DEVELOPMENT OF NATIVE AND RECOMBINANT MUMPS VIRUS SUBUNIT NASAL VACCINES USING PROTOLLIN TECHNOLOGY

by

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ABSTRACT

We sought to develop an inactivated nasal mumps virus (MuV) vaccine combined with the Protollin (Prl) adjuvant/delivery system. Antigen based on split Jones MuV was produced and characterized. Eight-week old BALB/c female mice were vaccinated with two or three doses of MuV antigen (4 or 8 μg) with or without 4 μg of Prl. Weight and behaviours were monitored to assess safety, and serum, respiratory secretions and splenocytes were obtained at study termination to assess MuV-specific immunity.

All vaccines were well-tolerated. Administration of 8 μg of MuV-Prl induced greater serum and mucosal antibodies than MuV antigen alone. MuV-Prl vaccines seemed to favour a Th1-type immune environment. Serum antibodies induced were capable of neutralizing MuV *in vitro*.

The intranasal MuV-Prl vaccine was safe and immunogenic. Future work will focus on the development of a trivalent MMR-Prl vaccine. Such a vaccine will be of great interest to the global health community.

RÉSUMÉ

Nous avons voulu explorer la faisabilité d'un vaccin contre le virus des oreillons (VdO) inactivé et administré par voie intra-nasale, et combiné avec l'adjuvant Protollin (Prl). Notre laboratoire a généré et a caractérisé des antigènes de virion entier désintégré utilisant un détergent. Des souris femelles de souche BALB/c âgées de huit semaines ont été vaccinées avec deux ou trois doses de VdO désintégré en antigène (4 ou 8 μg), avec ou sans 4 μg Prl. Les souris ont été suivies afin d'évaluer l'innocuité; des sérums et des sécrétions des muqueuses ont été obtenus à des intervalles afin d'évaluer l'immunité spécifique de VdO.

Tous les vaccins ont été bien tolérés chez les souris. Les vaccins VdO-Prl ont produit un plus grand taux d'IgG sériques et IgA au niveau de la muqueuse comparés aux vaccins VdO utilisés seuls. Les vaccins VdO-Prl ont tendance à générer une réponse immunitaire déviée sur Th1. Les anticorps sériques étaient capables de neutraliser le VdO.

Nous avons démontré que l'ensemble des vaccins de virus inactivés VdO-Prl administrés par voie intra-nasale est sans danger est immunogénique. Nous voulons générer un vaccin inactivé trivalent rougeole-oreillons-rubéole combiné avec le Prl. Un tel vaccin serait utile.

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STATEMENT OF CONTRIBUTIONS

Scientific contributions have been made by the following individuals: Dr. David Burt (ID Biomedical Corporation doing business as GlaxoSmithKline Biologicals of North America, Laval, QC), data analysis; Angela Brewer, Dr. Sazini Nzula, Dr. Carey Rodeheffer-Petrie (McGill University, Montreal, QC), technical assistance during animal procedures; Talik Mahir and Steven Rubin (Division of Viral Products, Food and Drug Administration, Bethesda, MD), in-laboratory experimental assistance and donation of MuV-containing plasmids. Funding for this project was provided by the Canadian Institutes for Health Research (CIHR), the Research Institute of the McGill University Health Center (RI-MUHC), and McGill University.

STATEMENT OF ORIGINALITY

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

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LIST OF ABBREVIATIONS

MMR	Measles-mumps-rubella
Prl	Protollin
MuV	Mumps virus
MeV	Measles virus
RV	Rubella virus
PIV	Parainfluenza virus
vRNAP	Viral RNA polymerase
vmRNA	Viral messenger RNA
OMP	Outer membrane protein
$TCID_{50}$	Tissue culture infectious dose 50
BAL	Bronchoalveolar lavage
MOI	Multiplicity of infection
NU	Neutralizing unit

INTRODUCTION

1. Introduction to Mumps virus

1.1 History

In 5th century B.C., Hippocrates first described a condition characterized by painful swelling of the parotid glands and testicles. This condition later became known as Mumps disease, a name thought to derive from the old English verb 'to grimace, to grin, or to mumble' (1). Johnson and Goodpasture have been credited with being the first to confirm mumps virus (MuV) as the causative agent of mumps disease by demonstrating that a filterable agent derived from the saliva of patients presenting with parotitis was able to cause clinical disease when transferred directly to the parotid gland of rhesus macaques (2). Furthermore, disease could be transmitted back into humans following transfer of infected monkey parotid gland tissue into infant patients (3).

