCL cells which had been cultured in the presence of lactacystin or brefeldin A (Figure 4A). Likewise, proliferation of NP/B27 CTL was reduced when B-LCL cells had been incubated with rNP derived from A/HK/2/68 in the presence of these inhibitors (Figure 4B).

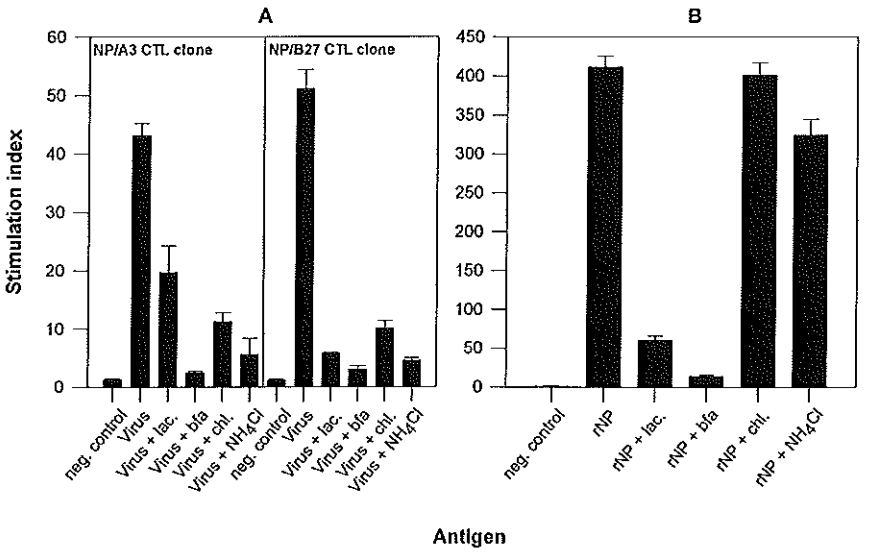

Figure 4. Effect of inhibitors of the conventional MHC class I pathway and endosomal antigen processing on MHC class I presentation of rNP. B-LCL cells of a HLA-A3 and -B27 positive donor were infected with influenza virus (m.o.i. = 0.1) in the presence or absence of lactacystin, brefeldin A, chloroquine or NH_4Cl and used as stimulator cells in LST with NP/A3 and NP/B27 CTL (A). B-LCL cells were also incubated with 100 µg rNP derived from A/HK/2/68 in the presence or absence of these inhibitors and used as stimulator cells in LST with NP/B27 CTL (B). LST were performed in triplicate. Mean stimulation indices are shown.

Effect of chloroquine and NH_4Cl on MHC class I presentation of rNP

A possible role for endosomal processing was studied using chloroquine and NH_4Cl which prevent acidification of endosomes and, consequently, proteolysis. The effect of these inhibitors was not tested in CTL assays since the required continuous presence of these agents affected CTL activity. Therefore, paraformaldehyde fixed B-LCL cells which had been infected with influenza virus Resvir-9 or incubated with rNP derived from A/HK/2/68 in the presence or absence of these inhibitors were used as stimulator cells in LST. Proliferation of NP/A3 and NP/B27 CTL was reduced after stimulation with infected B-LCL cells which had been cultured in the presence of chloroquine or NH_4Cl as compared to CTL stimulated with untreated infected B-LCL cells (Figure 4A). This is explained by the fact that a low pH in the endosomes is essential for conformational changes in the haemagglutinin allowing fusion of the viral membrane with the membrane of endosomes and subsequent release of viral antigens into the cytoplasm. In contrast, no significant difference in proliferation of NP/B27 CTL was observed upon stimulation with B-LCL cells incubated with 100 μg rNP in the presence or absence of these inhibitors (Figure 4B). When B-LCL cells were incubated with a lower amount of rNP (50 μg) only a limited reduction in proliferation of NP/B27 CTL was observed (data not shown).

MHC class I presentation of rNP in the absence of TAP

To study the role of TAP in the processing of rNP, BM28.7 and TAP-deficient BM36.1 cells were used. The recognition of virus-infected BM36.1 cells by NP/B27 CTL was significantly reduced ($p<0.001$) compared to the recognition of the corresponding TAP-competent BM28.7 cells (Figure 5A). This difference could not be attributed to differences in the infection rates of the respective cell lines since both cell lines were infected equally well as demonstrated by immunofluorescense using a NP-specific monoclonal antibody (data not shown). Surprisingly, the opposite was found for rNP derived from A/HK/2/68. BM36.1 cells incubated with rNP were lysed more efficiently by NP/B27 CTL than BM28.7 cells ($p<0.001$). To rule out that the observed differences between the BM28.7 and BM36.1 cells are the result of differences in the expression of peptide-receptive HLA-B27 molecules, the recognition of these cells by NP/B27

CTL was studied after incubation with limiting amounts of peptide (Figure 5B).

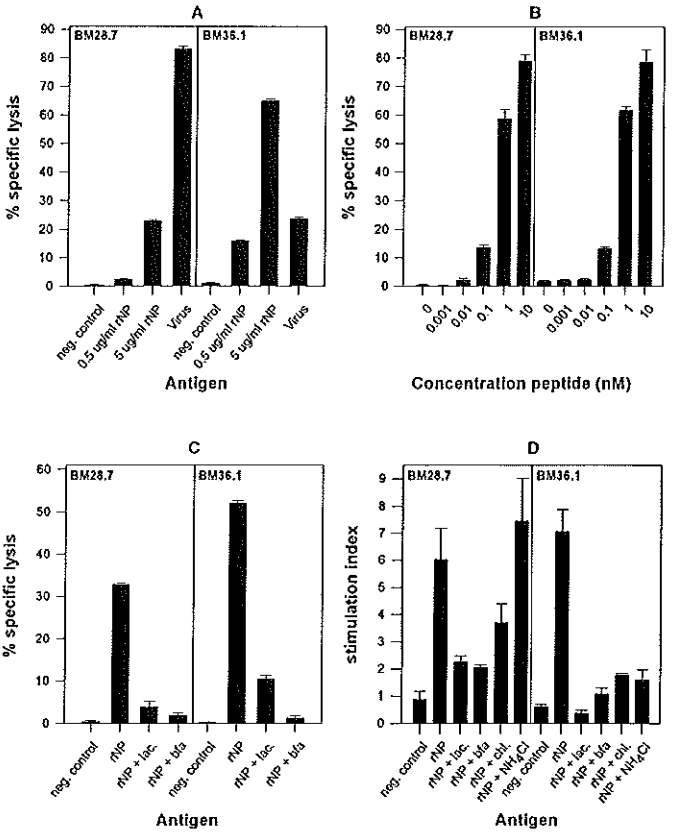

Figure 5. MHC class I presentation of rNP in the absence of TAP and the effect of inhibitors of the conventional MHC class I pathway and endosomal antigen processing. BM28.7 and BM36.1 cells were incubated with 0.5 or 5 μg rNP derived from A/HK/2/68 or infected with influenza virus (m.o.i. = 0.1) (A), incubated with various concentrations of the HLA-B27-restricted peptide SRYWAIRTR (B), or incubated with 5 μg rNP derived from A/HK/2/68 in the presence of lactacystin or brefeldin A (C). Cells were used as target cells in CTL assays with the NP/B27 CTL as effector cells. CTL assays were performed in tenfold. Mean percentages of specific lysis at an E:T ratio of 10:1 are shown. BM28.7 and BM36.1 cells incubated with 100 μg rNP derived from A/HK/2/68 in the presence or absence of lactacystin, brefeldin A, chloroquine or NH_4Cl were used as stimulator cells in LST with the NP/B27 CTL (D). LST were performed in triplicate. Mean stimulation indices are shown.

Both cell lines were recognized equally well by the NP/B27 CTL under these conditions indicating that HLA-B27 was expressed at comparable levels in both cell lines. In contrast to the CTL assay, no differences in proliferation of NP/B27 CTL stimulated with BM36.1 or BM28.7 cells incubated with rNP were observed (Figure 5D).
The effect of the MHC class I pathway inhibitors lactacystin and brefeldin A, as well as inhibitors of endosomal processing (chloroquine and NH_4Cl) was also studied for BM28.7 and BM36.1 cells incubated with rNP derived from A/HK/2/68 (Figure 5C and D). Specific lysis by NP/B27 CTL was reduced when cells had been incubated with rNP in the presence of either lactacystin or brefeldin A (Figure 5C). Similarly, proliferation of NP/B27 CTL was reduced under these circumstances (Figure 5D). Although proliferation of NP/B27 CTL stimulated with BM28.7 cells which had been incubated with rNP in the presence of chloroquine was reduced, no reduction was observed when BM28.7 cells had been incubated with rNP in the presence of NH_4Cl. In contrast, proliferation of NP/B27 CTL was severely reduced when TAP-deficient BM36.1 cells had been incubated with rNP in the presence of chloroquine or NH_4Cl (Figure 5D).

DISCUSSION

Although exogenous proteins are usually processed for MHC class II presentation to $CD4^+$ Th cells, it has been shown that some APC (e.g. macrophages and dendritic cells) are capable of processing these proteins for MHC class I presentation to $CD8^+$ CTL as well (8). With the exception of proteins that are delivered into the cytosol artificially (e.g. proteins associated with iscoms), or proteins that are internalized by receptor-mediated endocytosis, MHC class I presentation of exogenous protein by B-lymphocytes thus far has not been demonstrated[5-7,17,19,21,32,33,42]. Here, we show MHC class I presentation of the HLA-A3-restricted peptide ILRGSVAHK (aa 265-273) and the HLA-B27-restricted peptide SRYWAIRTR (aa 383-391) derived from exogenous rNP by B-LCL.
The NP/B27 CTL recognized B-LCL cells which had been incubated with rNP at a concentration of 200 ng/ml, while approximately 100 times higher concentrations of rNP were required to sensitize B-LCL cells for lysis by NP/A3 CTL. Since the expression levels of HLA-A3 and HLA-B27 in the B-LCL cells were virtually identical, the differences observed between

HLA-A3 and HLA-B27-associated presentation should result from differences in the efficiency of processing or presentation of the respective epitopes, or intrinsic differences between both CTL. Recently, it has been demonstrated that HLA-B27-restricted peptides have higher affinity for TAP than HLA-A3-restricted peptides[9] and, using peptides, we have shown that B-LCL cells could be sensitized for lysis by NP/B27 CTL at peptide concentrations much lower than needed for measuring HLA-A3-associated presentation. Peptides with higher affinities for their MHC class I molecule and/or TAP may be presented more efficiently than peptides possessing low affinity. The efficient presentation by HLA-B27 molecules may also be related to some unique features of these MHC class I molecules including their ability to bind peptides without associating with TAP or tapasin, the ability to bind peptides longer than nonamers and to form peptide binding homodimers[1,26,39].

The mode of processing of exogenous proteins for MHC class I-restricted presentation is not fully clear. MHC class I presentation of the HLA-A3 and HLA-B27-restricted peptides derived from exogenous rNP, like presentation of these peptides derived from de novo synthesized NP by virus-infected cells, was found to depend on proteasome activity and to be sensitive to brefeldin A. This suggests that rNP gained access to the cytoplasm, following endocytosis and subsequent leakage from endosomes, and that processing and presentation followed the conventional MHC class I pathway. It is unlikely that endocytsosis was receptor-mediated since the B-LCL cells did not express NP or MBP-specific Ig (data not shown). Previously, we have shown that influenza virus M1 protein fused to MBP only sensitized B-LCL cells for recognition by CTL specific for the M1 protein, HLA-A2-restricted, epitope GILGFVFTL (58-66) when the protein was associated with iscoms[42]. In addition, B-LCL cells incubated with high doses (100 μg) of this recombinant M1 protein (without iscoms) were not recognized by specific CTL (unpublished data). These results rule out a direct role of MBP in entry of rNP into the MHC class I pathway.

HLA-B27-associated presentation by B-LCL cells was shown to be insensitive to chloroquine and NH_4Cl at a high concentration of rNP, while at a lower concentration limited sensitivity to these agents was observed. This suggests that, in addition to the conventional MHC class I pathway, endosomal processing may be exploited by these cells to a limited extent. It is possible that in the endosomal compartments of B-LCL cells peptides were generated from rNP by proteolysis and subsequently loaded onto

peptide-receptive internalized MHC class I molecules, which are known to recycle through endosomal compartments[11,28]. This process could then be followed by presentation to specific CTL. While this mechanism may have contributed to the observed MHC class I presentation of rNP, it is most probably not the major route. Indeed, inhibition of presentation by chloroquine and NH_4Cl was less pronounced than by inhibitors of the conventional MHC class I pathway.

Surprisingly, HLA-B27-associated presentation of rNP also occured in the TAP-deficient cell line BM36.1. Since in cells lacking a functional TAP the conventional MHC class I pathway is hampered, as demonstrated by the fact that recognition of influenza virus-infected BM36.1 cells by NP/B27 CTL was reduced, processing and presentation of rNP in these cells must have followed alternative routes. TAP-independent MHC class I-restricted presentation has been described for peptides liberated from N-terminal signal sequences of proteins that enter the ER via translocation[2,4,10,12,16,37,43,45]. In addition, it has been suggested that entry of proteins or peptides in the ER of TAP-deficient cells may take place by (unknown) mechanisms other than translocation[37]. Since the influenza virus NP does not contain a signal sequence and because it was cloned C-terminal of the MBP, translocation-mediated entry in the ER is unlikely. MHC class I presentation of rNP by BM36.1 cells, but not BM28.7 cells, was found to be severely inhibited by chloroquine and NH_4Cl indicating that the endosomal route is involved in the processing of rNP in these cells. These results are in agreement with a study showing that processing and presentation of an ovalbumin epitope (257-264), contained within an ovalbumin fusion protein, was sensitive to chloroquine in TAP-deficient macrophages, but not in normal macrophages[44].

The observed MHC class I presentation of rNP by BM36.1 cells was also found to be sensitive to brefeldin A indicating that antigenic peptides were loaded onto MHC class I molecules prior to their transport to the cell surface. In addition to inhibition of transport of MHC class I molecules from the ER to the cell surface, brefeldin A is known to inhibit transport of MHC class II molecules from the endosomes to the cell surface[27]. It may be speculated that brefeldin A could also affect transport of internalized MHC class I molecules, loaded with peptide in the endosomes, back to the cell surface. Alternatively, brefeldin A treatment could have resulted in a reduced availability of MHC class I molecules for recycling in endosomes.

Interestingly, the processing of rNP in BM36.1 cells was sensitive to lactacystin implying that, at least in part, the peptides that were presented to the NP/B27 CTL were liberated by proteasome activity. Since BM36.1 cells are unable to transport these peptides in the ER by TAP, they were probably loaded onto MHC class I molecules through alternative, yet unkown, routes which may involve endosomes, the ER or a post ER compartment.
Collectively, the data presented show MHC class I presentation of exogenous rNP by B-LCL cells. While in normal cells the conventional MHC class I pathway seems to predominate, in TAP-deficient cells alternative pathways, including endosomal processing, may operate for HLA-B27-associated presentation. The results of this study also show that care should be taken in drawing conclusions with respect to the steps involved in processing and presentation of exogenous proteins.

ACKNOWLEDGEMENTS

Part of this work was supported by the Foundation for Respiratory Virus Infections, notably influenza (SRVI). We acknowledge Dr. W. Biddison, NIH, Bethesda, MD, for providing us with the NP/A3 CTL clone. The authors wish to thank Ger van der Water for continuous support.

REFERENCES

[01] Allen, R.L., O'Callaghan, C.A., McMichael, A.J. and Bowness, P. 1999. Cutting edge: HLA-B27 can form a novel β2-microglobulin-free heavy chain homodimer structure. *J.Immunol.* 162:5045-5048.

[02] Anderson, K., Cresswell, P., Gammon, M., Hermes, J., Williamson, A. and Zweerink, H. 1991. Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I-restricted cell-mediated lysis. *J.Exp.Med.* 174:489-492.

[03] Bachmann, M.F., Oxenius, A., Pircher, H., Hengartner, H., Ashton-Richardt, P.A., Tonegawa, S. and Zinkernagel, R.M. 1995. TAP1-independent loading of class I molecules by exogenous viral proteins. *Eur.J.Immunol.* 25:1739-1743.

[04] Bacik, I., Cox, J.H., Anderson, R., Yewdell, J.W. and Bennink, J.R. 1994. TAP (transporter associated with antigen processing)-independent presentation of endogenously synthesized peptides is enhanced by endoplasmic reticulum insertion sequences located at the amino- but not carboxyl-terminus of the peptide. *J.Immunol.* 152:381-387.

[05] Bansal, P., Mukherjee, P., Basu, S., George, A., Bal, V. and Rath, S. 1999. MHC class I-restricted presentation of maleylated protein binding to scavenger receptors. *J.Immunol.* 162:4430-4437.

[06] Barnaba, V., Franco, A., Alberti, A., Benvenuto, R. and Balsano, F. 1990. Selective killing of hepatitis B envelope antigen-specific B cells by class I-restricted, exogenous antigen-specific T lymphocytes. *Nature* 345:258-260.

[07] van Binnendijk, R.S., van Baalen, C.A., Poelen, M.C.M., de Vries, P., Boes, J., Cerundolo, V., Osterhaus, A.D.M.E. and Uytdehaag, F.G.C.M. 1992. Measles virus transmembrane fusion protein synthesized de novo or presented in immunostimulating complexes is endogenously processed for HLA class I- and class II-restricted cytotoxic t cell recognition. *J.Exp. Med.* 176:119-128.

[08] Brossart, P. and Bevan, M.J. 1997. Presentation of exogenous proteins on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* 90:1594-1599.

[09] Daniel, A., Brusic, V., Caillat-Zucman, S., Petrovsky, N., Harrison, L., Riganelli, D., Sinigaglia, F., Gallazzi, F., Hammer, J. and van Endert, P.M. 1998. Relationship between peptide selectivities of human transporters associated with antigen processing and HLA class I molecules. *J.Immunol.* 161:617-624.

[10] Elliot, T., Willis, A., Cerundolo, V. and Townsend, A. 1995. Processing of major histocompatibility class I-restricted antigens in the endoplasmic reticulum. *J.Exp.Med.* 181:1481-1491.

[11] Grommé, M., Uytdehaag, F.G.C.M., Janssen, H., Calafat, J., van Binnendijk, R.S., Kenter, M.J.H., Tulp, A., Verwoerd, D. and Neefjes, J. 1999. Recycling MHC class I molecules and endosomal peptide loading. *Proc.Natl.Acad.Sci.USA* 96:10326-10331.

[12] Guegen, M., Biddison, W.E. and Long, E.O. 1994. T cell recognition of an HLA-A2-restricted epitope derived from a cleaved signal sequence. *J.Exp.Med.* 180:1989-1994.

[13] Hahn, Y.S., Yang, B. and Braciale, T.J. 1996. Regulation of antigen processing and presentation to class I MHC restricted CD8+ T lymphocytes. *Immunol.Rev.* 151:31-49.

[14] Harding, C.V. and Song, R. 1994. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. *J.Immunol.* 153:4925-4933.

[15] Heemels, M.T. and Ploegh, H. 1995. Generation, translocation, and presentation of MHC class I-restricted peptides. *Annu.Rev.Biochem.* 64:463-491.

[16] Henderson, R.A., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D.F. and Engelhard, V.H. 1992. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science* 255:1264-1266.

[17] Ke, Y. and Kapp, J.A. 1996. Exogenous antigens gain access to the major histocompatibility complex class I processing pathway in B cells by receptor-mediated uptake. *J.Exp.Med.* 184:1179-1184.

[18] Kelly, A., Powis, S.H., Kerr, L.A., Mockridge, I., Elliot, T., Bastin, J., Uchanska-Ziegler, B., Ziegler, A., Trowsdale, J. and Townsend, A. 1992. Assembly and function of the two ABC transporter proteins encoded in the human histocompatibility complex. *Nature* 355:641-644.

[19] Kovacsovics-Bankowski, M., Clark, K., Benacerraf, B. and Rock K.L. 1993. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc.Natl.Acad.Sci.USA* 90:4942-4946.

[20] Kovacsovics-Bankowski, M. and Rock, K.L. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267:243-246.

[21] Lee, R., Tartour, E., van der Bruggen, P., Vantomme, V., Joyeux, I., Goud, B., Fridman, W.H. and Johannes. L. 1998. Major histocompatibility complex class I presentation of exogenous soluble tumor antigen fused to the B-fragment of shiga toxin. *Eur.J.Immunol.* 28:2726-2737.

[22] Liu, T., Chambers, B., Diehl, A.D., Van Kaer, L., Jondal, M. and Ljunggren, H. 1997. TAP peptide transporter-independent prsentation of heat-killed sendai virus antigen on MHC class I molecules by splenic antigen-presenting cells. *J.Immunol.* 159:5364-5371.

[23] Monaco, J.J. 1992. A molecular model of MHC class-I-restricted antigen processing. *Immunol.Today* 13:173-178.

[24] Neefjes, J.J. and Momburg, F. 1993. Cell biology of antigen presentation. *Current Opinion in Immunology* 5:27-34.

[25] Norbury, C.C., Hewlett, L.J., Prescott, A.R., Shastri, N. and Watts, C. 1995. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity* 3:783-791.

[26] Peh, C.A., Burrows, S.R., Barnden, M., Khanna, R., Cresswell, P., Moss, D.J. and McCluskey, J. 1998. HLA-B27-restricted antigen presentation in the absence of tapasin reveals polymorphism in mechanisms of HLA class I peptide loading. *Immunity* 8:531-542.

[27] Pond, L. and Watts, C. 1997. Characterization of transport of newly assembled, T cell-stimulatory MHC class II-peptide complexes from MHC class II compartments to the cell surface. *J.Immunol.* 159:543-553.

[28] Reid, P.A. and Watts, C. 1990. Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments. *Nature* 346:655-657.

[29] Reimann, J., Böhm, W. and Schirmbeck, R. 1994. Alternative processing pathways for MHC class I-restricted epitope presentation to CD8+ cytotoxic T lymphocytes. *Biol.Chem.Hoppe-Seyler.* 375:731-736.

[30] Reimann, J. and Schirmbeck, R. 1999. Alternative pathways for processing exogenous and endogenous antigens that can generate peptide for MHC class I-restricted presentation. *Immunol.Rev.* 172:131-152.

[31] Reis e Sousa, C. and Germain, R.N. 1995. Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigens by a subset of cells engaged in phagocytosis. *J.Exp.Med.* 182:841-851.

[32] Rock, K.L., Gamble, S. and Rothstein, L. 1990. Presentation of exogenous antigen with class I major histocompatibility complex molecules. *Science* 249:918-921.

[33] Rock, K.L., Rothstein, L., Gamble, S. and Fleischacker, C. 1993. Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. *J.Immunol.* 150:438-446.

[34] Schirmbeck, R., Melber, K. and Reimann, J. 1995. Hepatitis B virus small surface antigen particles are processed in a novel endosomal pathway for major histocompatibility complex class I-restricted epitope presentation. *Eur.J.Immunol.* 25:1063-1070.

[35] Schumacher, T.N.M. 1999. Accessory to murder. *Nature* 398:26-27.

[36] Sigal, L.J., Crotty, S., Andino, R. and Rock, K.L. 1999. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* 398:77-80.

[37] Snyder, H.L., Bacik, I., Bennink, J.R., Kearns, G., Behrens, T.W., Bächi, T., Orlowski, M. and Yewdell, J.W. 1997. Two novel routes of transporter associated with antigen processing (TAP)-independent major histocompatibility complex class I antigen processing. *J.Exp.Med.* 186:1087-1098.

[38] Song, R. and Harding, C.V. 1996. Roles of proteasomes, transporter for antigen presentation (TAP), and β_2-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. *J.Immunol.* 156:4182-4190.

[39] Urban, R.G., Chicz, R.M., Lane, W.S., Strominger, J.L., Rehm, A., Kenter, M.J.H., Uytdehaag, F.G.C.M. and Ploegh, H.L. 1994. A subset of HLA-B27 molecules contains peptides much longer than nonamers. *Proc.Natl.Acad.Sci.USA* 91:1534-1538.

[40] Voeten, J.T.M., Groen, J., van Alphen, D., Claas, E.C.J., de Groot, R., Osterhaus, A.D.M.E. and Rimmelzwaan, G.F. 1998. Use of recombinant nucleoproteins in enzyme-linked immunosorbent assays for detection of virus-specific immunoglobulin A (IgA) and IgG antibodies in influenza a virus- or B- infected patients. *J.Clin.Microbiol.* 36:3527-3531.

[41] Voeten, J.T.M., Bestebroer, T,M., Nieuwkoop, N.J., Fouchier, R.A.M., Osterhaus, A.D.M.E. and Rimmelzwaan, G.F. 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. *J.Virol.* 74:6800-6907.

[42] Voeten, J.T.M., Rimmelzwaan, G.F., Nieuwkoop, N.J., Lövgren-Bengtsson, K. and Osterhaus, A.D.M.E. 2000. Introduction of the haemagglutinin transmembrane region in the influenza virus matrix protein facilitates its incorporation into ISCOM and activation of specific CD8+ cytotoxic T lymphocytes. *Vaccine* 19:522-530.

[43] Wei, M.L. and Cresswell, P. 1992. HLA-A2 molecules in an antigen-presenting mutant cell contain signal sequence-derived peptides. *Nature* 356:443-446.

[44] Wick, M.J. and Pfeifer, J.D. 1996. Major histocompatibility complex class I presentation of ovalbumin peptide 257-264 from exogenous sources: protein context influences the degree of TAP-independent presentation. *Eur.J.Immunol.* 26:2790-2799.

[45] Yewdell, J.W., Snyder, H.L., Bacik, I., Anton, L.C. Deng, Y., Behrens, T.W., Bächi, T. and Bennink, J.R. 1998. TAP-independent delivery of antigenic peptides to the endoplasmic reticulum: therapeutic potential and insights into TAP-dependent antigen processing. *J.Immunother.* 21:127-131.

[46] Yewdell, J.W., Norbury, C.C. and Bennink, J.R. 1999. Mechanisms of exogenous antigen presentation by MHC class I molecules *in vitro* and *in vivo*: implications

for generating CD8+ T cell responses to infectious agents, tumors, transplants and vaccines. *Adv. Immunol.* 73:1-77.

[47] York, I.A. and Rock, K.L. 1996. Antigen processing and presentation by the class I major histocompatibility complex. *Annu.Rev.Immunol.* 14:369-396.

CHAPTER 5

Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes

J.T.M. Voeten, T.M. Bestebroer, N.J. Nieuwkoop, R.A.M. Fouchier, A.D.M.E. Osterhaus and G.F. Rimmelzwaan

Journal of Virology 74:6800-6807 (2000)

SUMMARY

Viruses exploit different strategies to escape immune surveillance, including the introduction of mutations in cytotoxic T-lymphocyte (CTL) epitopes. The sequence of these epitopes is critical for their binding to major histocompatibility complex (MHC) class I molecules and recognition by specific CTLs, both of which interactions may be lost by mutation. Sequence analysis of the nucleoprotein gene of influenza A viruses (H3N2) isolated in The Netherlands from 1989 to 1999 revealed two independent amino acid mutations at the anchor residue of the HLA-B27-specific CTL epitope SRYWAIRTR (383 to 391). A R384K mutation was found in influenza A viruses isolated during the influenza season 1989-1990 but not in subsequent seasons. In the influenza season 1993-1994, a novel mutation in the same CTL epitope at the same position was introduced. This R384G mutation proved to be conserved in all influenza A viruses isolated from 1993 onwards. Both mutations R384K and R384G abrogated MHC class-I presentation and allowed escape from recognition by specific CTLs.

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) of the $CD8^+$ phenotype control viral infections by recognizing antigenic peptides of viral proteins presented by infected cells in association with major histocompatibility complex (MHC) class I molecules. The interaction of specific CTLs with these complexes may lead to the elimination of infected cells. Viruses exploit several strategies to escape from immune surveillance by CTLs[21,32]. One strategy involves the introduction of amino acid mutations within CTL epitopes or in sequences flanking these epitopes. The flanking sequences are important for cytosolic processing of the viral proteins to yield the CTL epitopes, usually 9-mer peptides, while the epitope sequences themselves are critical both for association with MHC class I molecules and recognition by virus-specific CTLs. Mutations within or in close proximity of CTL epitopes, therefore, may be accompanied by loss of CTL-mediated lysis of target cells[15,46]. In addition, mutations in CTL epitopes may generate peptides that antagonize CTL function[2,10,18,20,38]. Mutations that affect CTL epitopes, resulting in escape from immune surveillance by specific CTLs, have been described for several viruses causing persistent infections, including lymphocytic choriomeningitis virus[28,35], Epstein-Barr virus[1,4,6,7], human

immunodeficiency virus[8,13,21,27,34,37], hepatitis B virus[3], and hepatitis C virus[45].

Influenza A viruses, causing acute infections, continuously escape from recognition by virus-neutralizing antibodies as a result of accumulation of mutations in their surface glycoproteins hemagglutinin and neuraminidase (antigenic drift) or by introduction of new subtypes of these glycoproteins (antigenic shift). The more conserved internal proteins of influenza viruses, such as the nucleoprotein (NP) and the matrix protein are important targets for CTLs[12,25]. Mutations in these proteins, which occur less frequently than in the surface glycoproteins, potentially could affect CTL-mediated immune surveillance.

Here we show that mutations at the anchor residue of the HLA-B27-restricted CTL epitope SRYWAIRTR (383-391), found in the NP of influenza A (H3N2) viruses isolated between 1989 and 1999 in The Netherlands, abrogate MHC class I presentation and recognition by specific CTLs.

MATERIALS AND METHODS

RNA isolation, RT-PCR and sequencing

RNA of 59 influenza A (H3N2) viruses of the influenza season 1989-1990, 16 of the season 1991-1992, 16 of the season 1992-1993, 56 of the season 1993-1994, and 15 of the season 1998-1999 (arbitrarily chosen) obtained from the Dutch National Influenza Centre originating from geographically distinct areas in The Netherlands, was isolated using a high-pure RNA isolation kit (Boehringer Mannheim) and dissolved in 50 µl diethylpyrocarbonaat-treated H_2O. The RNA was used as template to multiply all eight gene segments in a reverse transcriptase PCR (RT-PCR) using a single primer set. The RT-PCR reaction mixture contained 5 µl of RNA, 10 pmol of M13-uni12 primer (CAGGAAACAGCTATGACCAGCAAAAGCAGG), 10 pmol of M13-uni13 primer (TGTAAAACGACGGCCAGTAGTAGAAACAAGG), 0.01 M DTT, 0.25 mM deoxynucleoside triphosphates (dNTPs), 10 U of Rnasin (Promega), 8 U of avian myeloblastosis virus RT (Promega), and 1.25 U of *Pfu* polymerase (Stratagene) in a total of 25 µl 1 x *Pfu* polymerase buffer. The mixture was incubated for 60 min at 42°C followed by 4 min at 95°C, 2 min at 37°C, and 3 min at 72°C and 19 cycles of incubation for 1 min at 95°C, 1 min at 50°C, and 3 min at 72°C. The resulting cDNAs were used as template in a NP-specific PCR (nucleotides [nt] 696 to 1243). One microliter of template was added to 25 µl of reaction mixture containing 5 pmol of NP696 primer (TGCTTATGA-GAGAATGTGCAA), 5 pmol of NP1243 primer (TCTGTTGGTTGGTGTTTCCTCC), 1.5 mM $MgCl_2$, 20 µM dNTPs, and 2.5 U of *Taq* polymerase (Promega) in a total of 25 µl 1 x *Taq* polymerase buffer. The mixture was incubated for 2 min at 95°C followed by 1 min at 50°C, and 3 min at 72°C and 29 cycles of incubation for 1 min at 95°C, 30 s at 50°C, and 3 min at 72°C. Amplified DNA was diluted 1:5, and 10 µl was added to 10 µl of

sequencing reaction mixture (DYEnamic ET terminator cycle sequencing premix kit; Amersham Pharmacia Biotech, Inc.) containing 10 pmol of NP696 primer. The resulting mixture was incubated for 30 s at 95°C, 15 s at 45°C, and 2 min at 60°C for a total of 30 cycles. Then, 2 µl of 3M NaAc pH 4.8 and 80 µl of absolute ethanol were added followed by incubation on ice for 15 min and centrifugation at 2,400 x g for 30 min. Pellets were resuspended in 3 µl of sample buffer, and 0.8 µl was loaded on a sequence gel followed by automatic sequencing (ABI sequencer). Phylogenetic analysis was performed using DNAML software (Phylip version 3.5).

Isolation and analysis of NP-specific CTL clones

In round-bottomed microtiter plates, 1,000 peripheral blood mononuclear cells (PBMCs) of a selected donor (HLA-A01, A03, B07, B2705, Cw02, Cw07) were stimulated twice, with an interval of one week, with 2.5 x 10^4 gamma-irradiated (30 min, 3,000 rads) autologous phytohemagglutinin-stimulated PBMCs pulsed with the peptide SRYWAIRTR (an HLA-B27-restricted CTL epitope of the influenza A virus NP [amino acids (aa) 383 to 391]). The cells were cultured in RPMI 1640 medium containing L-glutamine (2 mM), streptomycin (100 µg/ml), penicillin (100 IU/ml), 2-mercaptoethanol (2 x 10^{-5} M), interleukin-2 (50 U/ml), and 10% pooled human serum at 37°C and 5% CO_2. One week after the second stimulation, expanded cells were analyzed for peptide-specific CTL activity. Cells from wells showing CTL activity were cloned by limiting dilution (0.3 , 1 and 3 cells per well) and stimulated non-specifically by adding 3 x 10^4 APD B-lymphoblastoid cell line (B-LCL) cells, 3 x 10^4 BSM B-LCL cells, and 6 x 10^5 allogeneic PBMCs (which were all gamma irradiated); 1 µg of phytohemagglutinin and 50 U of interleukin-2 per ml[14]. After incubation for 2 weeks, clones showing CTL activity were stimulated specifically with gamma-irradiated peptide-pulsed autologous PBMCs. After incubating the clones for 12 days, they were stimulated nonspecifically as described above in 75-cm^2 flasks. After 2 weeks, cells were harvested, aliquoted, and stored at -135°C until use. These CTLs will be referred to as the NP/B27 CTL clone. An HLA-A3-restricted CTL clone specific for the influenza A virus NP epitope ILRGSVAHK (aa 265 to 273) was kindly provided by W. Biddison, National Institutes of Health (NIH), Bethesda, Md., and will be referred to as the NP/A3 CTL clone. The phenotype of both CTL clones was determined by fluorescence-activated cell sorting (FACS) analysis using monoclonal antibodies specific for CD3, CD4, and CD8, and their specificity and HLA restriction was confirmed in CTL assays. To this end, 1 x 10^6 cells of an Epstein-Barr virus transformed B-LCL of an HLA-A3- and -B27 positive donor and mismatched (HLA-A3- and -B27 negative) B-LCL cells were incubated with the peptide ILRGSVAHK or SRYWAIRTR (10 µM) for one h at 37°C and used as target cells in CTL assays with the respective CTL clones as effectors.

Preparation of target cells

B-LCL cells (10^6) of one donor (HLA-A3- and -B27 positive) were incubated with the peptide ILRSGVAHK (HLA-A3), SRYWAIRTR (HLA-B27), or SKYWAIRTR or SGYWAIRTR (HLA-B27 mutant peptide) at a concentration giving the highest specific lysis (10 µM for the ILRGSVAHK peptide and 1 µM for the SRYWAIRTR and mutant peptides). In addition, the same B-LCL cells were infected with recombinant vaccinia viruses (RVV) expressing the NP of influenza virus A/Puerto Rico/8/34;H1N1 (A/PR/8/34) (kindly provided by B. Moss, NIH), A/Netherlands/018/94;H3N2 (A/Neth/18/94)

(generated essentially as previously described[39]), or a control vaccinia virus (VSC65), each at a multiplicity of infection of 10. Also, B-LCL cells were infected with the influenza A viruses A/PR/8/34; A/Netherlands/651/89;H3N2 (A/Neth/651/89), having an R384K mutation; or A/Neth/18/94, having an R384G mutation in the NP gene. Cells were cultured in RPMI 1640 medium containing L-glutamine (2mM), streptomycin (100 µg/ml), penicillin (100 IU/ml) and 10% fetal bovine serum at 37°C and 5% CO_2. After 16 h of incubation, cells were washed and used as target cells in CTL assays with the NP/A3 and NP/B27 CTL clones as effector cells.

CTL assays

Target cells (B-LCL cells) were labeled for 1 h with 75 µCi $Na_2[^{51}Cr]O_4$ in RPMI 1640 medium. Cells were washed three times in culture medium (see above) and resuspended in this medium at a concentration of 10^4 cells / 50 µl. Effector cells (CTLs) were suspended in this medium at a concentration of 2.5 x 10^4, 5 x 10^4 or 1 x 10^5cells / 100 µl (effector-to-target [E:T] ratios, 2,5:1, 5:1 and 10:1). Fifty microliters of target cells was incubated either with 100 µl of medium (spontaneous release), with 100 µl of 10% Triton X-100 (maximum release), or with 100 µl of effector cells (experimental release) for 4 h at 37° C. Supernatants were harvested, and radioactivity was measured by gamma counting. The percentage of specific lysis was calculated as: 100 x (experimental release - spontaneous release) / (maximum release - spontaneous release). CTL assays were performed in triplicate per target per E:T ratio.

Functional analysis of wild type and mutant NP

The NP coding sequences of influenza virus A/Hong Kong/2/68;H3N2 (A/HK/2/68), representing wild type virus, and A/Neth/18/94 (R384G mutant) were amplified by PCR using pBluescript plasmids containing the NP genes of both viruses as templates with a *Not*I-forward primer, CAGCGGCCGCATGGCGTCCCAAGGC, and an *Xho*I-reverse primer, CACTCGAGTTAATTGTCGTACTCCTCTGC (restriction endonuclease recognition sequences are underlined, and start and stop codons of the NP gene are in boldface). PCRs were performed with 10 ng of plasmid DNA, 10 pmol of each of the primers, 1.5 mM $MgCl_2$, 20 µM dNTPs and 5 U of *Pfu* polymerase in a total of 100 µl 1x *Pfu* polymerase buffer. This mixture was heated for 3 min at 94°C followed by a total of 20 cycles consisting of 1 min at 94°C, 2 min at 50°C, and 4 min at 72°C. The PCR products were cloned as *Not*I-*Xho*I fragments in a modified version of the eukaryotic expression plasmid pcDNA3 (Invitrogen) followed by large-scale production of plasmid DNA and purification by CsCl gradient centrifugation according to standard methods.
The respective plasmids were used for transfection into 293T cells. Plasmid DNA (1.5 µg) was mixed with equal amounts of the plasmids pHMG-PB1, pHMG-PB2 and pHMG-PA (encoding the polymerase proteins PB1, PB2, and PA, respectively; kindly provided by P. Palese, Mount Sinai School of Medicine, New York, NY[36]) and 0.5 µg of plasmid RF419 (constructed essentially as described previously[30]), from which the green fluorescent protein (GFP) gene flanked with the influenza A virus noncoding region of the NS gene segment is transcribed in a negative orientation. This plasmid mixture was transfected into 293T cells as described previously[33]. One day after transfection, cells were subjected to FACS analysis. Cells transfected with plasmid pcDNA3 without cloned NP sequences

served as a negative control, while cells transfected with plasmid pEGFP-N1 (encoding enhanced GFP; Clontech) served as a positive control.

Nucleotide sequence accession numbers

Nucleotide sequences have been submitted to GenBank and can be retrieved by the following accession numbers: AF225709-AF225764 (influenza season 1993-1994), AF225765-AF225823 (influenza season 1989-1990), AF225824-AF225839 (influenza season 1991-1992), AF225840-AF225855 (influenza season 1992-1993), AF225856-AF225869 and AF226872 (influenza season 1998-1999).

RESULTS

NP gene sequences of influenza A (H3N2) viruses

Sequence analysis of the NP genes of influenza A (H3N2) viruses isolated in The Netherlands from 1989 to 1999 was performed. The region of the NP genes sequenced encompasses aa 240 to 391 (or nt 720 to 1175) and harbours four previously described CTL epitopes: aa 265 to 273, aa 338 to 347, aa 380 to 388, and aa 383 to 391 presented, by HLA-A3, -B37, -B8, and -B27 molecules, respectively[9,16,26,43]. As shown in table 1; differences in the nucleotide sequences of viruses isolated in the same season were observed. Overall, 46 different nucleotide sequences were identified in 162 viruses isolated from 1989 to 1999. Nevertheless, within one season viruses were closely related, as is shown in the maximum likelyhood tree in figure 1. The amino acid sequences found in the influenza A viruses isolated in the respective influenza seasons are shown in figure 2, and nucleotide mutations underlying differences in these amino acid sequences are shown in table 2. Eleven different amino acid sequences were identified, and phylogenetic analysis based on these amino acid sequences revealed essentially the same distances between the recent influenza virus isolates (1993-1998) and older isolates (Figure 1). In the influenza season 1989-1990, 13 out of 59 isolated viruses had a R384K mutation affecting both the HLA-B8 and HLA-B27 epitopes (Figure 2). In fact, R384 is the anchor residue of the HLA-B27 epitope and critical for association with MHC class I molecules. This R384K mutation was not found in subsequent seasons. However, in the season 1993-1994, a novel mutation at the same position in these CTL epitopes was found. This R384G mutation was present in all 56 viruses tested of this season, and maintained in all viruses tested of the season 1998-1999 (Figure 2). In all mutant viruses, the G at position 384 was coded for by the same codon (Table 2). The abrupt introduction of the R384G

mutation in 1993-1994 was accompanied with two other amino acid mutations in the sequenced NP region, S259L and E375G. These mutations are located in close proximity to the HLA-A3 and the HLA-B8 and HLA-B27 epitopes, respectively (Figure 2). We also sequenced several viruses isolated between 1994 and 1998 which all showed the R384G, S259L, and E375G mutations (data not shown). In contrast to the overlapping HLA-B8 and HLA-B27 epitopes, the HLA-A3 epitope ILRGSVAHK (aa 265 to 273) proved to be conserved. Only three silent mutations were found in this epitope in 3 out of the 162 viruses tested (data not shown). Likewise, no mutations were found that affected the HLA-B37 epitope (338 to 347).