1.2 General virology of MuV

MuV is a member of the *Paramyxoviridae*, a family of small, negative-sense, single-stranded RNA viruses, many of which are the causative agents of serious childhood respiratory illnesses (4). MuV belongs to the subgroup *Rubulavirus*, and is closely related to the parainfluenza viruses (PIV) (Figure 1) (4;5).

Mumps virions consist of a nucleocapsid enclosed by a host cell-derived lipid bilayer, studded with MuV surface glycoproteins (Figure 2). Numerous copies of MuV N protein attach to the genome and confer upon it its helical

Figure 1. ***Paramyxoviridae*** **family tree.** Examples given for members of each respective genus do not represent an exhaustive list. Also, nine unclassified paramyxoviruses exist (e.g.: Fer-de-lance virus, Salem virus), all of which are not listed in the following genealogy (5).

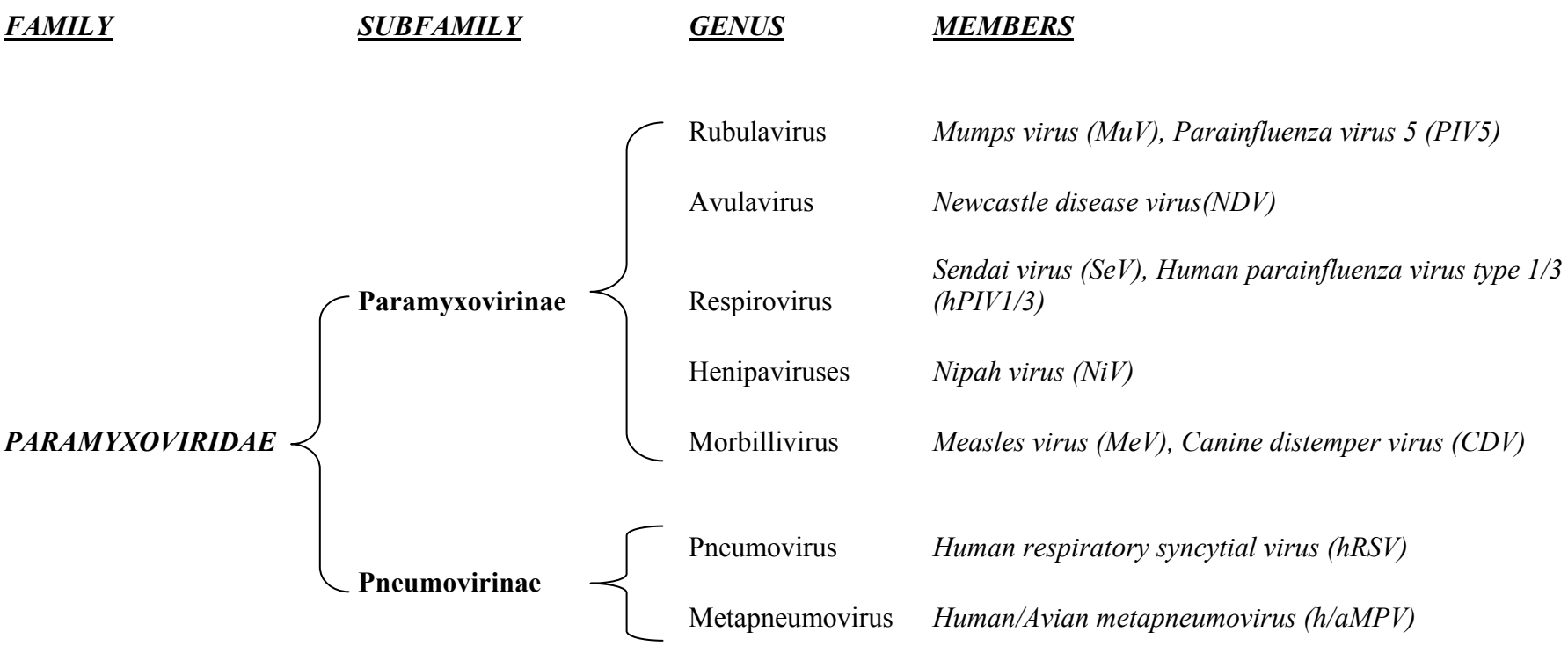
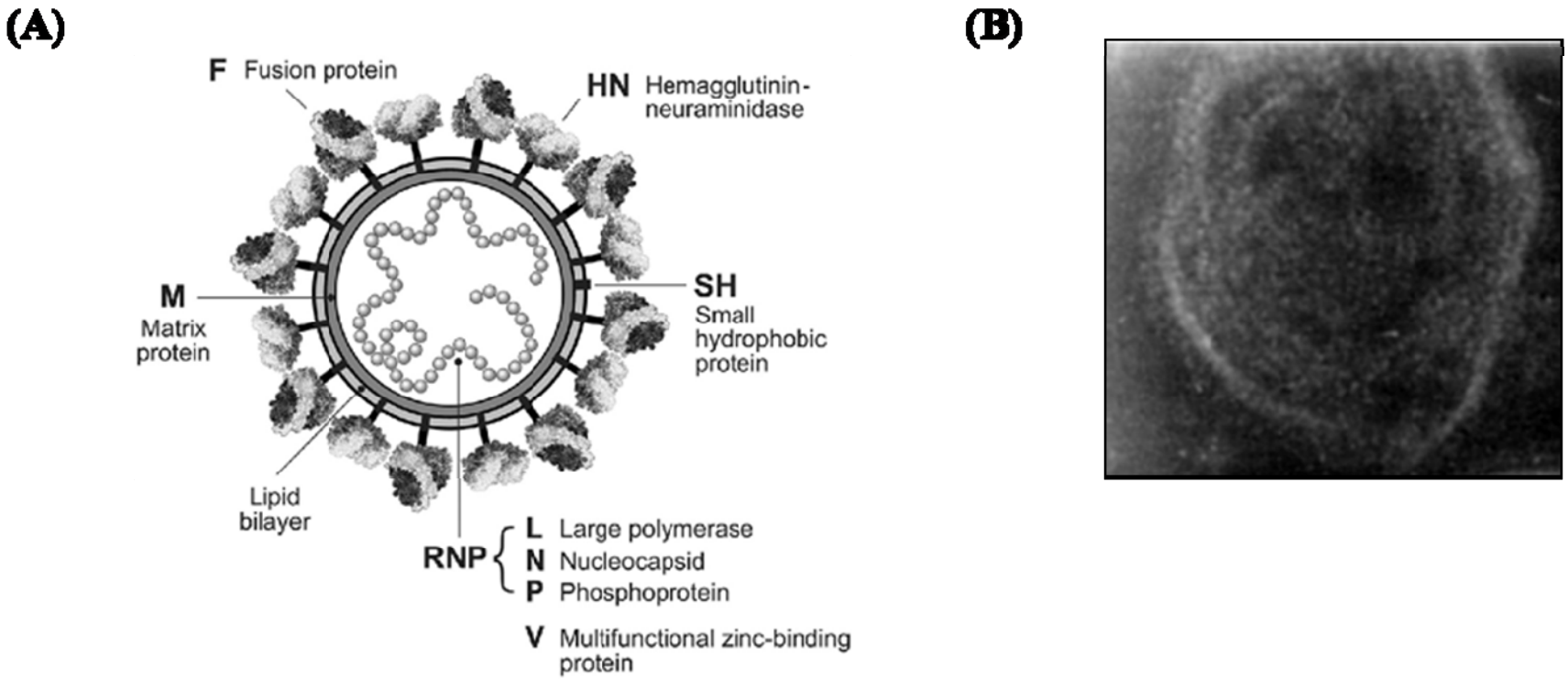