Season	Variant[a]	N[b]	Season	Variant	N	Season	Variant	N
1989-1990	1	18	1991-1992	15	8	1993-1994	27	35
n=59	2	14	n=16	16	3	n=56	28	3
	3	8		17	3		29	2
	4	7		18	1		30	2
	5	3		19	1		31	2
	6	1					32	1
	7	1	1992-1993	20	7		33	1
	8	1	n=16	21	4		34	1
	9	1		22	1		35	1
	10	1		23	1		36	1
	11	1		24	1		37	1
	12	1		25	1		38	1
	13	1		26	1		39	1
	14	1					40	1
			1998-1999	44	11		41	1
			n=15	45	3		42	1
				46	1		43	1

TABLE 1. Virus variants and number of each variant observed per influenza season. The nucleotide sequence of the NP gene (nt 720-1175) of each virus of a particular season was compared to the consensus sequence of that season. [a]Variant numbers correspond with the numbers shown in figure 1; [b]Number of each variant.

Recognition of influenza A virus NP by specific CTL clones

An HLA-B27-restricted CTL clone, designated NP/B27, with specificity for the NP epitope SRYWAIRTR (aa 383 to 391), was generated. This CTL clone lysed matched target cells, pulsed with peptide SRYWAIRTR (Figure

3B and D). As a control, an HLA-A3-restricted CTL clone, designated NP/A3, with specificity for the conserved NP epitope ILRGSVAHK (aa 265 to 273) was used. As shown in figure 3A and C, this CTL clone lysed matched target cells pulsed with the corresponding peptide. The phenotype of both CTL clones as determined by FACS analysis was $CD3^+$,$CD4^-$,$CD8^+$ (data not shown).

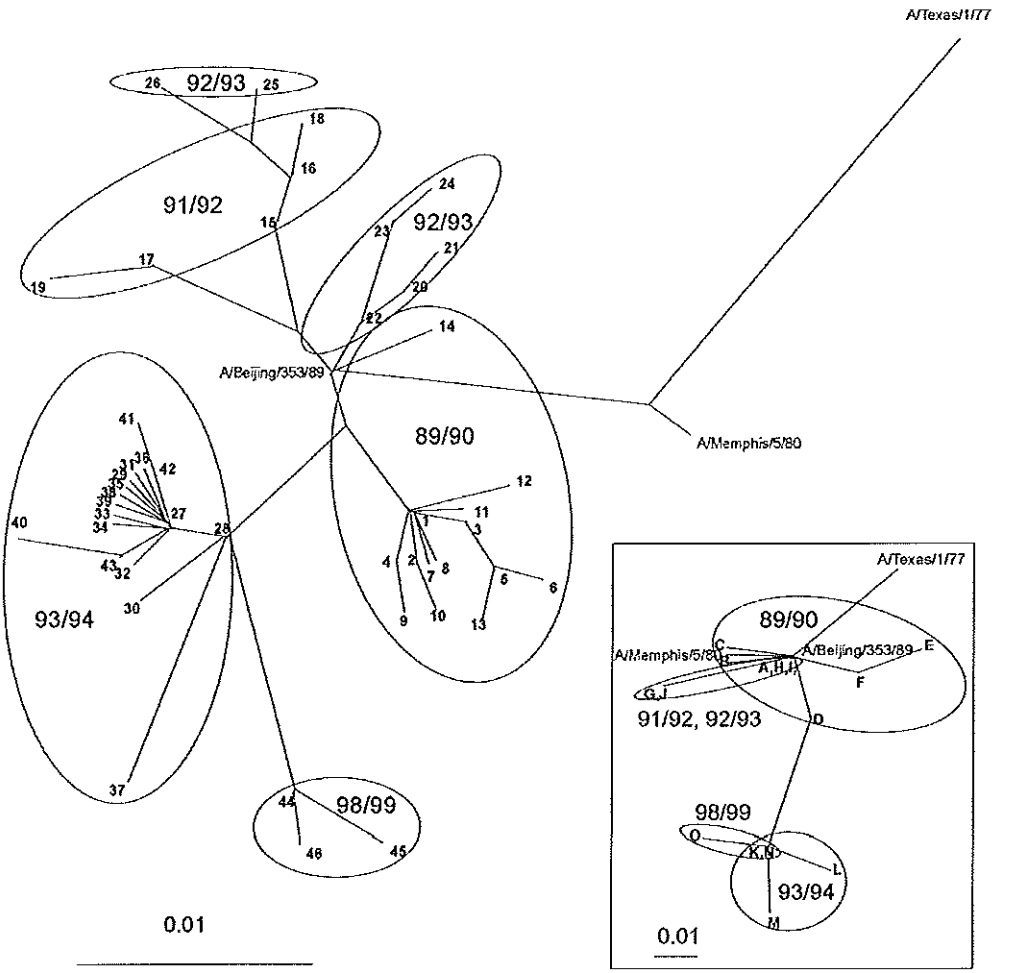

Figure 1. Maximum likelyhood tree based on nucleotide sequences of the NP gene. Part of the NP genes (nt 720-1175) of 162 influenza A (H3N2) viruses isolated from 1989 until 1999 was sequenced and subjected to phylogenetic analysis. Included in the figure are the influenza viruses A/Texas/1/77, A/Memphis/5/80 and A/Beijing/353/89. The numbers shown in the figure correspond to the numbers shown in table 1. The insert represents a protein distance tree (protdist, Fitch) based on the NP sequence of the representative influenza virus strains. The lettercode used for the respective amino acid sequences corresponds to that of figure 2.

```
                  240        250        260        270        280
                  |          |        ↓ |          |          |        ↓
1989-1990    A    DQVRESRNPG NAEIEDLIFS ARSALILRGS VAHKSCLPAC VYGPAVSSGY
             B    DQVRESRNPG NAEIEDLIFS ARSALILRGS VAHKSCLPAC VYGPAVSSGY
             C    DQVRESRNPG NAEIEDLIFS ARSALILRGS VAHKSCLPAC VYGPAVSSGY
             D    DQVRESRNPG NAEIEDLIFS ARSALILRGS VAHKSCLPAC VYGPAVSSGY
             E    DQVRESRNPG NAEIEDLIFS ARSALILRGS VAHKSCLPAC VYGPAVSSGY
             F    DQVRESRNPG NAEIEDLIFS ARSALILRGS VAHKSCLPAC VYGPAVSSGY

1991-1992    G    DQVRESRNPG NAEIEDLIFS ARSALILRGS VAHKSCLPAC VYGPAVSNGY
             H    DQVRESRNPG NAEIEDLIFS ARSALILRGS VAHKSCLPAC VYGPAVSSGY

1992-1993    I    DQVRESRNPG NAEIEDLIFS ARSALILRGS VAHKSCLPAC VYGPAVSSGY
             J    DQVRESRNPG NAEIEDLIFS ARSALILRGS VAHKSCLPAC VYGPAVSNGY

1993-1994    K    DQVRESRNPG NAEIEDLIFL ARSALILRGS VAHKSCLPAC VYGPAVSSGY
             L    DQVRESRNPG NAEIEDLIFL ARSALILRGS VAHKSCLPAC VYGPAVSSGY
             M    DQVRESRNPG NAEIEDLIFL ARSALILRGS VAHKSCLPAC VYGPAVSSGY

1998-1999    N    DQVRESRNPG NAEIEDLIFL ARSALILRGS VAHKSCLPAC VYGPAVSSGY
             O    DQVRESRNPG NAEIEDLIFL ARSALILRGS VAHKSCLPAC VYGPAVSSGY
                                                  HLA-A3
```

```
   290        300        310        320        330        340
   ↓  ↓     ↓ |          |          |          |          |
   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
   DFEREGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG

   GFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG

   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
   GFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG

   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
   DFEKEGYSLL GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG

   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
   DFEKEGYSLV GIDPFKLLQN SQVYSLIRPN ENPAHKSQLV WMACHSAAFE DLRLLSFIRG
                                                        HLA-B37
```

Figure 2. Continued on next page.

```
350         360         370         380         390
↓           ↓           ↓  ↓↓       ↓  ↓   ↓  ↓
TKVSPRGKLS TRGVQIASNE NMDNMESSTL ELRSRYWAIR TR (36) consensus
TKVSPRGKLS TRGVQIASNE NMDNMESSTL ELRSRYWAIR TR (1)
SKVSPRGKLS TRGVQIASNE NMDNMESSTL ELRSRYWAIR TR (8)
TKVSPRGKLS TRGVQIASNE NMDNMGSSTL ELRSRYWAIR TR (1)
TKVSPRGKLS TRGVQIASNE NMDNIESSTL ELRSKYWAIR TR (1)
TKVSPRGKLS TRGVQIASNE NMDNMESSTL ELRSKYWAIR TR (12)

TKVSPRGKLS TRGVQIASNE NMDNMESSTL ELRSRYWAIR TR (12) consensus
TKVSPRGKLS TRGVQIASNE NMDNMESSTL ELRSRYWAIR TR (4)

TKVSPRGKLS TRGVQIASNE NMDNMESSTL ELRSRYWAIR TR (14) consensus
TKVSPRGKLS TRGVQIASNE NMDNMESSTL ELRSRYWAIR TR (2)

TKVSPRGKLS TRGVQIASNE NMDNMGSSTL ELRSGYWAIR TR (52) consensus
TKVSPRGKLS TRGVQIASNE NMDNMGSSTL ELRSGYWAIR TR (2)
TKVSPRGKLS TTGVQIASNE NMDNMGSSTL ELRSGYWAIR TR (2)

TKVSPRGKLS TRGVQIASNE NMDNMGSSTL ELRSGYWAIR TR (14) consensus
TKVSPRGKLS TRGVQIASNE NMDNMGSSTL ELRSGYWAIT TR (1)
                                   HLA-B8/HLA-B27
```

Figure 2. Amino acid sequences of the NP (aa 240 to 391) of influenza A (H3N2) viruses isolated from 1989 until 1999. The consensus sequence of each season is shown in the upper rows whereas variant sequences are shown in the lower rows. CTL epitopes are underlined and shown in bold. Amino acid differences between seasons are shown in bold while differences within a season are shown in bold/italic. All mutations are marked with an arrow. The number in brackets refers to the number of isolates showing that sequence.

The effect of the R384K and R384G mutations in the HLA-B27-specific NP epitope SRYWAIRTR on CTL-mediated lysis was first studied with synthetic peptides. The NP/B27 CTL clone lysed target cells pulsed with the peptide SRYWAIRTR, whereas control untreated target cells or cells pulsed with the mutant peptides SKYWAIRTR or SGYWAIRTR were not recognized by this CTL clone (Figure 4B). Next, cells infected with influenza A viruses having the respective mutations in the NP were used as target cells. Target cells infected with influenza virus A/PR/8/34, A/Neth/651/89 or A/Neth/18/94 were all recognized by the NP/A3 CTL clone (Figure 4E). However, the NP/B27 CTL clone lysed only target cells infected with influenza virus A/PR/8/34, which had the non mutated epitope, and failed to recognize target cells infected with influenza virus A/Neth/651/89 or A/Neth/18/94 which had the R384K or the R384G mutation, respectively (Figure 4F). These data were further confirmed using RVV expressing the NP of A/PR/8/34 (non mutated epitope) or A/Neth/18/94 (R384G mutant epitope). The NP/A3 CTL clone recognized target cells infected with RVV expressing NP of A/PR/8/34 or

A/Neth/18/94 equally well and failed to recognize target cells infected with a control vaccinia virus (Figure 4C). The NP/B27 CTL clone, however, recognized target cells infected with RVV expressing NP of A/PR/8/34 but failed to recognize target cells infected with RVV expressing NP of A/Neth/18/94 (Figure 4D).

Season	Amino acid	Nucleotide mutation	N[a]
1989-1990	K293R	AAA→AGA	1
	T350S	ACC→TCC	8
	M374I	ATG→ATA	1
	E375G	GAA→GGA	1
	R384K	AGG→AAG	13
1991-1992	N287S	AAT→AGT	4
	G290D	GGC→GAC	4
1992-1993	S287N	AGT→AAT	2
	D290G	GAC→GGC	2
1993-1994	V299L	GTG→TTG	2
	R361T	AGA→ACA	2
	R384G	AGG→GGG[b]	56
1998-1999	R384G	AGG→GGG[b]	15
	R389T	AGG→ACG	1

TABLE 2. Amino acid mutations in the influenza NP and the corresponding nucleotide mutations.
[a]Number of viruses containing the mutation; see also figure 2; [b]Since all viruses from the influenza season 1993-1994 onwards contained the R384G mutation, the codon encoding the original R384 in the NP of influenza viruses of the previous season (1992-1993) was taken for comparison.

Functional analysis of NP sequences

In order to determine whether the R384G mutation affected the function of the NP, a eukaryotic expression plasmid encoding the NP of A/HK/2/68 (having an R at position 384) or the NP of A/Neth/18/94 (having a G at position 384) was cotransfected with expression plasmids encoding the three polymerase proteins of influenza A virus (PB1, PB2 and PA) and a plasmid expressing GFP RNA in the context of an influenza A virus NS gene segment. Others have shown previously that such negative-sense RNA molecules can serve as

templates for production of cRNA and mRNA in the presence of functional NP and polymerase proteins, ultimately resulting in synthesis of the encoded protein (29). GFP synthesis was measured by FACS analysis (Table 3). The percentage of positive cells and mean fluorescense did not differ significantly between cells transfected with the NP gene of A/HK/2/68 and those transfected with the NP gene of A/Neth/18/94, indicating that both NPs were equally functional. Furthermore, influenza viruses A/HK/2/68 and A/Neth/18/94 yielded comparable virus titers in MDCK cells, indicating that both viruses replicated equally well (data not shown).

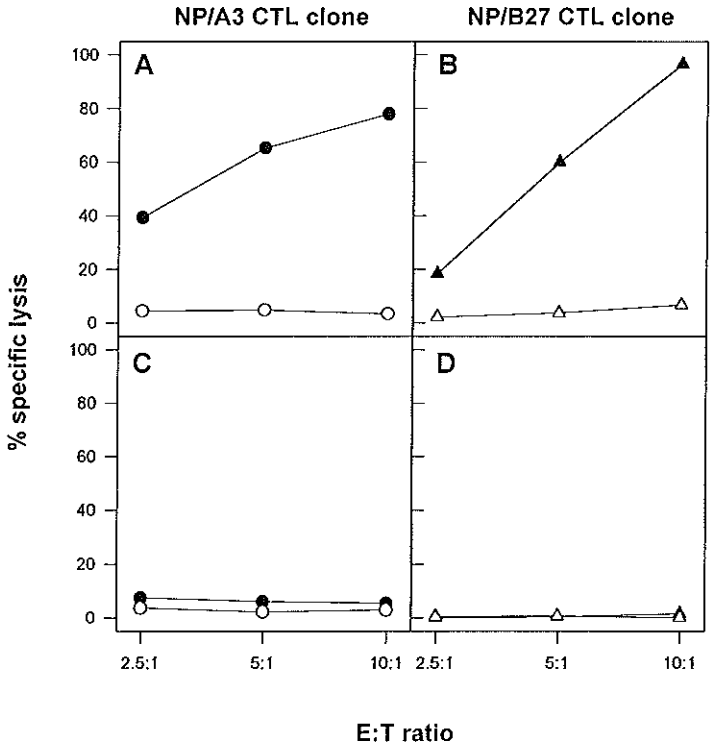

Figure 3. Confirmation of specificity and HLA restriction of CTL clones. HLA-A3 and -B27 positive (A and B) and HLA-A3 and -B27 negative (C and D) B-LCL were incubated with the HLA-A3-specific peptide ILRGSVAHK (solid circles), the HLA-B27-specific peptide SRYWAIRTR (solid triangles) or left untreated (open circles and triangles) followed by incubation with the NP/A3 (A and C) or NP/B27 (B and D) CTL clone. CTL assays were performed in triplicate at three E:T ratios. Mean percentages of specific lysis are shown.

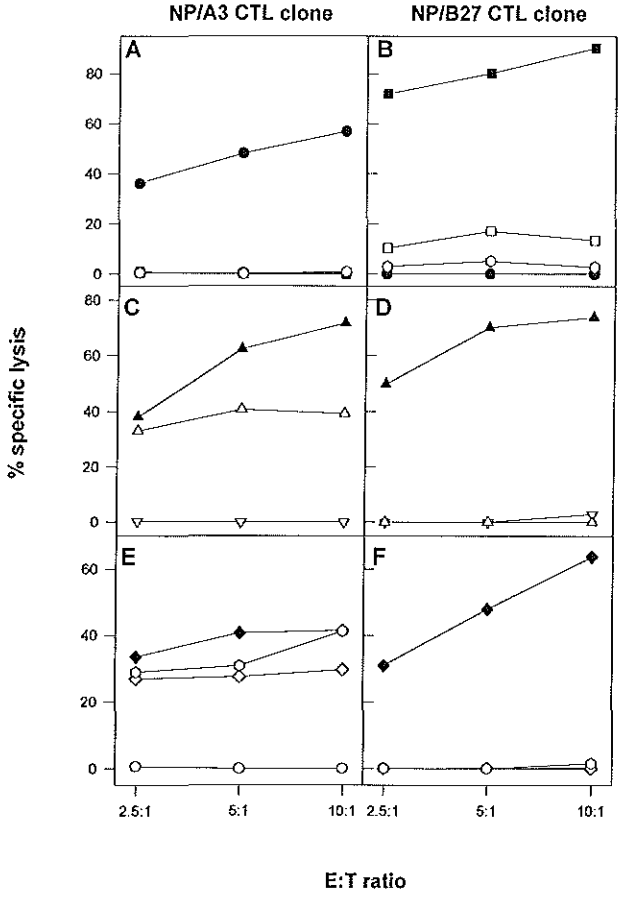

Figure 4. Effect of mutations in the HLA-B27 epitope on CTL-mediated lysis of target cells. HLA-A3 and -B27 positive B-LCL of one donor were incubated with the HLA-A3-specific peptide ILRGSVAHK (solid circles), the HLA-B27-specific peptide SRYWAIRTR (solid squares), the HLA-B27 mutant peptides SGYWAIRTR (open squares) or SKYWAIRTR (open hexagon) or left untreated (open circles) and used as targets in CTL assays with the NP/A3 (A) or NP/B27 (B) CTL clone as effector. The same B-LCL were infected with a control vaccinia virus (open triangle pointing downward), a vaccinia virus expressing the NP of A/PR/8/34 (solid triangle) or a vaccinia virus expressing NP of A/Neth/18/94 (open triangle) followed by incubation with the NP/A3 (C) or NP/B27 (D) CTL clone. Also, B-LCL were infected with influenza virus A/PR/8/34 (solid diamond), A/Neth/18/94 (open diamond), A/Neth/651/89 (open hexagon) or left untreated (open circles) followed by incubation with the NP/A3 (E) or NP/B27 (F) CTL clone. CTL assays were performed in triplicate at three E:T ratios. Mean percentages of specific lysis are shown.

NP	% Positive cells	Mean fluorescense
negative control	0.3	433
A/HK/2/68	26.4	2002
A/Neth/18/94	23.3	1944
positive control	95.0	6383

TABLE 3. Functional analysis of wild type and mutant NP.
293T cells were transfected with a mixture of plasmids including pHMG-PB1, pHMG-PB2, pHMG-PA, RF419 and a plasmid encoding NP of A/HK/2/68 or A/Neth/18/94. Cells transfected with plasmid pcDNA3 served as a negative control while cells transfected with plasmid pEGFP-N1 were used as a positive control. Percentage cells showing GFP expression and mean fluorescense of 293T cells were measured by FACS analysis. Results of a representative experiment are shown.

DISCUSSION

In the present paper, we show that a R384K or R384G mutation in the HLA-B27-specific epitope SRYWAIRTR (383-391) of influenza A virus NP abrogates MHC class I presentation and recognition by specific CTLs. In peptides that associate with HLA-B27, the second residue is often an arginine (R) and this so-called anchor residue is critical for binding to HLA-B27 molecules[19,23,24,41,44]. Mutations at this position are accompanied by loss of binding to HLA-B27 and hence loss of the activity of specific CTLs. This has previously been demonstrated for the CTL epitope KRWIILGLNK (263-272) in the HIV-1 Gag protein: exchanging R264 for K or G diminished binding to HLA-B27 and lysis of peptide pulsed target cells, with the R264G mutation having the greatest effect[31]. We here show that cells pulsed with mutant peptides, having identical mutations at the anchor residue of the epitope SRYWAIRTR of the influenza A virus NP (see Table 4 for comparison), were not lysed by HLA-B27-restricted CTLs. In addition, we show that cells infected either with influenza A viruses or with vaccinia virus expressing mutant NP were no longer recognized by specific CTLs.
The R384K mutation was found in several isolates of the influenza season 1989-1990, but not in later seasons. This mutation was previously found in two viruses isolated in 1971 and 1972[42]. In contrast, the R384G mutation was found in all influenza A virus isolates from the influenza season 1993-1994 onwards. A search in the influenza virus sequence data-base (Los Alamos National Laboratory) and in the literature revealed that from the introduction of H3N2 viruses in 1968 until the 1993 epidemics, all virus isolates (except for the R384K mutant viruses mentioned above) had the nonmutated HLA-B27

epitope SRYWAIRTR. Since most viruses have been selected for sequencing based on antigenic properties of their haemagglutinin, we assume that the NP sequences of influenza viruses in this data base are random with regard to CTL epitopes. Of note, the R384G mutation has never been found in H1N1 and H2N2 viruses. Since we sequenced influenza viruses that were isolated from patients living in geographically distinct areas in The Netherlands, it is unlikely that all viruses originated from a single source. Moreover, in the region of the NP that was sequenced (representing 152 aa) differences were found between viruses isolated within a single season. Interestingly, the R384G mutation has also been found in influenza A (H3N2) viruses isolated in Japan after 1993, although viruses lacking the R384G mutation cocirculated in this area after 1993[22].

HLA-B27 epitope	Amino acid sequence[a]	References fluorescense
HIV-1 gag (263-272)	KRWIILGLNK	(5)
HIV-1 gag mutant 1	KKWIILGLNK	(13,31[b])
HIV-1 gag mutant 2	KGWIILGLNK	(31[b])
Influenza NP (383-391)	SRYWAIRTR	(16)
Influenza NP mutant 1	SKYWAIRTR	present paper
Influenza NP mutant 2	SGYWAIRTR	present paper

TABLE 4. Comparison of wild type and mutant HLA-B27-restricted CTL epitopes of influenza A virus nucleoprotein with those of the HIV-1 gag protein.
[a] Mutations are shown in bold; [b] *In vitro* generated mutant.

The R384G mutation found in the influenza season 1993-1994 was accompanied with two other amino acid mutations in the NP, S259L and E375G. Also in the Japanese strains containing the R384G mutation (see above) the same accompanying mutations were found, which may indicate a more global spread of these viruses. The S259L mutation is only six aa N terminal of the HLA-A3 epitope ILRGSVAHK and, therefore, could have affected processing of this peptide. However, our results show that this mutation did not have an effect on MHC class I presentation of the HLA-A3 epitope. Although a G at position 384 was always accompanied with a L at position 259 and a G at position 375, the latter two amino acids have previously also been found with an R at position 384, indicating that the R384G mutation is not forced by the other two mutations or vice versa and that the mutations observed are not mutually compensatory. In addition, in the influenza season 1989-1990 we obtained a virus isolate having a G at position

375 and an R at position 384 of the NP.
The consequence of a mutation at the anchor residue with respect to virus escape from immunesurveillance by CTLs has been demonstrated previously[13]. The R264K mutation in the HIV-1 Gag HLA-B27 epitope KRWIILGLNK (263 to 272) was accompanied with progression to AIDS in HIV-1-infected patients wich showed strong CTL responses against the nonmutated epitope. Although the role of CTLs in protection from influenza virus infection is still controversial, CTLs are likely to contribute to virus clearance and inhibition of virus spread (40). Therefore, mutations at the anchor residue of the influenza A virus NP epitope SRYWAIRTR may have implications for HLA-B27 positive influenza virus-infected patients. At this point, it is not clear what the consequences of the R384G mutation are with respect to MHC class I binding and/or recognition by CTLs of the HLA-B8 epitope.
The observation that the R384G mutation was conserved in all sequenced influenza A (H3N2) viruses isolated after 1993 in The Netherlands suggests that this mutation is advantageous to the virus. We did not find differences between a wild-type virus and an R384G mutant virus with respect to replication properties *in vitro*. In addition, in transfection experiments, we have shown that an RNA molecule that resembles an influenza A virus gene segment was equally well transcribed and translated in the presence of wild-type and mutant NP. Since the R384G mutation completely abrogates the recognition of the HLA-B27 epitope by specific CTLs, influenza A viruses harbouring this mutation may escape from immunity mediated by virus-specific CTLs. HLA-B27 positive individuals constitute approximately 8% of the Caucasian population, which is predominant in The Netherlands. The immune pressure mediated by CTLs in these individuals, which recognize the wild-type HLA-B27 epitope in the NP, may have contributed to the emergence and continued circulation of escape mutant viruses. The mutant virus may have emerged from the quasispecies of influenza viruses in HLA-B27 positive individuals. Since the R384G mutation did not impose functional constraints on the NP, a selective pressure in 8% of the individuals may have been sufficient to drive the selection process. At present, it is unknown whether the HLA-B27 epitope is immunodominant. Conceivably, this would favour the emergence of the R384G mutant virus. Little is known about the *in vivo* rate of attack of target cells by specific CTL: an infected cell may be recognized by one CTL but not by another at the same time, allowing the virus to escape from the action of one CTL clone. Once emerged into the human population, viruses with the R384G

mutation are fully replication competent and ultimately have replaced the original virus having the non mutated epitope.

In contrast to the HLA-B8 and -B27 epitope, the HLA-A3 epitope proved to be conserved; we found only three silent mutations out of 162 sequenced influenza A (H3N2) viruses isolated over 10 years despite a higher prevalence of the HLA-A3 allele in the human population. With the exception of one virus having a I265V mutation[42], influenza virus sequence database searches (including H1N1, H2N2 and H3N2 viruses isolated over a period of more than 60 years) also did not reveal amino acid mutations within this epitope. A possible explanation is that mutations in this region of the NP are not or are less well tolerated by the virus because of functional constraints. For example, an R267A mutation in the HLA-A3 epitope has been shown elsewhere to affect RNA binding by the NP[11]. Recently, a second HLA-B27 epitope in the NP (174 to 184) has been described[17]. However, a sequence database search revealed that this HLA-B27 epitope is completely conserved.

CTL escape mutants have been shown to arise in individuals persistently infected with virus, e.g. HIV, as a result of continuous immune pressure mediated by CTLs. Influenza A viruses cause acute infections, affecting a large percentage of individuals each year, and therefore may be considered as persisting in the human population. We have provided epidemiological and immunological evidence for antigenic drift in the influenza A virus NP, possibly as a result of immune pressure mediated by CTLs. Thus, in addition to the introduction of mutations in the surface glycoproteins allowing escape from antibody-mediated immunity, the introduction of mutations in CTL epitopes may be a strategy exploited by influenza A viruses to escape from CTL-mediated immunity. This would be the first example of CTL-mediated antigenic drift in a virus that causes an acute infection.

ACKNOWLEDGEMENTS

Part of this work was supported by the Foundation for Respiratory Virus Infections, notably Influenza (SRVI). We acknowledge Dr. W. Biddison, NIH, Bethesda, MD for providing us with the NP/A3 CTL clone, Dr. B. Moss, NIH, Bethesda, MD for providing us with recombinant vaccinia virus expressing the NP of A/PR/8/34 and Dr. P. Palese, Mount Sinai School of Medicine, New York, NY for providing us with the HMG-PB1, PB2 and

PA expression plasmids. Finally, the authors wish to thank Ger van der Water for continuous support.

REFERENCES

[01] Apolloni, A., Moss, D., Stumm, R., Burrows, S., Suhrbier, A., Misko, I., Smidt, C. and Sculley, T. 1992. Sequence variation of cytotoxic T cell epitopes in different isolates of Epstein-Barr virus. *Eur.J.Immunol.* 22:183-189.

[02] Bertoletti, A., Sette, A., Chisari, F.V., Penna, A., Levrero, M., De Carli, M., Fiaccadori, F. and Ferrari, C. 1994. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature* 369:407-410.

[03] Bertoletti, A., Costanzo, A., Chisari, F.V., Levrero, M., Artini, M., Sette, A., Penna, A., Giuberti, T., Fiaccadori, F. and Ferrari, C. 1994. Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. *J.Exp.Med.* 180:933-943.

[04] Burrows, J.M., Burrows, S.R., Poulsen, L.M., Sculley, T.B., Moss, D.J. Khanna, R. 1996. Unusually high frequency of Epstein-Barr virus genetic variants in Papua New Guinea that can escape cytotoxic T-cell recognition: implications for virus evolution. *J.Virol.* 70:2490-2496.

[05] Buseyne, F., McChesney, M., Porrot, F., Kovarik, S., Guy, B. and Riviere, Y. 1993. Gag-specific cytotoxic T lymphocytes from human immunodeficiency virus type 1 infected individuals: gag epitopes are clustered in three regions of the p24 gag protein. *J.Virol.* 67:694-702.

[06] de Campos-Lima, P.O., Gavioli, R., Zhang, Q.J., Wallace, L.E., Dolcetti, R., Rowe, M., Rickinson, A.B. and Masucci, M.G. 1993. HLA-A11 epitope loss isolates of Epstein-Barr virus from a highly A11+ population. *Science* 260:98-100.

[07] de Campos-Lima, P.O., Levitsky, V., Brooks, J., Lee, S.P., Hu, F., Rickinson, A.B. and Masucci, M.G. 1994. T-cell responses and virus evolution: loss of HLA A11-restricted CTL epitopes in Epstein-Barr virus isolates from highly A11-positive populations by selective mutation of anchor residues. *J.Exp.Med.* 179:1297-1305.

[08] Couillin, I., Culmann-Penciolelli, B., Gomard, E., Choppin, J., Levy, J.P., Guillet, J.G. and Saragosti, S. 1994. Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus 1 NEF protein. *J.Exp.Med.* 180:1129-1134.

[09] DiBrino, M., Tsuchida, T., Turner, R.V., Parker, K.C., Coligan, J.E. and Biddison, W.E. 1993. HLA-A1 and HLA-A3 T cell epitopes derived from influenza virus proteins predicted from peptide binding motifs. *J.Immunol.* 151:5930-5935.

[10] Dong, T., Boyd, D., Rosenberg, W., Alp, N., Takiguchi, M., McMichael, A. and Rowland-Jones, S. 1996. An HLA-B35-restricted epitope modified at ananchor residue results in an antagonist peptide. *Eur.J.Immunol.* 26:335-339.

[11] Elton, D., Medcalf, L., Bishop, K., Harrison, D. and Digard, P. 1999. Identification of amino acid residues of influenza virus nucleoprotein essential for RNA binding. *J.Virol.* 73:7357-7367.

[12] Gotch, F., McMichael, A., Smith, G. and Moss, B. 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. *J.Exp.Med.* 165:408-416.

[13] Goulder, P.J., Phillips, R.E., Colbert, R.A., McAdam, S., Ogg, G., Nowak, M.A., Giangrande, P., Luzzi, G., Morgan, B., Edwards, A., McMichael, A.J. and Rowland-Jones, S. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat.Med.* 2:212-217.

[14] van de Griend, R.J., van Krimpen, B.A., Bol, S.J.L., Thompson, A. and Bolhuis, R.L.H. 1984. Rapid expansion of human cytotoxic T cell clones: Growth promotion by a heat-labile serum component and various types of feeder cells. *J.Immunol.Meth.* 66:285-298.

[15] Hahn, Y.S., Hahn, C.S., Braciale, V.L., Braciale, T.J. and Rice, C.M. 1992. CD8+ T cell recognition of an endogenously processed epitope is regulated primarily by residues within the epitope. *J.Exp.Med.* 176:1335-1341.

[16] Huet, S., Nixon, D.F., Rothbard, J.B., Townsend, A., Ellis, S.A. and McMichael, A.J. 1990. Structural homologies between two HLA B27-restricted peptides suggests residues important for interaction with HLA B27. *Int.Immunol.* 2:311-316.

[17] Jameson, J., Cruz, J. and Ennis, F.A. 1998. Human cytotoxic T-lymphocyte repertoire to influenza A virus. *J.Virol.* 72:8682-8689.

[18] Jameson, S.C., Carbone, F.R. and Bevan, M.J. 1993. Clone-specific T cell receptor antagonists of major histocompatibility complex class I-restricted cytotoxic T cells. *J.Exp.Med.* 177:1541-1550.

[19] Jardetzky, T.S., Lane, W.S., Robinson, R.A., Madden, D.R. and Wiley, D.C. 1991. Identification of self-peptides bound to purified HLA-B27. *Nature* 353:326-329.

[20] Klenerman, P., Rowland-Jones, S., McAdam, S., Edwards, J., Daenke, S., Lalloo, D., Koppe, B., Rosenberg, W., Boyd, D., Edwards, A., Giangrande, P., Phillips, R.E. and McMichael, A.J. 1994. Cytotoxic T-cell activiy antagonized by naturally occuring HIV-1 Gag variants. *Nature* 369:403-407.

[21] Koup, R.A. 1994. Virus escape from CTL recognition. 1994. *J.Exp.Med.* 180:779-782.

[22] Lindstrom, S.E., Hiromoto, Y., Nerome, R., Omoe, K., Sugita, S., Yamazaki, Y., Takahashi, T. and Nerome, K. 1998. Phylogenetic analysis of the entire genome of influenza A (H3N2) viruses from Japan: evidence for genetic reassortment of the six internal genes. *J.Virol.* 72:8021-8031.

[23] Madden, D.R., Gorga, J.C., Strominger, J.L. and Wiley, D.C. 1991. The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. *Nature* 353:321-325.

[24] Madden, D.R., Gorga, J.C., Strominger, J.L. and Wiley, D.C. 1992. The three-dimensional structure of HLA-B27 at 2,1 Å resolution suggests a general mechanism for tight peptide binding to MHC. *Cell* 70:1035-1048.

[25] McMichael, A.J., Mitchie, C.A., Gotch, F.M., Smith, G.L. and Moss, B. 1986. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. *J.Gen.Virol.* 67:719-726.

[26] McMichael, A.J., Gotch, F.M. and Rothbard, J. 1986. HLA B37 determines an influenza virus nucleoprotein epitope recognized by cytotoxic T lymphocytes. *J.Exp.Med.* 164:1397-1406.

[27] McMichael, A.J. and Phillips, R.E. 1997. Escape of human immunodeficiency virus from immune control. *Annu.Rev.Immunol.* 15:271-296.

[28] Moskophidis, D. and Zinkernagel, R.M. 1995. Immunobiology of cytotoxic T-cell escape mutants of lymphocytic choriomeningitis virus. *J.Virol.* 69:2187-2193.

[29] Neumann, G., Watanabe, T., Hiroshi, I., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D.R., Donis, R., Hoffmann, E., Hobom, G. and Kawaoka, Y. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *J.Virol.* 96:9345-9350.

[30] Neumann, G., Watanabe, T. and Kawaoka, Y. 2000. Plasmid-driven formation of influenza virus-like particles. *J.Virol.* 74:547-551.

[31] Nietfield, W., Bauer, M., Fevrier, M., Maier, R., Holzwarth, B., Frank, R., Maier, B., Riviere, Y. and Meyerhans, A. 1995. Sequence constraints and recognition by CTL of an HLA-B27-restricted HIV-1 gag epitope. *J.Immunol.* 154:2188-2197.

[32] Oldstone, M.B.A. 1997. How viruses escape from cytotoxic T lymphocytes: molecular parameters and players. *Virology* 234:179-185.

[33] Pear, W.S., Nolan, G.P., Scott, M.L. and Baltimore, D. 1993. Production of high-titer helper-free retrovirus by transient transfection. *Proc.Natl.Acad.Sci.USA* 90:8392-8396.

[34] Phillips, R.E., Rowland-Jones, E.S., Nixon, D.F., Gotch, F.M., Edwards, J.P., Ogunlesi, A.O., Elvin, J.G., Rothbard, J.A., Bangham, C.R., Rizza, C.R. and McMichael, A.J. 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T-cell recognition. *Nature* 354:453-459.

[35] Pircher, H., Moskophidis, D., Rohrer, U., Bürki, K., Hengartner, H. and Zinkernagel, R.M. 1990. Viral escape by selection of cytotoxic T cell-resistant virus variants *in vivo*. *Nature* 346:629-633.

[36] Pleschka, S., Jaskunas, R.S., Engelhardt, O.G., Zürcher, T., Palese, P. and Garcia-Sastres, A. 1996. A plasmid-based reverse genetics system for influenza A virus. *J.Virol.* 70:4188-4192.

[37] Price, D.A., Goulder, P.J., Klenerman, P., Sewell, A.K., Easterbrook, P.J., Troop, M., Bangham, C.R. and Phillips, R.E. 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc.Natl.Acad.Sci.USA* 94:1890-1895.

[38] Reid, S.W., Mc.Adam, S., Smith, K.J., Klenerman, P., O'Callaghan, C.A., Harlos, K., Jakobsen, B.K., McMichael, A.J., Bell, J.I., Stuart, D.L. and Jones, E.Y. 1996. Antagonist HIV-1 Gag peptides induce structural changes in HLA B8. *J.Exp.Med.* 184:2279-2286.

[39] Rimmelzwaan, G.F., Siebelink, K.H., Huisman, R.C., Moss, B., Francis, M.J. and Osterhaus, A.D.M.E. 1994. Removal of the cleavage site of recombinant feline immunodeficiency virus envelope protein facilitates incorporation of the surface glycoprotein in immune-stimulating complexes. *J.Gen.Virol.* 75:2097-2102.

[40] Rimmelzwaan, G.F. and Osterhaus, A.D.M.E. 1995. Cytotoxic T lymphocyte memory: role in cross-protective immunity against influenza ? *Vaccine* 13:703-705.

[41] Rötzschke, O., Falk, K., Stevanovic, S., Gnau, V., Jung, G. and Rammensee, H. 1994. Dominant aromatic/aliphatic C-terminal anchor in HLA-B2702 and B2705 peptide motifs. *Immunogenetics* 39:74-77.

[42] Shu, L.L., Bean, W.J. and Webster, R.G. 1993. Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. *J.Virol.* 67:2723-2729.

[43] Sutton, J., Rowland-Jones, S., Rosenberg, W., Nixon, D., Gotch, F., Gao, X.M., Murray, N., Spoonas, A., Driscoll, P., Smith, M., Willis, A. and McMichael, A. 1993. A sequence pattern for peptides presented to cytotoxic T lymphocytes by HLA B8 revealed by analysis of epitopes and eluted peptides. *Eur.J.Immunol.* 23:447-453.

[44] Tanigaki, N., Fruci, D., Vigneti, E., Starace, G., Rovero, P., Londei, M., Butler, R.H. and Tosi, R. 1994. The peptide binding specificity of HLA-B27 subtypes. *Immunogenetics* 40:192-198.

[45] Weiner, A., Erickson, A.L., Kansopon, J., Crawford, K., Muchmore, E., Hughes, A.K., Houghton, M. and Walker, C.M. 1995. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. *Proc.Natl.Acad.Sci.USA* 92:2755-2759.

[46] Yellen-Shaw, A.J., Wherry, E.J., Dubois, G.C. and Eisenlohr, L.C. 1997. Point mutation flanking a CTL epitope ablates in vitro and in vivo recognition of a full-length viral protein. *J.Immunol.* 158:3227-3234.

CHAPTER 6

Use of recombinant nucleoproteins in enzyme-linked immunosorbent assays for detection of virus-specific immunoglobulin A (IgA) and IgG antibodies in Influenza virus A- or B- infected patients

J.T.M. Voeten, J. Groen, D. van Alphen, E.C.J. Claas, R. de Groot, A.D.M.E. Osterhaus and G.F. Rimmelzwaan

Journal of Clinical Microbiology 36:3527-3531 (1998)

SUMMARY

The nucleoprotein genes of influenza virus A/Netherlands/018/94 (H3N2) and influenza virus B/Harbin/7/94 were cloned into the bacterial expression vector pMalC to yield highly purified recombinant influenza virus A and B nucleoproteins. With these recombinant influenza nucleoproteins, enzyme-linked immunosorbent assays (ELISAs) were developed for the detection of influenza virus A- and B-specific immunoglobulin A (IgA) and IgG serum antibodies. Serum samples were collected at consecutive time points after the onset of clinical symptoms from patients with confirmed influenza virus A or B infections. Nucleoprotein-specific IgA antibodies were detected in 41.2% of influenza virus A-infected patients and in 66.7% of influenza virus B-infected patients on day 6 after onset of clinical symptoms. In serum samples taken on day 21 (influenza virus A-infected patients) or day 28 (influenza virus B-infected patients), nucleoprotein-specific IgA antibodies could be detected in 58.8 and 58.3% of influenza virus A- and B-infected patients, respectively. At the same time, IgG antibody rises were detected in 88.2% of influenza virus A-infected patients and in 95.8% of influenza virus B-infected patients. On comparison, hemagglutination inhibition assays detected antibody titre rises in 81.3 and 72.7% of patients infected with influenza virus A and B, respectively. In contrast to the detection of nucleoprotein-specific IgG antibodies or hemagglutination inhibiting antibodies, the detection of nucleoprotein-specific IgA antibodies does not require paired serum samples and therefore can be considered an attractive alternative for the rapid serological diagnosis of influenza.