Figure 2. ***Paramyxoviridae*** **structure.** (A) Schematic of the paramyxoviruses. The helical nucleocapsid is encapsulated by a virus-modified host-derived cellular membrane. Multiple copies of the matrix protein underlie the viral envelope. Proteins necessary for viral transcription and replication (L, P) are found associated with the nucleocapsid. Image sourced from Lamb *et al.* (5). (B) Electron micrograph of MuV virion. Surface glycoproteins can be visualized as outward projections on the envelope and helical nucleocapsids can be seen within the structure. Image sourced from Horne *et al.* (6).



shape. Virion size can vary and has been reported to range anywhere from 100 to 600 nm (1;6). The 15 kb non-segmented MuV genome consists of seven genes in the following order 5'--L-HN-SH-F-M-P/V-NP--3', which can be roughly categorized into three general groups: the surface glycoproteins, the structural proteins, and the non-structural proteins (7).

The hemagglutinin/neuraminidase (HN) and fusion (F) proteins constitute the surface glycoproteins of MuV; both are acetylated and anchored in the viral envelope (1;5). The HN protein, a 74-80 kDa molecule, possesses hemagglutinin and neuraminidase activities and mediates the initial binding of virus to sialic acid residues on host cells (8-12). The F protein, slightly smaller than the HN protein at 65-74 kDa, mediates fusion of the viral envelope with the host cell membrane. The protein exists naturally in its inactive form, F_0, which is incapable of fusion. Upon cleavage, immature F_0 yields two separate subunits, F_1 and F_2, which are held together by a disulfide bond. Cleavage exposes the fusion peptide at the C' terminus of F_1 which acts to guide the two membranes together (8-10;13-16). Once fusion has occurred, the nucleocapsid is released into the host cell cytoplasm (1;5).

Among the non-structural proteins, the large (L) protein, at 180-200 kDa, is the largest and possesses domains which infer the presence of a catalytic polymerase site, an ATP binding site, and an RNA template contact region. Thus, it is thought to comprise the active enzyme of the viral RNA polymerase (vRNAP) and to play an essential role in role in MuV genome transcription and replication (1;5;17-19).

Other non-structural proteins include V, P, and I proteins, which are all

generated from the P/V gene by post-transcriptional addition of guanosine residues at an editing site found within the gene (20). The V protein is a faithful transcript of the P gene, and is involved in MuV evasion of the immune response by targeting interferon (IFN) signalling pathways (20;21). The P protein is produced by addition of two guanosine nucleotides at the editing site and serves as an essential cofactor for the L protein. Studies with other closely related paramyxoviruses have shown that the L and P proteins form the whole vRNAP and associate with the nucleocapsid (20;22). The functions of the two remaining MuV non-structural proteins (SH and I) remain unknown. The I protein is produced by addition of one or four guanosine nucleotides at the editing site within the P/V gene (23). The SH molecule is a small hydrophobic protein, associated with the surface of the viral envelope (approximately 5 kDa). Though its role remains unknown, the SH protein has been shown to be unnecessary for viral replication but has been useful for genotyping purposes (24-27).

The structural proteins of MuV include the matrix (M) and nucleoprotein (NP) proteins. The M protein is a 41-42 kDa membrane-associated protein, found on the underside of the viral envelope. It plays a critical role in virion assembly and mediates alignment of the nucleocapsid within the host cell below regions of virus-modified host cell membrane. This phenomenon occurs immediately before budding (1;28;29).

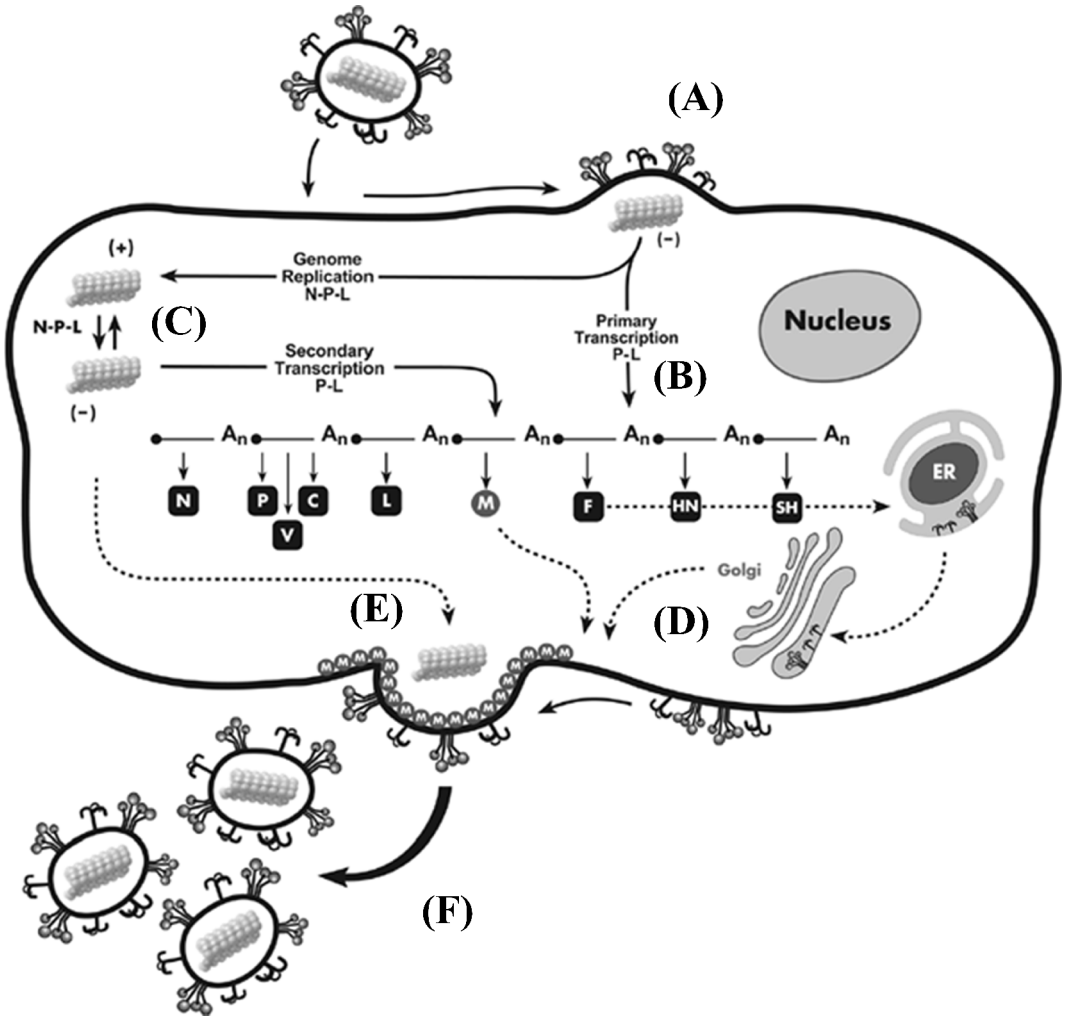
The NP protein is the most abundant MuV protein. Multiple copies bind to the RNA genome forming the helical nucleocapsid. The NP protein has a molecular weight of 61-73 kDa and is essential for protection of the genetic material from degradation within the host cell (1).