INTRODUCTION

Influenza viruses (family *Orthomyxoviridae*) are the causal agents of recurrent epidemics of acute respiratory disease in man. For the laboratory diagnosis of influenza virus infections, several methods which detect either viral antigens or antigen-specific serum antibodies are used. For the quantification of influenza virus-specific serum antibodies, the hemagglutination inhibition (HI) assay and complement fixation (CF) assay are routinely used. However, these assays suffer from some disadvantages. They are laborious to perform, difficult to incorporate into automated procedures and require a continuous source of the appropriate erythrocytes. Alternatively, enzyme-linked immunosorbent assays (ELISAs) have been used for the detection of influenza virus-specific antibodies. ELISAs measuring influenza virus-specific serum

IgG antibodies have been shown to be more sensitive than the HI or the CF assay[1,10,11,13-17,22,23]. In addition, ELISAs enable the detection of antibodies of different isotypes[3,6,18,19]. For example, the demonstration of virus-specific IgA antibodies after influenza virus infections has been shown to be of diagnostic value[5,6,19]. The preparation of viral antigens to be used in these ELISAs usually requires the concentration and purification of virus conventionally propagated in embryonated chicken eggs or cell culture. However, ELISAs with purified (recombinant) viral proteins have also been described[8,9,12,20]. In the present paper we describe the production of recombinant nucleoproteins (NPs) of influenza viruses A and B as a virtually unlimited source of viral antigen. By using highly purified recombinant NPs of influenza viruses A and B, ELISAs were developed for the detection of virus-specific immunoglobulin A (IgA) and IgG serum antibodies. With serum samples obtained from patients with confirmed influenza virus A and B infections, the value of these recombinant NP-based ELISA systems was demonstrated.

MATERIALS AND METHODS

Cloning of the NP genes of influenza viruses A and B

The influenza viruses A/Netherlands/018/94 (H3N2) and B/Harbin/7/94 were obtained from the repository of the Dutch National Influenza Centre. Viral RNA was extracted from these viruses as described previously[4]. A reverse transcriptase (RT) reaction was performed to obtain single-stranded DNA copies of gene segment 5, which encodes the NP. To 10 µl of viral RNA 2 µl forward primer (10 pmol/µl) was added and the mixture was incubated at 80°C for 2 min, followed by 5 min of incubation on ice. Then, deoxynucleoside triphosphates (0.5 mM each), dithiothreitol (10 mM), RNasin (40 U) and Moloney murine leukemia virus RT (200 U) were added in a total volume of 25 µl of 1 x RT buffer followed by incubation at 42°C for 45 min. The reaction was stopped by heating the mixture to 95°C for 3 min. The DNA obtained was used as a template in a PCR. Besides the DNA, the PCR mixture contained 20 pmol of forward and 20 pmol of reverse primer, deoxynucleoside triphosphates (0.2 mM each), and *Pfu* polymerase (5 units) in a total of 100 µl 1x *Pfu* buffer. The PCR cycles consisted of 1 min at 94°C, 2 min at 52°C, and 4 min at 72°C for a total of 40 cycles. Primer sequences were based on the consensus sequence of the NP genes of recent influenza virus A and B strains obtained from the Wisconsin Sequence Analysis Package and designed in such a way that the ultimate PCR product contained an *Eco*RI (influenza virus A) or *Xba*I (influenza virus B) restriction endonuclease recognition sequence upstream of the start codon and a *Sal*I restriction endonuclease recognition sequence downstream of the stop codon of the NP genes. The PCR products of the NP gene of both viruses were cloned into the bacterial expression vector pMalC (New England Biolabs) in frame with the gene encoding the maltose binding protein (MBP) by using the *Eco*RI or *Xba*I and *Sal*I sites in the multiple cloning site of this plasmid. Restriction

endonuclease digestion, ligation, transformation in *Escherichia coli* (*E. coli*) DH5α, plasmid DNA isolation and agarose gel electrophoresis were performed by standard procedures[21].

Production, isolation and purification of recombinant NP

A total of 500 ml of SOB medium[21] containing ampicillin (50 µg/ml) and supplemented with glucose (2g/L) was inoculated with 5 ml of an overnight culture of recombinant *E. coli*, and incubated at 37°C in a shaking incubator. The optical density at wavelength 600 nm was monitored, and at a value between 0.5 and 0.6 1 mM isopropyl ß-D-thiogalactopyranoside was added to induce expression of the fusion gene. Four hours after induction, the bacteria were pelleted by centrifugation, resuspended in 25 ml column buffer (20 mM Tric-Cl pH 7.4, 200 mM NaCl, 1 mM EDTA) containing Pefablock protease inhibitor (Boehringer Mannheim), and lysed by sonication. The lysate was diluted to 100 ml in column buffer and run through an amylose resin column (New England Biolabs). After extensive washing of the column, recombinant protein was eluted with columnbuffer containing 25 mM maltose. Peak fractions were pooled and the purified proteins were stored at -70°C until use. Protein concentrations were determined using the Bradford reagent[2]. The procedure was carried out for recombinant *E. coli* carrying the pMalC plasmid without cloned sequences to obtain recombinant MBP (rMBP) and for recombinant *E. coli* carrying the pMalC plasmid in which the NP gene of influenza virus A or B was cloned to obtain recombinant fusion proteins consisting of MBP and influenza virus A NP (rNPA) or influenza virus B NP (rNPB), respectively.

SDS-PAGE and Western blotting

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed according to standard procedures[21]. Blots were incubated with blocking buffer (2% nonfat milk powder, 0.05% Tween 20 in phosphate-buffered saline [PBS] for 1 h, followed by 1 h of incubation with 1:100 diluted polyclonal rabbit antisera specific for influenza virus A or B. After washing the blots with PBS, the blots were incubated for 1h with 1:500 diluted horse radish peroxidase (HRP)-labeled swine-anti-rabbit IgG antibodies (Dako, Glostrup, Denmark). Then, the blots were washed with PBS followed by incubation in diaminobenzidine/H_2O_2 in PBS (250 µg diaminobenzidine/ml, 0.002% H_2O_2). The reaction was stopped with H_2O when protein bands became visible.

Sera

Influenza virus A- and B-specific polyclonal rabbit and ferret antisera were obtained from rabbits injected with sucrose gradient-purified influenza virus A/Hong Kong/2/68 (H3N2) or B/Harbin/7/94 and from ferrets experimentally infected with influenza virus A/Netherlands/018/94 (H3N2) or B/Harbin/7/94.

Human sera were obtained from adult patients with acute influenza virus B (n=24) and influenza virus A (H1N1; n=2, H3N2; n=15) infection; the patients were enrolled in clinical studies during the respiratory season in March 1995 and December 1995, respectively. Influenza virus infection was confirmed by an immunofluoresence test or by virus isolation from cell culture. Sera were collected on the day of onset of clinical symptoms (day 1) and at several time points thereafter. For the influenza virus A-infected patients, additional serum samples collected on days 6, 21, and 60 were available. For the patients with influenza virus B

infection, additional serum samples collected on days 6 and 28 were available. Sera were stored at -20°C until use.

Hemagglutination inhibition assay (HI assay)

One volume of serum was mixed with five volumes of cholera filtrate, and the mixture was incubated at 37°C for approximately 16 h, followed by 1 h of incubation at 56°C. To 50 µl of twofold dilution series of serum in PBS, 25 µl of a solution of influenza virus A/Singapore/6/86 (H1N1), A/Johannesburg/33/94 (H3N2), or B/Harbin/7/94 containing 4 hemagglutinating units (HAU) was added, and the mixture was incubated at 37°C for 30 min. Then, 25 µl of a 1% turkey erythrocyte suspension in PBS was added, followed by 1 h of incubation at 4°C. Subsequently, the hemagglutination pattern was examined and expressed as the reciprocal value of the highest serum dilution inhibiting hemagglutination. A fourfold titer rise for paired serum samples was considered indicative of a recent influenza virus infection.

Enzyme-linked immunosorbent assay (ELISA)

ELISA for detection of IgA serum antibodies (capture IgA NP-ELISA)

Ninety-six-well plates coated with rabbit anti-human IgA antibodies (Meddens Diagnostics, Brummen, The Netherlands) were washed with demineralized H_2O containing 0.05% Tween 80, followed by incubation with patient sera diluted 1:100 in ELISA buffer (Meddens Diagnostics). After 1 h of incubation at 37°C, the plates were washed and incubated with rNPA or rNPB, which were conjugated with HRP by previously described methods (24). Following 1 h of incubation at 37°C, the plates were washed again and incubated with tetramethylbenzidine substrate (Meddens Diagnostics) for 10 min. The reaction was stopped with 2M H_2SO_4, and the OD was measured at 450 nm. NP-specific reactivities were expressed as the following ratio: OD_{450nm} for patient serum/OD_{450nm} for negative control serum. The negative control serum consisted of a pool of sera negative for influenza virus A- and B-specific IgA antibodies. Ratios greater than 2.0 were considered positive.

ELISA for detection of IgG serum antibodies (indirect IgG NP-ELISA)

For rabbit and ferret sera, 96 wells plates were coated overnight at room temperature with 50 ng rNPA or rNPB in 100 µl 0.1 M sodium carbonate buffer (pH 9.6). The plates were washed with demineralized H_2O containing 0.05% Tween 80. Influenza virus A- and B-specific rabbit and ferret antisera were twofold diluted from 1:100 to 1:6,400 in ELISA buffer. A total of 50 µl of each dilution was incubated in the recombinant NP-coated plates for 1 h at 37°C. After washing of the plates, 50 µl of 1:500 diluted goat anti-ferret IgG antibodies (Kirkegaard & Perry) or 1:500 diluted swine anti-rabbit IgG antibodies (Dako, Glostrup, Denmark) conjugated with HRP was added, and incubated for 1 hour at 37°C. The plates were washed again and incubated with 50 µl of tetramethylbenzidine substrate for 10 min. The reaction was stopped by adding 50 µl of 2M H_2SO_4, and the OD_{450} was measured.

For human sera, ELISA was performed as described above. Human sera were diluted 1:100,000 and IgG antibodies were detected with 1:5,000-diluted HRP-labeled goat anti-human IgG antibodies (Biosource Europe, Fleurus, Belgium). In addition to reactivities with rNPA and rNPB, the reactivity of human sera with rMBP was also measured. NP-specific

reactivities were expressed as the following ratio: OD_{450} measured with rNPA or rNPB/OD_{450} measured with rMBP. An increase in this ratio for paired serum samples of at least a factor 2.0 was considered indicative of a recent influenza virus infection.

RESULTS

Recombinant NPs of influenza viruses A and B

After induction of expression and purification by affinity chromatography, the recombinant proteins rNPA and rNPB were analyzed by SDS-PAGE. As shown in figure 1A, highly purified protein preparations with molecular masses of 100 kD for rNPA and 107 kD for rNPB (including 40 kD for MBP) were obtained. The difference in the molecular masses between rNPA and rNPB is in accordance with the difference in the lengths of the coding sequences for both proteins (1,494 bp for the NP of influenza virus A and 1,680 bp for the NP of influenza virus B). The identities of rNPA and rNPB were confirmed by Western blot analysis. Rabbit antiserum raised against an influenza virus A reacted only with rNPA and not with rNPB, whereas a rabbit antiserum directed against an influenza virus B showed reactivity with rNPB but not with rNPA (Figure 1B and C, respectively). The identities of the recombinant proteins were further confirmed in indirect ELISAs with rabbit and ferret antisera raised against influenza viruses A and B which showed reactivity only with the homologous rNPA and rNPB, respectively (Figure 2).

Detection of IgA antibodies in patient sera by capture IgA NP ELISA

Serum samples collected at consecutive time points from patients with confirmed influenza virus A and B infections were analyzed for the presence of NP-specific IgA antibodies. For the group of patients infected with influenza virus A, an IgA response against the NP of influenza virus A but not influenza virus B was measured (Figure 3A). The IgA response peaked at day 21 and subsequently declined. Sera from 10 of 17 patients (58.8%) showed reactivity with rNPA at day 21, while serum from only 1 patient (5.9%) showed reactivity with rNPB at this time point (Figure 4A). For the group of patients infected with influenza virus B, a type-specific IgA response against NP was observed. The response showed a peak 6 days after the onset of clinical symptoms and slowly declined by day 28 (Figure 3B). For this group of patients, sera from two patients (8.3%) showed reactivity with rNPB on day 1 (Figure 4B). This number increased until sera from 16 patients (66.7%) showed reactivity by day 6. Sera from four patients (16.7%) showed reactivity

with rNPA on day 1 but this number did not increase during the course of infection.

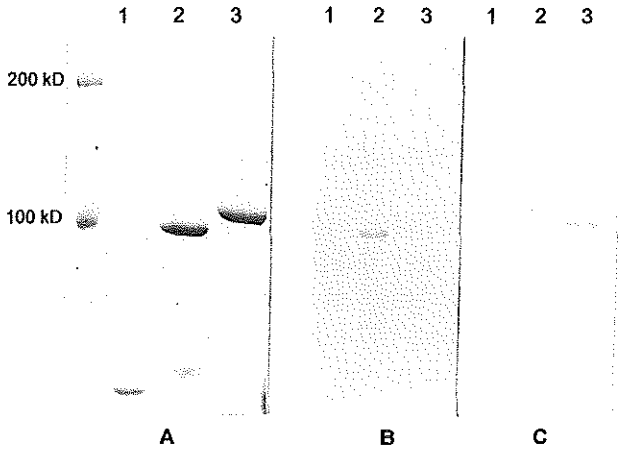

Figure 1. Analysis of rNPA and rNPB by SDS-PAGE and Western blot. rMBP (lane 1), rNPA (lane 2) and rNPB (lane 3) were separated on a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue (A) or transferred to nitrocellulose membranes which were incubated with a rabbit serum specific for influenza virus A (B) or influenza virus B (C).

Detection of IgG antibodies in patient sera by indirect IgG NP ELISA

The same serum samples were also analyzed for NP-specific IgG antibodies. For the group of patients infected with influenza virus A, a type-specific IgG response against NP was observed (Figure 3C). No reactivity was measured with the heterotypic rNPB. The NP-specific IgG response reached a maximum at 21 days after the onset of clinical symptoms and subsequently declined. For sera from 15 of 17 patients (88.2%) an increase in reactivity with rNPA was observed on day 21, whereas sera from none of these patients showed reactivity with rNPB (Figure 4A). In the influenza virus B-infected patients, a strong IgG response against the homologous NP was observed, and this response increased at least until day 28 after the onset of clinical symptoms (Figure 3D). For this group, sera from 23 of 24 patients (95.8%) showed an increase in reactivity with rNPB on day 28, while only 3 patients (12.5%) showed an increase in reactivity with rNPA (Figure 4B). In addition to rNPA and rNPB, the reactivities of sera with rMBP were also measured. The reactivity with MBP did not change during the time course of influenza virus A or B infection (data not shown).

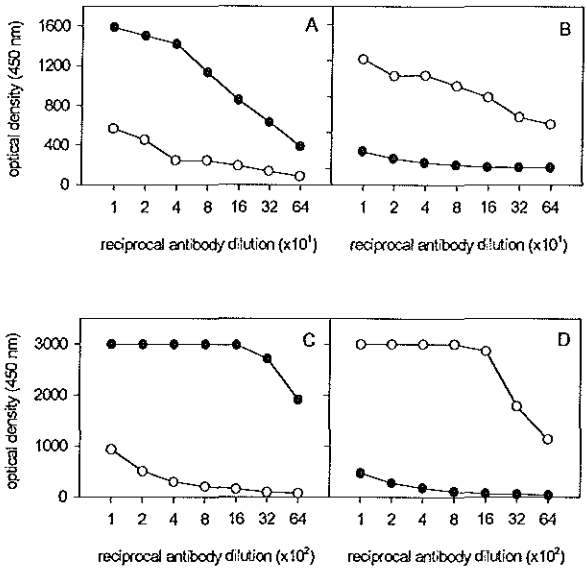

Figure 2. Confirmation of the identity of rNPA and rNPB in indirect IgG NP-ELISA's. Plates were coated with rNPA (A and C) or rNPB (B and D) and incubated with ferret (A and B) or rabbit (C and D) antisera raised against influenza virus A (solid circles) or influenza virus B (open circles).

Comparison of the indirect IgG NP ELISA and the HI assay

In the HI assay, 13 of 16 (81.3%) patients infected with influenza virus A showed a fourfold rise in serum antibody titer (between day 1 and day 21) against influenza virus A, while 88.2% showed an NP-specific IgG response (Figure 4A). Three patients who showed NP-specific IgG responses did not show a titer rise in the HI assay, whereas in two patients a rise in the HI titer was observed but no NP-specific IgG response was observed. None of the influenza virus A-infected patients showed an influenza virus B NP-specific IgG response, whereas by the HI assay the serum of one patient showed a rise in titer against influenza virus B. Among the patients infected with influenza virus B, the sera of 16 of 22 (72.7%) patients showed rises in titers against influenza virus B (between day 1 and day 28) by the HI assay, while the sera of 95.8% showed NP-specific IgG responses (Figure 4B). For five patients,

IgG responses against NP were observed in the absence of at least fourfold rises in titer by the HI assay. The serum of one patient did not show an NP-specific IgG response and no rise in titer by the HI assay. The sera of three patients showed influenza virus A and B NP-specific IgG responses. Serum from one of those three patients and sera from another two patients showed rises in titer to influenza viruses A and B by the HI assay.

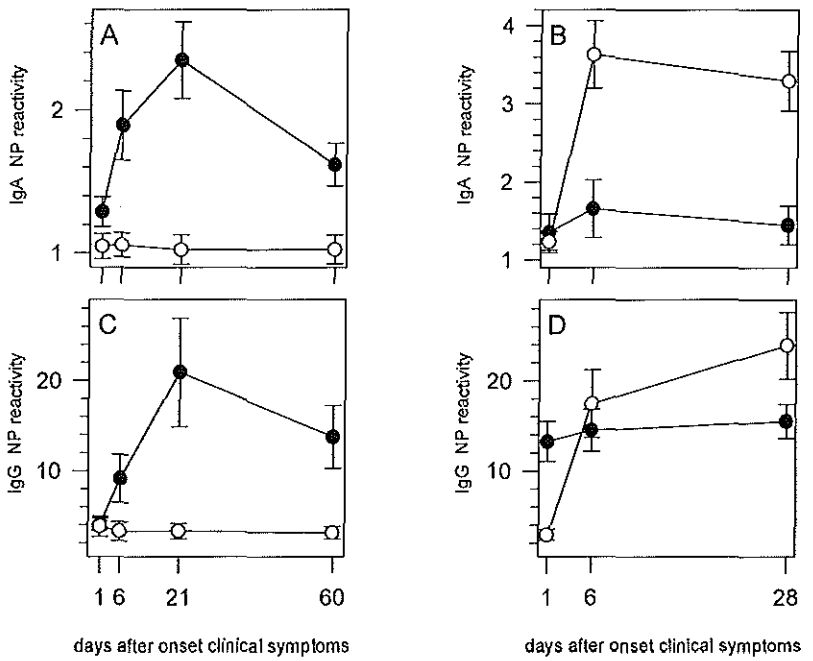

Figure 3. IgA (A and B) and IgG (C and D) responses in influenza virus A (A and C) or B (B and D) -infected patients as measured by capture IgA NP-ELISA's and indirect IgG NP-ELISA's using rNPA (solid circles) and rNPB (open circles). In the capture IgA NP-ELISA's, sera were measured against rNPA and rNPB and NP-specific reactivities expressed as the ratio: OD_{450nm} patient serum/OD_{450nm} negative control serum. In the indirect IgG NP-ELISA's, sera were measured against rMBP, rNPA and rNPB and NP-specific reactivities expressed as the ratio: OD_{450nm} measured on rNPA or rNPB/OD_{450nm} measured on rMBP. The mean values of the influenza virus A (n=17) and B (n=24) -infected patients on each time point are presented (+/- SEM).

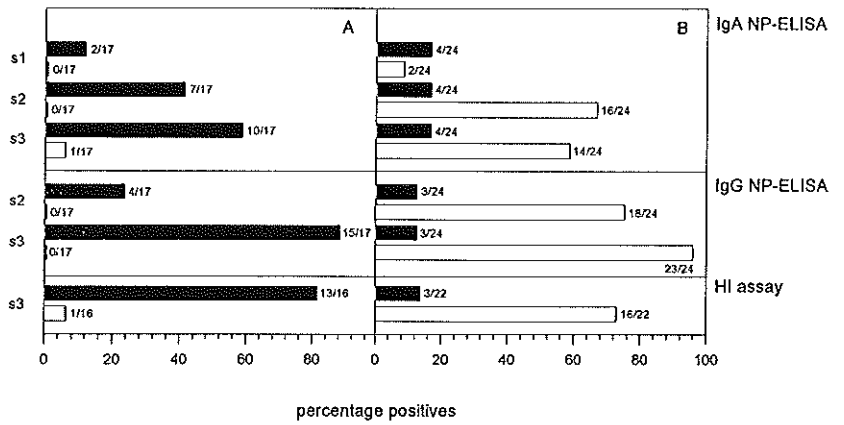

Figure 4. Percentages influenza virus A (A) or influenza virus B (B) -infected patients showing NP-specific antibody responses as measured by the capture IgA NP-ELISA's and indirect IgG NP-ELISA's, and percentages infected patients showing titre rises in the HI assay. Results of sera s1 (day 1), s2 (day 6) and s3 (day 21 for influenza virus A-infected patients and day 28 for influenza virus B-infected patients) are shown for the capture IgA NP-ELISA's. For the indirect IgG NP-ELISA's , results of paired samples (s2 and s3 compared to s1) are shown. In the HI assays s3 was compared to s1. Sera were measured against influenza virus type A (solid bars) and influenza virus type B (open bars).