Although clinical mumps is similar regardless of the virus strain, many MuV genotypes exist and have been classified based on the SH protein gene sequence, as this gene demonstrates the greatest variability within the MuV genome (27). To date, thirteen different genotypes have been identified, denoted by the letters A through M (30). Many different strains fall into each genotypic category, and can vary in terms of antigenic content. These antigenic differences can have an effect on the ability of strain-specific antibodies to cross-neutralize and might help to explain why re-infection occurs in some situations. Despite these antigenic and genotypic differences, it is currently accepted that only one MuV serotype exists. Re-infection is still considered a rare event, and natural infection by MuV confers life-long protection in most cases (31;32).

1.3 MuV life cycle

Life-cycle studies for the *Paramyxoviridae* have generally not used MuV as a model system, though it is logical to assume that most features are generally shared among paramyxoviruses (Figure 3). It is known that MuV binds to sialic acid residues on target cells via its HN surface glycoprotein. Sialic acid modification is a common characteristic of many mammalian glycoproteins and glycolipids (including cell surface molecules) (1), which most likely explains the diverse tissue tropism demonstrated by MuV. Once bound to the host cell, the F protein mediates the fusion of the viral envelope with the cellular plasma membrane via a pH-independent process (1;5). The HN protein has been shown to be required for this process, most likely through an energy-transfer mechanism

<u>Figure 3.</u> Schematic representation of the paramyxovirus life-cycle. (A) Upon fusion of the virus to the target cell membrane, nucleocapsids are released into the cytoplasm. (B) Early transcription (or primary transcription) occurs first. (C) Once gene products build up within the cytoplasm, genome replication (and subsequently secondary transcription) can occur. (D) F, HN and SH gene products travel to the outer leaflet of the cell membrane by hijacking the secretory pathway, and the remaining non-structural proteins carry out their respective functions. (E) New negative-sense nucleocapsids are brought to regions of virus-modified cell membrane via interactions with the matrix protein. (F) New virions are released from the cell by budding. Image sourced from Lamb *et al*. (5).
**Note: the following features shown within this life-cycle are different for MuV: (i) MuV does not produce a C protein, but rather an I protein; (ii) MuV nucleocapsid protein is usually denoted as NP as opposed to N.*



(1;33;34). This membrane fusion releases the nucleocapsid into the cytoplasm of the host cell, and M protein uncoating proceeds in an as of yet undetermined manner. Once uncoated, transcription and replication of the viral genome can begin (5).