DISCUSSION

In the present paper, recombinant NPs of influenza viruses A and B were used for the development of ELISA systems which can detect virus-specific IgA and IgG serum antibodies. By using serum samples from laboratory animals experimentally immunized with influenza viruses A and B and from humans with confirmed influenza virus A or B infections, the specificities of these ELISAs were confirmed. In the majority of the patients with influenza, virus type-specific antibodies were detected, demonstrating the diagnostic value of these recombinant NP-based ELISAs. These assays may replace the commonly used HI and CF assays for the serodiagnosis of influenza virus infections and can be performed when respiratory specimens are not available or to confirm results obtained by culture procedures with respiratory specimens.

Capture IgA NP-ELISAs were developed for the detection of influenza virus A and B NP-specific IgA serum antibodies with virus type-specific recombinant NP directly labeled with HRP. IgA responses were detected within 21 days after the onset of clinical symptoms in 58.8% of the influenza virus A-infected patients and 66.7% of the influenza virus B-infected patients. These perecentages are comparable to the percentages of patients with virus-specific IgA responses reported in other studies (3,6,18). The sera of four patients with confirmed influenza virus B infections had IgA antibodies directed to influenza virus A from the first day of clinical onset onward. These patients may have suffered from a recent infection with an influenza A virus. Since influenza viruses of type A have also circulated in the 1994 and 1995 influenza season, this is a likely explanation. Since the level of preexisting influenza NP-specific IgA antibody levels is low, the capture IgA NP-ELISAs do not require paired serum samples and, therefore, allow rapid serodiagnosis of influenza virus infections. This ELISA can be considered an alternative to assays that measure IgG serum antibodies when only one serum sample is available.

In addition to the capture IgA NP-ELISAs, virus type-specific recombinant NP was also used for the detection of influenza virus A and B NP-specific IgG serum antibodies in indirect IgG NP-ELISAs. By these ELISAs, IgG antibody rises could be detected in almost all of the influenza virus A and B-infected patients. Although in these ELISAs serum antibodies were measured against influenza virus NP, while in the HI assay serum antibodies directed against the hemagglutinin (HA) were measured, the results of both assays were compared to evaluate the diagnostic value of the IgG NP-ELISAs. The results of the IgG NP-ELISAs for the detection of influenza virus A-specific antibodies compared well with the results obtained by the HI assay. The IgG NP-ELISA for the measurement of influenza virus B-specific antibodies, however, detected a higher percentage of patients with increased antibody titers than the HI assay, which is in agreement with the results of earlier studies[2,11,14,16,17,22,23].

In contrast to antibodies of the IgA and IgG isotypes, the diagnostic value of IgM antibodies in influenza virus infection seems to be limited. Although the measurement of IgM responses has been shown to be of diagnostic value in primary influenza infection[3,18], IgA and IgG antibody responses predominate in influenza virus-infected patients[5,7,11]. Therefore, the measurement of IgG and IgA antibody responses is preferred for the serologic confirmation of influenza virus infections.

The division of influenza A and B viruses is based on antigenic differences in the NPs and matrix proteins. Indeed, no rises in influenza virus B NP-specific IgG antibody titers were measured in the influenza virus A-infected patients.

However, rises in influenza virus A and B NP-specific IgG antibodies titers were measured in 12.5% of the influenza virus B-infected patients. A recent influenza virus A infection or simultaneous infection with influenza viruses A and B may explain this observation.
Since the NP is well conserved within the influenza A viruses, the IgG NP-ELISA enables the detection of antibodies induced by influenza A viruses of both circulating subtypes (H1N1 and H3N2). Furthermore, this assay does not require the annual adjustment of the viral antigen preparations, in contrast to the HI assay, which measures antibodies against the highly variable hemagglutinin. Two of the influenza virus A-infected patients seemed to be infected with an H1N1 virus since in the HI assay only rises in titer against an H1N1 virus were measured (data not shown). Rises in NP-specific IgG antibody levels could be measured in these individuals, even though the recombinant NP used in the IgG NP-ELISA was derived from an H3N2 virus.
Although the reactivities with MBP differed between patients, none of them showed increases in MBP-specific IgG antibody levels during the time course after the infection. Thus, it does not seem to be necessary to measure MBP antibody titers separately to be able to detect rises in the levels of influenza virus-induced antibodies in the IgG NP-ELISAs.
In conclusion, recombinant influenza virus NPs were produced in virtually unlimited quantities and were purified to a high degree. These bacterially expressed viral antigens proved to be valuable reagents for the development of ELISA systems for the detection of virus-specific IgA and IgG antibodies which can be used for the serodiagnosis of influenza virus type A and B infections. Especially for the detection of NP-specific IgA antibodies, the IgA NP-ELISA proved to be valuable since it allows early diagnosis and does not require paired serum samples.

ACKNOWLEDGEMENTS

Part of this work was supported by the SRVI: Foundation for Respiratory Virus Infections. We thank Glaxo Wellcome for kindly providing us with serum samples. We also thank Ger van der Water for continuous support and Cedrick Copra for technical assistance.

REFERENCES

[01] Bishai, F.R. and Galli, R. 1978. Enzyme-linked immunosorbent assay for detection of antibodies to influenza A and B and parainfluenza type 1 in sera of patients. *J. Clin. Microbiol.* 8:648-656.

[02] Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-25.

[03] Burlington, D.B., Clements, M.L., Meiklejohn, G., Phelan, M. and Murphy, B.R. 1983. Hemagglutinin-specific antibody responses in immunoglobulin G, A, and M isotypes as measured by enzyme-linked immunosorbent assay after primary or secondary infection of humans with influenza A virus. *Infect. Immun.* 41:540-545.

[04] Claas, E.C.J., Sprenger, M.J.W., Kleter, G.E.M., van Beek, R., Quint, W.G.W. and Masurel, N. 1992. Type-specific identification of influenza A, B and C by the polymerase chain reaction. *J. Virol. Methods.* 39:1-13.

[05] Doller, G., Doller, P.C. and Gerth, H.J. 1986. Diagnostic significance of influenza subtype-specific IgG, IgA, and IgM antibodies. *J. Biol. Standard.* 14:163-175.

[06] Groen, J., Claas, E.C.J., Balentien, E. and Osterhaus, A.D.M.E. 1998. High influenza morbidity and mortality in unvaccinated elderly people in curacao. *J. Infect.* 36:241-242.

[07] Harmon, M.W., Phillips, D.J., Reimer, C.B. and Kendal, A.P. 1986. Isotype-specific enzyme immunoassay for influenza antibody with monoclonal antibodies to human immunoglobulins. *J. Clin. Microbiol.* 24:913-916.

[08] Harmon, M.W., Jones, I., Shaw, M., Keitel, W., Reimer, C.B., Halonen, P. and Kendal, A.P. 1989. Immunoassay for serological diagnosis of influenza type A using recombinant DNA produced nucleoprotein antigen and monoclonal antibody to human IgG. *J. Med. Virol.* 27:25-30.

[09] Joassin, L., Reginster, M. and Vaira, D. 1983. Anti M-protein antibody responses to type A or B natural influenza detected by solid phase enzyme linked immunosorbent assay and by complement fixation. *Arch. Virol.* 76:15-23.

[10] Julkunen, I., Kleemola, M. and Hovi, T. 1984. Serological diagnosis of influenza A and B infections by enzyme immunoassay. Comparison with the complement fixation test. *J. Virol. Methods.* 9:7-14.

[11] Julkunen, I., Pyhala, R. and Hovi, T. 1985. Enzyme immunoassay, complement fixation and hemagglutination inhibition tests in the diagnosis of influenza A and B virus infections. Purified hemagglutinin in subtype-specific diagnosis. *J. Virol. Methods.* 10:75-84.

[12] Khan, M.W., Bucher, D.J., Koul, A.K., Kalish, G., Smith, H. and Kilbourne, E.D. 1982. Detection of antibodies to influenza virus M protein by an enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 16:813-820.

[13] Koskinen, P., Vuorinen, T. and Meurman, O. 1987. Influenza A and B virus IgG and IgM serology by enzyme immunoassays. *Epidemiol. Infect.* 99:55-64.

[14] Madore, H.P., Reichman, R.C. and Dolin, R. 1983. Serum antibody responses in naturally occurring influenza A virus infection determined by enzyme-linked immunosorbent assay, hemagglutination inhibition, and complement fixation. *J. Clin. Microbiol.* 18:1345-1350.

[15] Masihi, K.N and Lange, W. 1980. Enzyme-linked immunosorbent assay for the detection of influenza type-specific antibodies. *J. Immunol. Methods.* 36:173-179.

[16] Murphy, B.R., Tierney, E.L., Barbour, B.A., Yolken, R.H., Alling, D.W., Holley, H.P., Mayner, R.E. and Chanock, R.M. 1980. Use of the enzyme-linked immunosorbent assay to detect serum antibody responses of volunteers who received attenuated influenza A virus vaccines. *Infect. Immun.* 29:342-347.

[17] Murphy, B.R., Phelan, M.A., Nelson, D.L, Yarchoan, R., Tierney, E.L., Alling, D.W. and Chanock, R.M. 1981. Hemagglutinin-specific enzyme-linked immunosorbent assay for antibodies to influenza A and B viruses. *J. Clin. Microbiol.* 13:554-560.

[18] Murphy, B.R., Nelson, D.L., Wright, P.F., Tierney, E.L., Phelan, M.A. and Chanock, R.M. 1982. Secretory and systemic immunological response in children infected with live attenuated influenza A virus vaccines. *Infect. Immun.* 36:1102-1108.

[19] Rimmelzwaan, G.F., Baars, M., van Beek, R., van Amerongen, G., Lovrgen-Bengtsson, K., Claas, E.C.J. and Osterhaus, A.D.M.E. 1997. Induction of protective immunity against influenza virus in a macaque model: comparison of conventional and iscom vaccines. *J. Gen. Virol.* 78:757-765.

[20] Rota, P.A., Black, R.A., De, B.K., Harmon, M.W. and Kendal, A.P. 1990. Expression of influenza A and B virus nucleoprotein antigens in baculovirus. *J. Gen. Virol.* 71:1545-1554.

[21] Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning. A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

[22] Turner, R., Lathey, J.L., Van Voris, L.P. and Belshe, R.B. 1982. Serological diagnosis of influenza B virus infection. Comparison of an enzyme-linked immunosorbent assay and the haemagglutination inhibition test. *J. Clin. Microbiol.* 15:824-829.

[23] Van Voris, L.P., Betts, R.F., Menegus, M.A., Murphy, B.R., Roth, F.K. and Douglas Jr., R.G. 1985. Serological diagnosis of influenza A/USSR/77 H1N1 infection: value of ELISA compared to other antibody techniques. *J. Med. Virol.* 16:315-320.

[24] Wilson, M.B. and Nakane, P.K. 1978. Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In: W. Knapp (ed.) *Immunofluorescense and related staining techniques*, pp. 215-224. Elsevier, Amsterdam.

CHAPTER 7

Summarizing discussion

Influenza viruses continue to be a major pathogen to man affecting a considerable percentage of the human population every year. While most influenza virus infected individuals completely recover from infection, infection with influenza virus can be accompanied with severe complications which even may result in death. This is especially true for people belonging to influenza risk groups. Vaccination remains the major means to prevent infection or to reduce influenza-associated illness in these people[30]. The currently used influenza vaccines are trivalent, i.e. contain representatives of influenza virus A H1N1 and H3N2 as well as influenza virus B and are administered as inactivated whole virus, as split virus or as purified haemagglutinin (HA) and neuraminidase (NA). Due to antigenic drift of influenza viruses, influenza vaccines need to be reformulated regularly to ensure a close match between the viruses represented in the vaccine and those circulating in humans. Partly as a result of this antigenic drift, vaccination needs to be repeated annually. Influenza vaccines are produced using embryonated chicken eggs and the vaccine viruses usually are 'high-growth' reassortant viruses. These reassortant viruses are generated after dual infection of eggs with a selected vaccine strain and a virus adapted to replicate to high titers in eggs (a virus with a high-growth phenotype). Those reassortant viruses which contain the appropriate surface glycoproteins and display a high-growth phenotype are selected for vaccine production.

The use of embryonated chicken eggs for vaccine production suffers from major disadvantages. Most importantly, virus production in eggs lacks flexibility. The use of eggs requires long-term planning by vaccine manufacturers and the availability of eggs may become a problem in case of a sudden need for large quantities of vaccine, for example in pandemic situations. One cannot just order chickens to lay more eggs! A more flexible source for production of influenza vaccines would thus be needed. The use of continuous cell lines for production of influenza vaccines may fulfil this need as they are easily maintained and their production can be scaled up in short periods of time, allowing vaccine production at any time in any quantities. Other advantages of the use of continuous cell lines would be that thus propagated influenza viruses usually more closely resemble the original human isolate than do viruses propagated in eggs. In general, they also constitute a more homogenous population of influenza viruses[17,39,40,50]. A disadvantage of most cell lines is the requirement to grow cells in the presence of fetal bovine serum which interferes with the activity of trypsin,

necessary for propagation of most influenza A viruses, and complicates standardization of culture conditions. A novel MDCK cell line, adapted to grow without serum, MDCK-SF1, has been developed and vaccine studies already have shown that MDCK-SF1 cell-derived vaccines are equally efficient as egg-derived vaccines[8,9,31]. Chapter 2 describes the use of these MDCK-SF1 cells for generation and propagation of high-growth reassortant influenza A viruses as a possible alternative to the current influenza vaccine production procedure in embryonated chicken eggs. It was shown that with these cells, like with eggs, high-growth reassortant influenza A viruses containing the appropriate surface glycoproteins can be generated relatively easily by infection of MDCK-SF1 cells with a virus containing the desired surface glycoproteins and a second virus with a high-growth phenotype. The use of a H1N1 virus with a high-growth phenotype (A/PR/8/34) in dual infections with H3N2 viruses for generation of high-growth H3N2 viruses, and, conversely, the use of a H3N2 high-growth virus (A/HK/2/68) in dual infections with H1N1 viruses to yield high-growth H1N1 viruses, were shown to be advantageous with respect to both selection of reassortant viruses containing the desired surface glycoproteins (using antisera raised against H1N1 and H3N2 viruses) and subsequent genetic characterization of these viruses. The origin of HA was determined in haemagglutination inhibition (HI) assays while the origin of the other gene segments was determined by RT-PCR and restriction enzyme analysis, both of which assays allow easy discrimination between viruses belonging to different subtypes. In this way, it was shown that reassortant viruses obtained from dual infections of MDCK-SF1 cells with A/PR/8/34 and H3N2 viruses contained the HA and NA of the H3N2 viruses while the matrix protein and polymerase PA protein-encoding gene segments were derived from the high-growth parent. Reassortant viruses obtained from dual infection of MDCK-SF1 cells with A/HK/2/68 and H1N1 viruses contained the HA and NA of the H1N1 viruses while the matrix protein and polymerase PB1 protein-encoding gene segments originated from the high-growth parent. Reassortant viruses propagated in MDCK-SF1 cells, in general, yielded higher amounts of haemagglutinating units (HAU) per volume than the corresponding non high-growth parent. The procedure described to generate and characterize high-growth reassortant influenza A viruses in combination with the advantages of a continuous cell line in general, as well as the ability to culture cells in the absence of serum in particular, makes the use of

MDCK-SF1 cells an attractive alternative to the current vaccine production procedure in embryonated chicken eggs.

A relatively novel method to generate reassortant influenza viruses is reverse genetics. By this method, RNP complexes (RNPs) are transfected to influenza virus-infected cells allowing reassortment between the gene segments of the virus and the gene segment(s) contained within the transfected RNPs. While in double infected eggs or MDCK-SF1 cells in principle 256 (2^8) different combinations of gene segments can be made (and as many different viruses), by reverse genetics reassortment can be limited to the gene segments encoding HA and NA and this more controlled reassortment can be considered an advantage in the selection of reassortant viruses for vaccine purposes. However, infection of cells with a high-growth virus followed by transfection with RNPs containing the desired HA and NA gene segments only, may not always be suffcient to obtain (high-growth) reassortant viruses. Indeed, results obtained from studies in which reassortant viruses were generated by dual infection suggest that there are limitations towards reassortment[12,19,42,52,this thesis] and not all combinations of gene segments may yield viable viruses. If reverse genetics will prove to be a universally applicable method, then it may replace current procedures for generating high-growth reassortant viruses for vaccine purposes.

Besides studies aiming at improvement of current influenza vaccine production procedures, numerous studies are foccussing on novel influenza vaccines making use of several adjuvant formulations and antigen presentation forms. One antigen presentation form that has received considerable attention is the immune stimulating complex (ISCOM). ISCOM-based experimental vaccines have been shown to be potent inducers of both humoral and cellular immune responses which, in animal models, have been shown to confer protection against challenge infection[36,37], and ISCOM-based influenza vaccines are currently being evaluated in clinical phase III trials in humans[5,13,38]. In addition, an ISCOM-based influenza vaccine has already been licensed for use in horses. ISCOMs were originally developed as an antigen presentation form for viral membrane proteins[28] and most studies concerning the development of ISCOM-based vaccines for influenza have focussed on the surface glycoprotein HA as a model antigen. HA is considered the most important influenza virus antigen with regard to protection and, due to its highly hydrophobic membrane-spanning region, is incorporated well into the ISCOM structure. This feature is thought to be essential for optimal induction of immune responses. Incorporation of less

hydrophobic antigens usually requires modification of the antigen with hydrophobic sequences which can be achieved by coupling fatty acids to the antigen or by recombinant DNA technology. In chapter 3, this latter possibility was explored. The necessity of a hydrophobic sequence and incorporation into the ISCOM structure for ISCOM-facilitated entry of antigen into the MHC class I processing and presentation pathway was evaluated using the influenza virus A matrix M1 protein. Recombinant M1 protein (rMA) was modified with the transmembrane region of HA to yield rMAHA protein. rMAHA protein seemed to incorporate better into ISCOM than rMA protein and *in vitro* analysis of MHC class I presentation of the M1 protein-derived, HLA-A2-restricted, peptide GILGFVFTL (aa 58 to 66) showed that the addition of the transmembrane region of HA to the M1 protein favoured MHC class I presentation to specific cytotoxic T lymphocytes (CTL). To determine whether or not for this purpose incorporation into the ISCOM structure was essential, pre-formed 'empty' ISCOMs were mixed with the respective proteins. MHC class I processing and presentation was observed for both rMA and rMAHA protein mixed with empty ISCOMs indicating that incorporation of antigen is not necessary for its delivery into the MHC class I pathway. In fact, MHC class I presentation of both antigen preparations was more efficient than rMAHA protein incorporated into ISCOMs and was best for rMAHA protein mixed with empty ISCOMs. Furthermore, it was shown that mixing of the respective proteins with empty ISCOMs was necessary for entry of the proteins into the MHC class I pathway, since pre-incubation of cells with empty ISCOMs followed by washing the cells and subsequent incubation with the proteins did not result in MHC class I presentation. Thus, presumably rMA and rMAHA protein associate with empty ISCOMs and the presence of the transmembrane region of HA added to this. In another experiment using recombinant nucleoprotein (rNP) it was found that rNP mixed with empty ISCOMs was processed for MHC class I presentation of the HLA-A3-restricted peptide ILRGSVAHK (aa 265 to 273) equally well as rNP incorporated into ISCOMs (unpublished data). These results seem to conflict with those obtained with the measles virus fusion (F) protein which was shown to be processed for MHC class I presentation when it was incorporated into ISCOMs but not after mixing with empty ISCOMs[7]. This discrepancy most likely reflects differences in the preparation and nature of the antigens. The results of the studies with recombinant matrix protein and NP strongly suggest that incorporation of antigen into ISCOMs per se is not

necessary for ISCOM-facilitated entry into the MHC class I pathway and implicates that the use of ISCOM as an antigen presentation form, for the induction of humoral and cellular immune responses, is not restricted to antigens containing highly hydrophobic sequences like membrane-spanning envelope proteins.

The observation that ISCOMs facilitate the processing of matrix protein and NP for MHC class I presentation to specific CTL *in vitro*, indicates that ISCOM-based vaccines may be used to induce *in vivo* CTL responses against these proteins, which are the most abundant viral antigens and important targets for CTL[6,15,25,26,47,53]. Since the matrix protein and NP are relatively well conserved, vaccines based on these proteins would not suffer from the necessity of regular reformulation as is the case for the currently used vaccines. However, although experimental NP-based vaccines have been shown to induce CTL which conferred protective immunity in animal models[14,48,49,51], it is generally believed that in humans protective immunity is primarily mediated by virus-neutralizing antibodies raised against HA and NA, and that CTL merely contribute to the clearance of virus-infected cells and reduce spread of virus[1,2,10,43,46]. Thus, ISCOM-based vaccines aiming at the induction of CTL responses against conserved influenza virus antigens only, probably would not suffice to establish long-lasting immunity against influenza in humans. ISCOM-based vaccines containing matrix protein and NP in addition to the influenza virus surface glycoproteins may be useful for the induction of both CTL and virus-neutralizing antibody responses against the major influenza virus antigens.

The conventional MHC class I processing and presentation pathway usually requires endogenous protein synthesis. In virus-infected cells, viral proteins produced in the cytoplasm are degraded by the proteasome to yield peptides which are transported to the ER by TAP. In the ER, the peptides associate with MHC class I molecules and the resulting complexes traverse the Golgi system to be presented at the cell surface for recognition by specific $CD8^+$ CTL. However, numerous studies have already shown that exogenous proteins, which normally are processed in endosomes for MHC class II presentation to $CD4^+$ Th cells, can be processed for MHC class I presentation to $CD8^+$ CTL as well, and several alternative mechanisms for MHC class I processing and presentation have been proposed[18,22,41,45,54]. These studies mainly focussed on macrophages and dendritic cells and the antigen used was often particulate (e.g. bacteria or antigen coupled to beads). MHC class I processing and presentation by B-lymphoblastoid cell

line (B-LCL) cells has been described for exogenous proteins incorporated into or associated with ISCOM[7,this thesis]. Alternatively, exogenous proteins have been shown to enter the MHC class I pathway of B-lymphocytes by receptor-mediated endocytosis[3,4,20,23]. Chapter 4 describes MHC class I processing and presentation of exogenous protein by B-LCL cells not attributable to these mechanisms. MHC class I presentation of exogenous rNP by B-LCL cells was demonstrated in CTL assays and lymphocyte stimulation tests (LST) using HLA-A3 and HLA-B27-restricted CTL with specificity for the NP-derived epitopes ILRGSVAHK (aa 265 to 273) and SRYWAIRTR (aa 383 to 391), respectively. HLA-B27-associated presentation required low amounts of protein (as little as 0.2 μg/ml was sufficient) and was demonstrated for rNP containing the proper epitope but not for rNP with a mutated epitope, for rNP of influenza virus B or for control rMBP. MHC class I presentation associated with HLA-A3 required significantly higher doses of protein and, like HLA-B27, was observed only for rNP containing the appropriate epitope. Using inhibitors of the conventional MHC class I pathway (the proteasome inhibitor lactacystin and the ER transport inhibitor brefeldin A), and inhibitors of proteolysis in endosomes (chloroquine and NH_4Cl), it was demonstrated that processing and presentation of rNP most likely followed the conventional MHC class I pathway. In contrast, results obtained with cells lacking a functional TAP suggested alternative mechanisms for MHC class I processing and presentation. Processing of rNP in endosomes and loading of recycled MHC class I molecules with peptide in these compartments may be a major route exploited by these cells. The results of this study thus not only demonstrate that B-LCL cells can process exogenous rNP for presentation to CTL but also that multiple pathways may exist for MHC class I processing and presentation of exogenous proteins.

Although NP is relatively well conserved, mutations in this protein do occur. Since NP harbours many CTL epitopes (at least eight epitopes are known), amino acid mutations in this protein may affect processing and presentation of these epitopes and/or recognition by specific CTL. In chapter 5, the effect of mutations, found within a CTL epitope of the influenza virus A NP, with respect to MHC class I presentation and recognition by specific CTL was studied. Part of the NP gene, containing four different CTL epitopes, of influenza A H3N2 viruses isolated from 1989 to 1999 in The Netherlands was sequenced. A R384K mutation in the HLA-B8 and HLA-B27-restricted CTL epitopes ELRSRYWAI (aa 380 to

388) and SRYWAIRTR (aa 383 to 391), respectively, was found in part of the influenza A viruses isolated in the influenza season 1989-1990, but not in the influenza A viruses isolated in later seasons. This mutation has previously been described for two viruses isolated in 1971 and 1972[44]. In the influenza season 1993-1994, another mutation at the same position (R384G), that did not occur before 1993, was found in all sequenced isolates of this season and showed to be conserved. Indeed, this mutation was found in all sequenced influenza A viruses isolated from 1993 onwards, and has also been found in viruses isolated in Japan after 1993[24]. In contrast to the HLA-B8 and HLA-B27 epitopes, no mutations were found that affected the other two CTL epitopes (HLA-A3 and HLA-B37) present in the sequenced NP region. In CTL assays, it was shown that B-LCL cells infected with mutant influenza viruses or recombinant vaccinia virus expressing NP with a R384G mutation, or B-LCL cells pulsed with a 9-mer peptide (representing the epitope) containing the R384K or R384G mutation were no longer recognized by HLA-B27-restricted CTL. Exactly the same type of mutations (R264K and R264G) have been associated with loss of recognition of the HLA-B27-restricted HIV-1 Gag epitope KRWIILGLNK (aa 263 to 272) by specific CTL[29]. Thus, a mutation at the second residue of HLA-B27-restricted epitopes, which is a critical anchor residue position, completely abrogates MHC class I presentation and subsequent recognition by HLA-B27-restricted CTL.

The introduction of mutations in CTL epitopes as a possible mechanism to escape CTL-mediated immunity has thus far only be described for viruses causing persitent infections, like HIV-1, and a mutation in a HLA-B27-restricted epitope of the HIV-1 Gag protein has been associated with progression to AIDS[11,16,21,27,33,34]. It has been questioned whether such a mechanism could be exploited by influenza viruses[32]. First, influenza viruses cause acute infections in immunocompetent individuals and, as a result, there may be little time for evolution of influenza viruses and the emergence of CTL escape mutants within an individual. Second, most of the currently known CTL epitopes are located within NP. This protein plays a crucial role in transcription and replication of the virus and mutations at a certain position will not be tolerated because of functional constraints, again limiting the oppertunities for the emergence of CTL escape mutants. The fact that we did not find any amino acid mutations in the HLA-A3- and HLA-B37-restricted epitopes may reflect this limitation. Nevertheless, the results of our study suggest that mutations introduced in CTL epitopes

within NP, that do not impose functional constraints on NP, are selected for by influenza virus to allow escape from CTL-mediated immunity. In support of this, CTL escape mutant viruses with amino acid changes in a CTL epitope of NP have been isolated recently from influenza virus-infected mice transgenic for a NP-specific T cell receptor[35]. Although it is unknown from where the R384G mutant virus originated, it may have emerged in HLA-B27 positive individuals which constitute approximately 8% of the Caucasian population. The immune pressure mediated by HLA-B27-restricted CTL in these individuals may have been sufficient to drive the selection process. Since the R384G mutation did not have a negative effect on virus replication, the mutant viruses may have spread in the human population ultimately replacing viruses containing the intact epitope.

In addition to studies related to vaccine development and to MHC class I processing and presentation of influenza virus antigens, one chapter deals with influenza serology. Chapter 6 describes the development of ELISAs for detection of NP-specific antibodies of the IgA and IgG isotypes in sera of influenza virus A or B-infected patients using rNP derived from A/Netherlands/18/94;H3N2 and B/Harbin/7/94. Both the IgA and IgG ELISA were shown to be specific and the IgG ELISA compared well with the commonly used HI assay. While in the HI assay serum antibodies against the highly variable HA are measured, the IgG ELISA measures antibodies against the relatively well conserved NP. The use of NP in ELISAs can be considered an advantage since these ELISAs, in contrast to the HI assay, do not require annual adjustments of the antigen used. Moreover, these ELISAs can be used to measure NP-specific antibodies in patients infected with either of the currently circulating influenza virus A subtypes (H1N1 and H3N2), while in the HI assay representatives of both these subtypes need to be included. In addition, there are some practical advantages of the NP-based ELISAs over the HI assay. The recombinant NPs of influenza virus A and B can be produced in large quantities with high purity and the amount of antigen used can be defined accurately. In contrast, in the HI assay whole virus and erythrocytes are used of which the quality may vary and the appropriate amount of antigen used is more difficult to determine since it is related to the amount of haemagglutinating units (HAU). Also, the read out of ELISAs, unlike that of HI assays, can be incorporated easily into automated procedures. While for influenza serology, NP-based ELISAs may be a useful alternative to the currently used HI assays, the latter still will be required for influenza virus subtype-

specific surveillance purposes. Indeed, unlike the NP-based ELISAs, the HI assay can be used to discriminate between influenza virus A subtypes and variants of influenza virus A subtypes as well as influenza virus B and thus provide information about antigenic properties of the circulating influenza viruses.

In conclusion, the studies described in this thesis have largely focussed on vaccine development and on MHC class I processing and presentation of influenza virus antigens. It was shown that a continuous cell line offers opportunities to improve current vaccine production procedures and that the main goal of current vaccines, the induction of virus-neutralizing antibodies against the surface glycoproteins HA and NA, may be extended to the induction of CTL responses against the internal matrix protein and NP by using ISCOM as an antigen presentation form. The observation that incorporation of these influenza virus antigens in the ISCOM structure is not a prerequisite for entry into the MHC class I pathway adds value to the use of ISCOMs as the basis of alternative influenza vaccines. The efficacy of vaccines aiming at the induction of virus-neutralizing antibodies and CTL responses, however, would not only depend on the antigenic match between the surface glycoproteins contained within the vaccine virus and those of the circulating viruses, but also on the match of CTL epitopes between these viruses. Indeed, like mutations in HA and NA allowing escape from antibody-mediated immunity, mutations in CTL epitopes may allow escape from CTL-mediated immunity and the emergence of CTL escape mutant viruses in the population may limit the efficacy of such vaccines. Thus, in developing influenza vaccins aiming at the induction of both virus-neutralizing antibodies and CTL responses one has to take into account both antigenic drift of HA and NA and the possibility of CTL escape mutants. Although the variability of influenza viruses will remain and will continue to limit the efficacy of vaccination against influenza, the development of novel generations of influenza vaccines as well as alternative production procedures undoubtedly will contribute to the prevention and control of influenza.

REFERENCES

[01] Ada, G.L. and Jones, P.D. 1986. The immune response to influenza infection. *Curr.Top.Microbiol.Immunol.* 128:1-54.

[02] Askonas, B.A., McMichael, A.J. and Webster, R.G. 1982. The immune response to influenza viruses and the problem of protection against infection. In: Beare, A.S. (ed.) *Basic and applied influenza research*, pp. 159-188. CRC Press, Boca Raton, Florida.

[03] Bansal, P., Mukherjee, P., Basu, S., George, A., Bal, V. and Rath, S. 1999. MHC class I-restricted presentation of maleylated protein binding to scavenger receptors. *J.Immunol.* 162:4430-4437.

[04] Barnaba, V., Franco, A., Alberti, A., Benvenuto, R. and Balsano, F. 1990. Selective killing of hepatitis B envelope antigen-specific B cells by class I-restricted, exogenous antigen-specific T lymphocytes. *Nature* 345:258-260.

[05] Bates, J., Ackland, J., Coulter, A., Cox, J., Drane, D., Macfarlan, R., Varigos, J., Wong, T-Y. and Woods, W. 1996. Iscom adjuvant – a promising adjuvant for influenza virus vaccines. In: Brown, L.E., Hampson, A.W. and Webster, R.G. (eds.) *Options for the control of influenza III*, pp. 661-667. Elsevier, Amsterdam.

[06] Bennink, J.R. and Yewdell, J.W. 1990. Recombinant vaccinia viruses as vectors for studying T lymphocyte specificity and function. *Curr.Top.Microbiol.Immunol.* 163:153-183.

[07] van Binnendijk, R.S., van Baalen, C.A., Poelen, M.C.M., de Vries, P., Boes, J., Cerundolo, V., Osterhaus, A.D.M.E. and Uytdehaag, F.G.C.M. 1992. Measles virus transmembrane fusion protein synthesized de novo or presented in immunostimulating complexes is endogenously processed for HLA class I- and class II-restricted cytotoxic t cell recognition. *J.Exp. Med.* 176:119-128.

[08] Brands, R., Palache, A.M. and van Scharrenburg, G.J.M. 1996. Madin Darby Canine Kidney (MDCK)-cells for the production of inactivated influenza subunit vaccine. Safety characteristics and clinical results in the elderly. In: Brown, L.E., Hampson, A.W. and Webster, R.G. (eds.) *Options for the Control of Influenza III*, pp. 683-693. Elsevier, Amsterdam.

[09] Brands, R., Palache, A.M. and van Scharrenburg, G.J.M. 1997. Development of influenza subunit vaccine produced using mammalian cell culture technology. In: Carrondo, M.J.T., Griffiths, B. and Moreira, J.L.P. (eds.) *Animal Cell Technology: From vaccines to genetic medicine*, pp. 165-167. Kluwer Academic Publishers, Dordrecht.

[10] Couch, R.B. and Kasel, J.A. 1983. Immunity to influenza in man. *Annu.Rev.Microbiol.* 37:529-549.

[11] Couillin, I., Culmann-Penciolelli, B., Gomard, E., Choppin, J., Levy, J.P., Guillet, J.G. and Saragosti, S. 1994. Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus 1 NEF protein. *J.Exp.Med.* 180:1129-1134.

[12] Cox, N.J., Maassab, H.F. and Kendal, A.P. 1979. Comparative study of wild-type and cold-mutant (temperature-sensitive) influenza viruses: nonrandem reassortment of genes during preparation of live virus vaccine candidates by recombination at 25°C between recent H3N2 and H1N1 epidemic strains and cold-adapted A/Ann Arbor/6/60. *Virology* 97:190-194.

[13] Ennis, F.A., Cruz, J., Jameson, J., Klein, M., Burt, D. and Thipphawong, J. 1999. Augmentation of human influenza A virus-specific cytotoxic T lymphocyte

memory by influenza vaccine and adjuvanted carriers (ISCOMS). *Virology* 259:256-261.

[14] Fu, T.M., Friedman, A., Ulmer, J.B., Liu, M.A. and Donnelly, J.J. 1997. Protective cellular immunity: cytotoxic T-lymphocyte responses against dominant and recessive epitopes of influenza virus nucleoprotein induced by DNA immunization. *J.Virol.* 71:2715-2721.

[15] Gotch, F., McMichael, A., Smith, G. and Moss, B. 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. *J.Exp.Med.* 165:408-416.

[16] Goulder, P.J., Phillips, R.E., Colbert, R.A., McAdam, S., Ogg, G., Nowak, M.A., Giangrande, P., Luzzi, G., Morgan, B., Edwards, A., McMichael, A.J. and Rowland-Jones, S. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat.Med.* 2:212-217.

[17] Gubareva, L.V., Wood, J.M., Meyer, W.J., Katz, J.M., Robertson, J.S., Major, D. and Webster, R.G. 1994. Codominant mixtures of viruses in reference strains of influenza virus due to host cell variation. *Virology* 199:89-97.

[18] Harding, C.V. and Song, R. 1994. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. *J.Immunol.* 153:4925-4933.

[19] Kaverin, N.V., Rudneva, I.A., Smirnow, A. and Finskaya, N.N. 1988. Human-avian influenza virus reassortants: effect of reassortment pattern on multi-cycle reproduction in MDCK cells. *Arch.Virol.* 103:117-126.

[20] Ke, Y. and Kapp, J.A. 1996. Exogenous antigens gain access to the major histocompatibility complex class I processing pathway in B cells by receptor-mediated uptake. *J.Exp.Med.* 184:1179-1184.

[21] Koup, R.A. 1994. Virus escape from CTL recognition. *J.Exp.Med.* 180:779-782.

[22] Kovacsovics-Bankowski, M. and Rock, K.L. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267:243-246.

[23] Lee, R., Tartour, E., van der Bruggen, P., Vantomme, V., Joyeux, I., Goud, B., Fridman, W.H. and Johannes, L. 1998. Major histocompatibility complex class I presentation of exogenous soluble tumor antigen fused to the B-fragment of shiga toxin. *Eur.J.Immunol.* 28:2726-2737.

[24] Lindstrom, S.E., Hiromoto, Y., Nerome, R., Omoe, K., Sugita, S., Yamazaki, Y., Takahashi, T. and Nerome, K. 1998. Phylogenetic analysis of the entire genome of influenza A (H3N2) virus from Japan: evidence for genetic reassortment of the six internal genes. *J.Virol.* 72:8021-8031.

[25] McMichael, A.J., Mitchie, C.A., Gotch, F.M., Smith, G.L. and Moss, B. 1986. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. *J.Gen.Virol.* 67:719-726.

[26] McMichael, A. 1994. Cytotoxic T lymphocytes specific for influenza virus. *Curr.Top.Microbiol.Immunol.* 189:75-91.

[27] McMichael, A.J. and Phillips, R.E. 1997. Escape of human immunodeficiency virus from immune control. *Annu.Rev.Immunol.* 15:271-296.

[28] Morein, B., Sundquist, B., Hoglund, S., Dalsgaard, K. and Osterhaus, ADME. 1984. Iscom, a novel structure for antigenic presentation of membrane proteins of enveloped viruses. *Nature* 308:457-459.

[29] Nietfield, W., Bauer, M., Fevrier, M., Maier, R., Holzwarth, B., Frank, R., Maier, B., Riviere, Y. and Meyerhans, A. 1995. Sequence constraints and recognition by CTL of an HLA-B27-restricted HIV-1 gag epitope. *J.Immunol.* 154:2188-2197.

[30] Osterhaus, A.D.M.E. and de Jong, J.C. 1999. The control of influenza: antivirals as an adjunct to vaccines. *Vaccine* 18:779-780.

[31] Palache, A.M., Brands, R. and van Scharrenburg, G.J.M. 1997. Immunogenicity and reactogenicity of influenza subunit vaccines produced in MDCK cells or fertilized chicken eggs. *J. Infect. Dis.* 176:S20-S23.

[32] Parker. C.E. and Gould, K.G. 1996. Influenza A virus-a model for viral antigen presentation to cytotoxic T lymphocytes. *Sem.Virol.* 7:61-73.

[33] Phillips, R.E., Rowland-Jones, E.S., Nicon, D.F., Gotch, F.M., Edwards, J.P., Ogunlesi, A.O., Elvin, J.G., Rothbard, J.A., Bangham, C.R., Rizza, C.R. and McMichael, A.J. 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T-cell recognition. *Nature* 354:453-459.

[34] Price, D.A., P.J. Goulder, P.J., Klenerman, P., Sewell, A.K., Easterbrook, P.J., Troop, M., bangham, C.R. and Phillips, R.E. 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc.Natl.Acad.Sci.USA* 94:1890-1895.

[35] Price, G.E., Ou, R., Jiang, H., Huang, L. and Moskophidis, D. 2000. Viral escape by selection of cytotoxic T cell-resistant variants in influenza A virus pneumonia. *J.Exp.Med.* 191:1853-1867.

[36] Rimmelzwaan, G.F. and Osterhaus, A.D.M.E. 1995. A novel generation of viral vaccines based on the ISCOM matrix. In: Powell, M.F. and Newman, M.J. (eds.) *Vaccine Design: The Subunit and Adjuvant Approach*, pp. 543-558. Plenum Press, New York.

[37] Rimmelzwaan, G.F. and Osterhaus, ADME. 1997. ISCOMs: Materials, Preparation, Antigen Delivery and Immune Response. In: Gander, B., Merkle, H.P. and Corradin, G. (eds.) *Antigen delivery systems. Immunological and technological issues*, 8: 123-128. Harwood academic publishers, Amsterdam.

[38] Rimmelzwaan, G.F., Nieuwkoop, N., Brandenburg, A., Sutter, G., Beyer, W.E.P., Maher, D., Bates, J. and Osterhaus, A.D.M.E. 2000. A randomized double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM vaccines and conventional vaccines. *Vaccine* in press.

[39] Robertson, J.S., Bootman, J.S., Nicolson, C., Major, D., Robertson, E.W. and Wood, J.M. 1990. The hemagglutinin of influenza B virus present in clinical material is a single species identical to that of mammalian cell-grown virus. *Virology* 179:35-40.

[40] Robertson, J.S., Cook, P., Nicolson, C., Newman, R. and Wood, J.M. 1994. Mixed populations in influenza virus vaccine strains. *Vaccine* 12:1317-1320.

[41] Rock, K.L., Rothstein, L., Gamble, S. and Fleischacker, C. 1993. Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. *J.Immunol.* 150:438-446.

[42] Rudneva, I.A., Kovaleva, V.P., Varich, N.L., Farashyan, V.R., Gubareva, L.V., Yamnikova, S.S., Popova, I.A., Presnova, V.P. and Kaverin, N.V. 1993. Influenza A virus reassortants with surface glycoprotein genes of the avian parent viruses: effects of HA and NA gene combinations on virus aggregation. *Arch.Virol.* 133:437-450.

[43] Shaw, M.W., Arden, N.H. and Maassab, H.F. 1992. New aspects of influenza viruses. *Clin.Microbiol.Rev.* 5:74-92.

[44] Shu, L.L., Bean, W.J. and Webster, R.G. 1993. Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. *J.Virol.* 67:2723-2729.

[45] Song, R. and Harding, C.V. 1996. Roles of proteasomes, transporter for antigen presentation (TAP), and β_2-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. *J.Immunol.* 156:4182-4190.

[46] Steinhoff, M.C., Fries, L.F., Karron, R.A., Clements, M.L. and Murphy, B.R. 1993. Effect of heterosubtypic immunity on infection with attenuated influenza A virus vaccines in young children. *J.Clin.Microbiol.* 31:836-838.

[47] Townsend, A.R.M. and Skehel, J.J. 1984. The influenza A nucleoprotein gene controls the induction of both subtype-specific and crossreactive cytotoxic T cells. *J.Exp.Med.* 160:552-563.

[48] Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., DeWitt, C.M., Friedman, A., Hawe, L.A., Leander, K.R., Martinez, D., Perry, H.C., Shiver, J.W., Montgomery, D.L. and Liu, M.A. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259:1745-1749.