1.3.1 Transcription and replication

Paramyxovirus transcription is usually divided into two stages: (1) primary transcription and (2) secondary transcription. Primary (or early) transcription occurs when viral gene products are at a low concentration in the cell, and focuses mainly on the production of viral messenger RNA (vmRNA). The virus brings along the necessary machinery required for viral transcription, most importantly the vRNAP. Transcription of vmRNA begins at a single entry site on the 3' end of the genome, where the vRNAP is able to dock. Individual viral gene mRNA transcripts are formed as the vRNAP proceeds along the length of the genome (in a 3'→5' direction) by a start-stop mechanism. Once the transcription complex reaches the end of the upstream gene, the vRNAP has the option to shuttle along the genome until it reaches the leader sequence of the downstream gene and continue transcription. This is mainly regulated by a group of *cis*-acting RNA sequences, though other mechanisms of regulation (specifically *trans*-acting protein effectors (5;35;36)) are thought to be able to occur. This process is not always perfect, and in many instances the vRNAP falls off the genome, thus creating a gradient of viral mRNAs within the cell. Consequently, transcripts for genes at the 3' end are produced in greater abundance than those at the 5' end. All vmRNAs are 5'-capped and 3'-poly-adenylated, and the host protein-synthesis machinery is hijacked to create gene products from the transcripts. MuV

structural and non-structural proteins accumulate in the cytoplasm, while MuV surface membrane proteins (HN, F, SH) exploit the cellular exocytic pathway to reach the cell surface (5).

Once MuV proteins reach a poorly understood 'threshold' concentration within the cell, the vRNAP reads through the regulatory sequences at the end of each gene and focuses on genome replication, initially producing the positive-sense antigenome and subsequently the negative-sense genome (5). Both of these processes require abundant levels of NP protein, as replication and NP coating of the antigenome/genome occur concurrently (37). Three options then exist for the newly-produced genome: (a) to act as a template for more vmRNA transcripts, in a process known as secondary transcription; (b) to act as a template for more antigenome transcripts; or (c) to be packaged into new virions (5).

1.3.2 Budding and release of virions

If situation (c) arises, newly generated nucleocapsids associate with L/P complexes and are transported to the host-cell membrane to regions of high MuV surface glycoprotein content and virion budding can occur. This transport is mediated by interactions with the M protein, thus highlighting the importance of this protein in the packaging of new virions (5). The exact mechanism of paramyxovirus budding has not been fully elucidated, though it has been observed that only the matrix protein and either of the surface glycoproteins are absolutely required for the budding process (38). As surface glycoproteins are transported to the host cell surface, F_0 is cleaved by a host cell protease into its F_1 and F_2 components within the *trans* Golgi (1;15;39). The neuraminidase activity of the HN protein is thought to prevent premature fusion to other cellular membrane

components and to other nascent virions. The surface glycoproteins accumulate in regions on the external leaf of the cell membrane, though it is presently a matter of debate whether or not this occurs within lipid rafts. Well-characterized lipid rafts have been shown to occur in some paramyxovirus infections but not in others (5). The cytoplasmic tails of the surface glycoproteins are thought to interact directly with the M protein, and to its associated genome/vRNAP complex (5;38;40;41). Once all viral components are in place, nascent MuV virions are released to continue further rounds of infection and replication (5).

2. Mumps disease: pathogenesis and epidemiology

MuV is the causative agent of mumps, or epidemic parotitis, a respiratory disease most commonly affecting children between five and nine years of age. MuV is transmitted via droplet, and spread either through the air or by direct contact (3;42). The virus initially infects and replicates in epithelial cells of the upper respiratory tract, after which it spreads to the mastoid and sub-occipital lymph nodes of the head and the cervical lymph nodes of the neck, where it undergoes further replication. The primary viremia, defined as the first entry of virus into the bloodstream, typically occurs at this stage, and is characterized by dissemination of virus to various regions of the body, such as the salivary glands, the male and female gonads, the central nervous system, the pancreas, as well as the heart and the breast. After additional replication within these tissues, the virus enters the bloodstream once again (secondary viremia), and is localized primarily within the kidneys. At this stage of illness, large amounts of virus are shed in the urine, known as viruria (1;43).

Initial clinical manifestations to MuV infection are non-specific and include symptoms such as fever, headache, anorexia, fatigue, and general malaise. The classical symptom of mumps is swelling of the parotid gland, or parotitis, which is responsible for the characteristic swelling of the neck associated with infection. Clinical parotitis is typically accompanied by fever and pain. A range of secondary complications can occur, including aseptic meningitis, hearing problems or deafness, orchitis/oophoritis, and pancreatitis. Aseptic meningitis is the most serious complication, and is characterized by headache, fever, and inflammation of the meninges (1;43). Mumps has also been associated with spontaneous abortion in mothers who acquire the infection during the first trimester of pregnancy. Although live virus has been retrieved from the aborted fetal tissue, a causal relationship between mumps and spontaneous abortion has yet to be proved (44).