[49] Ulmer, J.B., Fu, T.M., Deck, R.R., Friedman, A., Guan, L., DeWitt, C., Liu, X., Wang, S., Liu, M.A., Donnelly, J.J. and Caulfield M.J. 1998. Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. *J. Virol.* 72:5648-5653.

[50] Wang, M., Katz, J.M. and Webster, R.G. 1989. Extensive heterogeneity in the hemagglutinin of egg-grown influenza viruses from different patients. *Virology* 171:275-279.

[51] Wraith, D.C., Vessey, A.E. and Askonas, B.A. 1987. Purified influenza virus nucleoprotein protects mice from lethal infection. *J.Gen.Virol.* 68:433-440.

[52] Yasuda, J., Toyoda, T., Nakayama, M. and Ishihama, A. 1993. Regulatory effects of matrix protein variations on influenza virus growth. *Arch.Virol.* 133:283-294.

[53] Yewdell, J.W., Bennink, J.R., Smith, G.L. and Moss, B. 1985. Influenza A virus nucleoprotein is a major target antigen for cross reactive anti influenza A virus cytotoxic T lymphocytes. *Proc.Natl.Acad.Sci.USA* 82:1785-1789.

[54] Yewdell, J.W., Norbury, C.C. and Bennink, J.R. 1999. Mechanisms of exogenous antigen presentation by MHC class I molecules *in vitro* and *in vivo*: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants and vaccines. *Adv. Immunol.* 73:1-77.

SAMENVATTING

Influenza A en B virussen zijn de veroorzakers van een acute respiratoire infectieziekte bekend als influenza of griep. Influenza A virussen worden onderverdeeld op basis van de typen oppervlakteglycoproteïnen haemagglutinine (HA) en neuraminidase (NA) waarvan respectievelijk 15 (H1 t/m H15) en 9 typen (N1 t/m N9) kunnen worden onderscheiden. Op dit moment komen bij mensen, naast influenza virus B, de influenza virus A subtypen H1N1 en H3N2 voor. Het continu veranderen van influenza virussen door introductie van mutaties in HA en NA (antigene drift) is de drijvende kracht achter de jaarlijks in de winter terugkerende griepepidemieën. Hoewel de meeste mensen zonder blijvende schade herstellen van griep, kan infectie met het influenzavirus gepaard gaan met complicaties met verstrekkende gevolgen, met name bij mensen die tot de influenzarisicogroepen gerekend worden zoals 65^{+}-ers, diabetici, mensen met chronische hart of luchtwegaandoeningen en mensen met een verminderde werking van het immuunsysteem. Om de ernst en het voorkomen van infectie met het virus te voorkomen en eventuele complicaties te verminderen, wordt vaccinatie als preventieve maatregel aanbevolen. Thans gebruikte influenzavaccins bevatten geïnactiveerd virus of virale antigenen (HA en NA) en zijn trivalent, dat wil zeggen dat de beide typen influenza A virussen (H1N1 en H3N2) en influenzavirus B er in vertegenwoordigd zijn. De in dit proefschrift beschreven studies zijn in de meest algemene zin gerelateerd aan vaccinontwikkeling met speciale aandacht voor MHC klasse I-gerestricteerde processing en presentatie van virale antigenen. Eén hoofdstuk is gewijd aan serologische diagnostiek van influenzavirusinfectie.

Influenzavirussen die in het vaccin worden opgenomen worden geproduceerd in geëmbryoneerde kippeneieren. Hiervoor worden veelal 'high-growth' reassortant influenza A virussen gebruikt die zich tot hoge titers vermeerderen. Deze virussen worden verkregen door dubbelinfectie van geëmbryoneerde kippeneieren met het virus waartegen bescherming moet worden opgewekt (vaccinvirus) en een virus met een high-growth fenotype, en die reassortant virussen welke de oppervlakteglycoproteïnen (HA en NA) van het vaccinvirus én een high-growth fenotype bezitten worden geselecteerd voor vaccinproductie. Het gebruik van kippeneieren voor de productie van influenzavaccins kent een aantal nadelen waarvan het gebrek aan flexibiliteit, met als consequentie een noodzakelijke lange-

termijn planning, de belangrijkste is. Hoofdstuk 2 beschrijft het gebruik van een continue cellijn, geadapteerd aan groei in serum-vrij medium (MDCK-SF1), als mogelijk alternatief voor de thans toegepaste influenzavaccin-productieprocedure in kippeneieren. Analoog aan de methode in kippeneieren werden reassortant influenza A virussen gegenereerd in MDCK-SF1 cellen door dubbelinfectie met geselecteerde H1N1 of H3N2 vaccinvirussen en H3N2 of H1N1 high-growth virussen. De origine van elk van de gensegmenten van de reassortantvirussen alsmede hun 'groei-eigenschappen' in MDCK-SF1 cellen werden bepaald. Uit deze studie is gebleken dat reassortant influenza A virussen in deze cellen, net als in kippeneieren, gegenereerd kunnen worden door dubbelinfectie, dat de genetische samenstelling van deze virussen vrij eenvoudig en snel kan worden bepaald, en dat de aldus verkregen reassortant virussen, die allemaal het MA eiwit coderende RNA segment van het high-growth virus bezitten, meestal tot hogere virusproduktie leiden.
Naast studies gericht op het verbeteren van huidige vaccinproductie-procedures, wordt veel aandacht besteed aan de ontwikkeling van alternatieve influenzavaccins waarbij gebruik wordt gemaakt van verschillende adjuvantia en antigeenpresentatievormen. Een voorbeeld hiervan zijn vaccins gebaseerd op de zogenaamde immuunstimulerend complex (ISCOM) structuur, opgebouwd uit lipiden, cholesterol en het adjuvans QuilA waarin virale eiwitten geïncorporeerd kunnen worden. Talrijke studies met ISCOM-gebaseerde experimentele vaccins in diermodellen hebben aangetoond dat zowel humorale als cellulaire immuunresponsen geïnduceerd kunnen worden die meestal een excellente bescherming bieden tegen challenge infecties. Bovendien zijn ISCOM-gebaseerde influenzavaccins reeds getest in fase III studies in mensen en is een ISCOM-gebaseerd influenzavaccin voor paarden op de markt. De meeste studies met ISCOM-gebaseerde vaccins zijn uitgevoerd met virale envelopeiwitten (bijvoorbeeld influenzavirus HA en NA) omdat deze, dankzij de hydrofobe transmembraansequenties, gemakkelijk te incorporeren zijn in de ISCOM structuur. Incorporatie van de meer hydrofiele interne virale eiwitten is doorgaans alleen mogelijk na modificatie van het eiwit met hydrofobe sequenties bijvoorbeeld door koppeling van vetzuren aan het eiwit of door middel van recombinant DNA technologie. Hoofdstuk 3 beschrijft een studie waarbij deze laatste mogelijkheid werd onderzocht. ISCOMs werden gemaakt in aanwezigheid van recombinant matrixeiwit met (rMAHA) of zonder (rMA) transmembraansequentie van HA. De noodzaak van een hydrofobe sequentie en incorporatie in de ISCOM structuur van het matrixeiwit met

betrekking tot MHC klasse I processing en presentatie van het HLA-A2-gerestricteerde epitoop GILGFVFTL (aminozuren 58 t/m 63) werd onderzocht gebruikmakend van cytotoxische T lymphocyten (CTL) die dit epitoop herkennen. rMAHA leek beter geïncorporeerd te worden in ISCOMs dan rMA, en MHC klasse I presentatie aan specifieke CTL *in vitro* werd alleen waargenomen voor rMAHA-ISCOMs. Echter, MHC klasse I presentatie werd ook waargenomen voor rMA en rMAHA gemengd met ISCOMs, waarbij de combinatie van ISCOMs en rMAHA de beste bleek. De conclusie van deze studie is dat incorporatie van influenzavirus matrixeiwit in ISCOMs niet noodzakelijk is, maar dat associatie met ISCOMs voldoende is voor ISCOM-gemedieerde processing en presentatie via de MHC klasse I pathway. Het feit dat incorporatie niet noodzakelijk is betekent dat het gebruik van ISCOMs als antigeenpresentatievorm niet gerestricteerd is tot de hydrofobe oppervlakteglycoproteïnen HA en NA, maar ook toegepast zou kunnen worden voor het induceren van immuunresponsen tegen de meer hydrofiele interne influenzaviruseiwitten zoals matrixeiwit en het nucleoproteine (NP) welke waarschijnlijk belangrijke targets zijn voor CTL responsen. Theoretisch zou incorporatie van dergelijke eiwitten in ISCOM-gebaseerde influenzavaccins een bredere bescherming kunnen geven en wellicht zelfs de jaarlijkse aanpassing aan circulerende stammen minder noodzakelijk maken.

Processing van eiwitten voor MHC klasse I-gerestricteerde presentatie aan CTL vereist in het algemeen eiwitsynthese in het cytoplasma van cellen. Echter, exogene eiwitten (niet gesynthetiseerd door de cel), welke normaal worden geprocessed voor MHC klasse II-gerestricteerd presentatie aan T helpercellen, kunnen via alternatieve routes geprocessed worden voor MHC klasse I-gerestricteerde presentatie. Hoofdstuk 4 beschrijft MHC klasse I processing en presentatie van exogeen recombinant influenzavirus A NP (rNP) door B-lymphoblastoide cellijnen (B-LCL). MHC klasse I presentatie van het HLA-A3-gerestricteerde epitoop ILRGSVAHK (265 t/m 273) en het HLA-B27-gerestricteerde epitoop SRYWAIRTR (383 t/m 391) werd aangetoond gebruikmakend van specifieke CTL clones. HLA-A3-geassocieerde presentatie vereiste ongeveer 100 maal hogere concentraties exogeen rNP dan HLA-B27-geassocieerde presentatie. De route waarlangs exogeen rNP werd geprocessed voor MHC klasse I presentatie werd onderzocht gebruikmakend van remmers van de conventionele MHC klasse I processing en presentatie pathway (lactacystine en brefeldine A), remmers van processing in endosomen (chloroquine en NH_4Cl) en een cellijn zonder een functionele transporter geassocieerd met antigeenprocessing (TAP). De resultaten die hiermee werden verkregen duiden erop dat in normale B-LCL

cellen processing en presentatie voornamelijk verloopt via de conventionele MHC klasse I pathway en dat in cellen zonder functioneel TAP processing van rNP en associatie van peptiden met MHC klasse I moleculen in endosomen waarschijnlijk de meest belangrijke route is.
De invloed van een mutatie in een CTL epitoop met betrekking tot MHC klasse I presentatie en herkenning door specifieke CTL is beschreven in hoofdstuk 5. Van 162 influenza A (H3N2) virussen die tussen 1989 en 1999 in Nederland werden geïsoleerd, werd van een deel van het NP gen dat vier CTL epitopen bevat, de aminozuurvolgorde bepaald. Een deel van de influenzavirussen uit het seizoen 1989-1990 bevatte een R384K mutatie in het HLA-B8-gerestricteerde epitoop ELRSRYWAI (380 t/m 388) en het HLA-B27-gerestricteerde epitoop SRYWAIRTR (384 t/m 391). Deze R384K mutatie werd niet meer aangetroffen bij influenzavirussen geïsoleerd na 1990. Een andere mutatie op dezelfde positie, R384G, werd gevonden in alle influenza A H3N2 virussen geïsoleerd vanaf het seizoen 1993-1994 en leek dus geconserveerd. Geen aminozuurmutaties werden aangetroffen in de HLA-A3 en HLA-B37-gerestricteerde epitopen aanwezig in het NP. In CTL assays werd getoond dat cellen geïnfecteerd met influenza A virussen of recombinant vacciniavirus welke mutant NP tot expressie brengen, of geïncubeerd waren met mutante peptides, niet herkend werden door HLA-B27-gerestricteerde CTLs. Het introduceren van mutaties in CTL epitopen is een strategie die gebruikt word door virussen die persistente infecties veroorzaken (bv. HIV) om CTL-gemedieerde immuniteit te omzeilen en mutaties op het tweede aminozuur (ankerresidu) van een HLA-B27 epitoop van het HIV-1 Gag eiwit zijn in verband gebracht met verlies van specifieke CTL activiteit en progressie naar AIDS. De resultaten van deze studie suggereren eenzelfde strategie voor influenzavirussen. Dit zou betekenen dat net zoals de continue introductie van mutaties in de oppervlakteglycoproteïnen HA en NA influenzavirussen de mogelijkheid bieden antilichaam-gemedieerde immuniteit te omzeilen, mutaties in CTL epitopen influenzavirussen in staat stellen te ontsnappen aan CTL-gemedieerde immuniteit.
Tenslotte beschrijft hoofdstuk 6 de ontwikkeling van enzyme-linked immunosorbent assays (ELISAs) voor het meten van NP-specifieke IgA en IgG antistoffen in sera van influenzavirus A of B-geïnfecteerde patiënten gebruikmakend van rNP van influenzavirus A en B. Zowel de IgA als de IgG ELISA bleken antistoffen zeer specifiek te kunnen aantonen en de resultaten van de IgG ELISA verhielden zich goed tot de thans toegepaste haemagglutinatie-inhibitie (HI) test, die HA-specifieke antistoffen meet. NP-gebaseerde ELISAs zijn voor bepaalde doeleinden dus een bruikbaar

alternatief voor de HI test en hebben daarnaast nog een aantal voordelen. NP is een relatief geconserveerd eiwit en derhalve is het niet nodig om NP regelmatig te vervangen en kan in het geval van influenza A virussen hetzelfde NP gebruikt worden voor het meten van antistoffen geïnduceerd door zowel influenza A H1N1 en H3N2 virussen. Bovendien kan recombinant NP in ongelimiteerde hoeveelheden en met een constante kwaliteit worden geproduceerd. Bovendien en is het aflezen van ELISAs eenvoudiger en meer objectief dan het aflezen van de HI test.
Samenvattend kan gesteld worden dat de studies beschreven in dit proefschrift hebben getoond dat het gebruik van een continue cellijn goede mogelijkheden biedt als alternatief voor de huidige vaccinproductie-procedure in kippeneieren en dat een mogelijke nieuwe generatie influenzavaccins gebaseerd op de ISCOM structuur de mogelijkheid biedt CTL activiteit te induceren tegen de interne virale antigenen. Hoewel deze antigenen relatief goed geconserveerd zijn, dient bij de ontwikkeling van influenzavaccins gericht op het induceren van CTL responsen rekening te worden gehouden met de mogelijkheid op het ontstaan van CTL escape mutanten. Ondanks dat influenzavirussen continu veranderen en dit de ontwikkeling van een uniform vaccin moeilijk, zo niet onmogelijk, maakt, zullen nieuwe vaccinproductieprocedures en nieuwe generaties influenzavaccins bijdragen aan preventie en controle van influenza.

DANKWOORD

Op deze plaats wil ik een ieder bedanken die direct of indirect betrokken is geweest bij de totstandkoming van dit proefschrift. Op de eerste plaats bedank ik Ab Osterhaus die mij in de gelegenheid heeft gesteld promotie-onderzoek te verrichten binnen de afdeling virologie. Mede dankzij zijn aanmoedigingen: "ga eens aan je werk sloompie!" en zijn doortastende optreden in het laatste half jaar is het gelukt dit boekje nog in 2000 af te krijgen. Guus Rimmelzwaan wil ik bedanken voor de uitstekende en enthousiaste begeleiding gedurende mijn promotie-onderzoek. Dankzij zijn publicatiedrift werden manuscripten snel gecorrigeerd en ingestuurd. Voor de begeleiding tijdens de eerste jaren van mijn promotie-onderzoek bedank ik tevens Eric Claas. Nella Nieuwkoop bedank ik voor het mij bijbrengen van het cellulaire (met name CTL) werk dat na jaren op het VMT lab gezeten te hebben een welkome afwisseling bood, en voor haar niet geringe bijdrage aan het onderzoek, met name gedurende de laatste paar jaar. Theo Bestebroer bedank ik voor de korte, maar vruchtbare samenwerking. Zonder de hulp van deze sequence-goeroe was ik waarschijnlijk nooit tot sequencen gekomen. Ron Fouchier bedank ik voor zijn adviezen bij het beoordelen van manuscripten en Jacco Boon voor het leveren van de juiste literatuur op het juiste moment. De overige leden van het influenza team, Ruud van Beek, Marianne Baars, Liane van der Kemp, Gerrie de Mutsert, Jan de Jong, Sander Herfst, Monique Spronken, Emmie de Wit en Tiny Geelhoed wil ik bedanken voor de prettige samenwerking en hun belangstelling gedurende het onderzoek. Hetzelfde geldt voor Willem Huisman, Marlinda Dings en Hans Kruining. De mensen van het management team, te weten Conny Kruyssen, Tineke Jongenotter, Wim Leep en Ger van der Water, alsmede Robert Dias D'Ullois bedank ik voor hun inspanningen achter de schermen. Dankzij de doortastendheid die Ger tentoonspreidde bij bestellingen werd de continuïteit van het onderzoek gewaarborgd. Hij was bovendien één van de weinigen die begreep waar ik het over had bij de koffiepauzes. Verder wil ik de mensen bedanken bij wie het allemaal begonnen is, Kees Siebelink en Jos Karlas. Met veel plezier denk ik terug aan mijn stage bij de FIV groep welke een mooie afsluiting was van mijn studie biologie en tevens een goede basis vormde voor mijn promotie-onderzoek. Alle niet met name genoemde collega's wil ik bedanken voor de prettige samenwerking. Tot slot bedank ik mijn familie voor hun niet aflatende belangstelling en met name mijn ouders, die mij in de gelegenheid hebben gesteld (eindeloos) door te studeren met dit proefschrift als eindresultaat.

CURRICULUM VITAE

De auteur van dit proefschrift is op 13 januari 1968 te Bosschenhoofd geboren. Na het behalen van het MAVO diploma in 1985 aan de toenmalige Pastoor Hellemons MAVO in Oudenbosch, werd begonnen met een MLO opleiding aan het toenmalige Dr. Struycken Instituut te Etten-Leur. Deze studie werd in 1989 afgerond na een stage bij het pathologisch laboratorium van het voormalige Bergweg Ziekenhuis in Rotterdam. Vervolgens werd begonnen met een HLO opleiding aan de Hogeschool West Brabant in Etten-Leur die in 1993 werd afgerond met een stage bij de afdeling Virologie van de Landbouwuniversiteit Wageningen. Tenslotte werd begonnen met een studie Biologie aan de Universiteit Utrecht die in 1996 werd afgerond na een stage bij het Instituut Virologie van de Erasmus Universiteit Rotterdam. Binnnen ditzelfde instituut werd in datzelfde jaar begonnen met het promotie-onderzoek dat in 2000 werd afgerond met dit proefschrift.

PUBLICATIONS

Kool, M., Voeten, J.T.M., Goldbach, R.W., Tramper, J. and Vlak, J.M. 1993. Identification of seven putative origins of *Autographa californica* multiple nucleocapsid polyhedrosis virus DNA replication. *Journal of General Virology* 74:2661-2668.

Kool, M., Voeten, J.T.M., Goldbach, R.W. and Vlak, J.M. 1994. Functional mapping of regions of the *Autographa californica* nuclear polyhedrosis virus genome required for DNA replication. *Virology* 198:680-689.

Voeten, J.T.M., Groen, J., van Alphen, D., Claas, E.C.J., de Groot, R., Osterhaus, A.D.M.E. and Rimmelzwaan, G.F. 1998. Use of recombinant nucleoproteins in enzyme-linked immunosorbent assays for detection of virus-specific immunoglobulin A (IgA) and IgG antibodies in influenza virus A- or B- infected patients. *Journal of Clinical Microbiology* 36:3527-3531.

Voeten, J.T.M., Brands, R., Palache, A.M., van Scharrenburg, G.J.M., Rimmelzwaan, G.F., Osterhaus, A.D.M.E. and Claas, E.C.J. 1999. Characterization of high-growth reassortant influenza A viruses generated in MDCK cells cultured in serum-free medium. *Vaccine* 17:1942-1950.

Voeten, J.T.M., Claas, E.C.J., Brands, R., Palache, A.M., van Scharrenburg, G.J.M., Rimmelzwaan, G.F. and Osterhaus, A.D.M.E. 1999. Generation and characterization of reassortant influenza A viruses propagated in serum-free cultured MDCK-SF1 cells. *Developments in Biological Standardization* 98:77-87.

Voeten, J.T.M., Bestebroer, T.M., Nieuwkoop, N.J., Fouchier, R.A.M., Osterhaus, A.D.M.E. and Rimmelzwaan, G.F. 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. *Journal of Virology* 74:6800-6807.

Voeten, J.T.M., Rimmelzwaan, G.F., Nieuwkoop, N.J., Lövgren-Bengtsson, K. and Osterhaus, A.D.M.E. 2000. Introduction of the haemagglutinin transmembrane region in the influenza virus matrix protein facilitates its incorporation into ISCOM and activation of specific cytotoxic T lymphocytes. *Vaccine* 19:522-530.

Voeten, J.T.M., Rimmelzwaan, G.F., Nieuwkoop, N.J., Fouchier, R.A.M. and Osterhaus, A.D.M.E. 2000. Multiple routes of antigen processing for HLA-B27-associated presentation of exogenous influenza A virus nucleoprotein by B-lymphoblastoid cells. *Submitted for publication.*