Humans are the only known natural hosts for MuV, though experimentally-induced infection has been reported in various animal models (43;45;46). MuV usually circulates in areas of high population density, and survives best in crowded settings (47). In non-vaccinated populations, mumps disease has a variable annual incidence, with epidemics usually occurring every two to five years, and is at its highest during the late winter/early spring (42). The precise incidence of mumps can be difficult to determine however, as ~30% of individuals seropositive for MuV-specific antibodies show no history of clinical mumps disease (48;49).

3. Immunologic responses to MuV

MuV infection elicits both humoral and cell-mediated immunity. Early antibody responses can be detected systemically (serum IgM) and within the saliva (secretory IgA; sIgA) (50;51). Clearance of viral load in the saliva is associated with an increase in sIgA, most likely due to the neutralizing capacity of this Ig subset. In later responses, there is the typical switch in virus-specific serum antibodies from IgM to IgG. The distribution of IgG isotypes has not been determined for natural infection, but in the case of vaccination, IgG1 (and some IgG3) is usually favoured. The extent to which this response wanes over time has not been fully explored (52-54). Cross-protection between MuV strains is common, though such protection is imperfect and re-infection has been reported. Serum antibodies elicited by MuV can also display cross-reactivity with other *Paramyxoviridae* (e.g.: PIV-2) (55-57).

Though it is well known that cellular immunity is important for the clearance of intracellular pathogens, cell-mediated immune responses to MuV have not been well defined. It has been demonstrated that MuV-specific CD8+ and γ:δ cytotoxic T lymphocytes are present within the cerebrospinal fluid and circulating in the blood during acute infection (58;59). Also, in terms of cellular immune memory, peripheral blood mononuclear cells from patients naturally infected with MuV demonstrate robust cellular responses (lymphoproliferation, IFN-γ production) in response to MuV exposure (60).

4. MuV and vaccines

To date, no specific therapy exists for mumps disease and the focus of public health authorities has mainly been on disease control through vaccination.

Before the introduction of MuV-containing vaccines, mumps disease in Canada was a common childhood ailment, with approximately 34,000 cases recorded each year (61). Currently, 110 countries have implemented a MuV vaccine into their national vaccination programs (42), thereby successfully reducing the incidence of MuV infection within these regions. The first MuV vaccine was based on a formalin-inactivated virus formulation, licensed in the US in 1948 and used between 1950 and 1978. However, this vaccine failed to provide long-term immunity (≤ 1year), resulting in its removal from the market (1). A number of live-attenuated MuV vaccines were subsequently developed in various countries, including the United States, Japan, Switzerland, and the former Soviet Union. Today, all available vaccine strains are based on live-attenuated MuV strains, which include Jeryl-Lynn, Leningrad 3, L-Zagreb, and Urabe AM9 (42).

Mumps vaccines are usually offered within national vaccine programs as a trivalent formulation, with measles and rubella vaccines (known as the MMR). In Canada, MMR was initially introduced as a single-dose vaccine, and brought disease incidence down to fewer than 400 cases/year by the 1990's. A second dose of measles vaccine was recommended in 1996 following reports of primary measles vaccine failure and suboptimal protection with the single-dose vaccines. In regions/countries that implemented a second dose of MMR, mumps disease rates dropped below 90 cases/year (61) . Many nations implementing this type of vaccine regimen have experienced a similar decreasing trend in disease incidence (42).

5. Rationale for continued research into MuV vaccination

Though the different vaccines developed worldwide vary somewhat in terms of protective efficacy and side effect profiles, each has been accepted by international health organizations as safe and effective (42). However, recent MuV outbreaks worldwide have demonstrated limitations with both vaccination programs and the vaccines themselves, emphasizing the need for new vaccine technologies.

In 2007, Canada experienced the most severe mumps outbreak since vaccine introduction, with a total number of 1,284 cases reported (62). Other large outbreaks have occurred in recent years, most notably in the Netherlands (2007-2008) (63), the United States (2006) (64) and the United Kingdom (2004-2006) (65). These outbreaks were characterized by a high proportion of cases in young adults, indicating a shift in disease epidemiology (66). Secondary complications associated with the disease, namely orchitis, were more frequent and more severe. While the Canadian and British outbreaks seemed to occur in individuals who had received either no or only a single vaccine dose, a large proportion of reported cases in the United States and Netherlands occurred in individuals who had received two doses (31;66) . This indicates that current vaccine programs based on live attenuated vaccines are not wholly effective at generating life-long protection against MuV. Furthermore, rare (but serious) adverse events following MuV vaccination have been reported in children, such as aseptic meningitis, orchitis, sensorineural deafness , and post-infectious encephalomyelitis (42;67;68). These problems associated with current MuV vaccines provide strong support for continued research and development efforts to improve MuV vaccination.

6. Rationale for use of Protollin for MuV vaccines

An exciting new option for intranasal vaccine delivery has recently been developed. Proteosome adjuvants, licensed by ID Biomedical (doing business as GlaxoSmithKline Biologicals of North America), have been described as effective adjuvant/delivery vehicles for intranasal administration of various inactive respiratory virus vaccines (e.g.: influenza virus, measles virus (MeV) and respiratory syncytial virus (RSV)) (69). These candidate inactivated vaccines typically consist of two components: viral antigen against which the immune response is desired and the 'proteosomes'. Proteosomes are particles formed by outer membrane proteins (OMP) derived from *Neisseria* species bacteria. About 80% of the protein content of proteosomes is Porin B, which can act as a Toll-like receptor (TLR)-2 ligand. OMP are highly amphipathic and associate with one another via hydrophobic protein-protein interactions. When isolated via detergent extraction, OMP have the property of spontaneously self-assembling into clusters. These structures can be up to several hundred nanometers in size, with an average diameter of ~200 nm and are hence considered to be nanoparticles (69).

To produce proteosome-based vaccines, equal amounts of detergent-solubilized proteosome particles are mixed with purified soluble viral antigens and dialyzed to permit OMP-antigen clusters to form. For successful association of the antigen with the OMP vesicles, it is currently thought that the antigen(s) must be amphipathic. It is thought that simple mixing of these two components leads to the intercalation of the viral antigen into the OMP clusters, as the

hydrophobic regions of the former 'anchor' or insert into hydrophobic pockets of OMP clusters. This is a spontaneous non-covalent interaction that is believed to continue until all available 'sites' within the proteosome particles are occupied. Theoretically, this interaction permits the hydrophilic moieties of the antigen to remain exposed and creates a hydrophilic micro-environment at the periphery of the nanoparticles, effectively stabilizing them (69).

OMP have also been shown to spontaneously associate with bacterial lipopolysaccharide (LPS) like any other amphipathic molecule. Proteosomes bearing LPS represent a somewhat modified version of the proteosome adjuvant/delivery vehicle. Termed Protollin (Prl), these particles are composed of meningococcal OMP and LPS derived from *Shigella flexneri* (70). Unlike proteosome-based vaccines in which the formulation process includes a combined OMP-antigen assembly, Prl-based vaccines are produced by simple mixing of Prl with viral antigen immediately prior to injection. In this situation, the antigens may or may not integrate into the OMP clusters. However, adjuvancy is markedly enhanced with Prl vaccines, likely because the LPS acts as an additional immune-stimulator via TLR-4 ligation (71).

Although the basis for the effectiveness of proteosome/Prl-based vaccines is not yet fully understood, several factors likely contribute. These include (a) the particulate nature of the clusters, a characteristic commonly believed to benefit immunogenicity; (b) *in vitro* evidence that porins can translocate and insert themselves into cell membranes (72;73) thus possibly providing physical assistance in antigen delivery; (c) inhibition of antigen degradation; (d) the possible generation of physiologically relevant antigen arrays within the clusters,

thus providing a basis for pattern recognition by the immune system (if the antigen(s) physically integrate into the clusters); and (e) the presence of both OMP and LPS in the formulations therefore stimulating both TLR-2 and TLR-4 signaling (72-74).

Although all of these characteristics may plausibly contribute, the role of TLR ligation in proteosome and Prl adjuvancy has recently been directly studied. In a murine intestinal model, it was demonstrated that the presence proteosomes as an adjuvant enhanced vaccine uptake by (and subsequent migration of) local dendritic cells, leading to a more robust immune response. Based on mouse knockout and receptor agonist studies, these effects were mediated primarily by TLR-2 (75). Further mechanistic studies in mice using Prl-based vaccines specific for RSV demonstrated that signaling through the TLR-4 pathway was required to generate virus-specific antibody and cell-mediated immune responses. It was further discovered that different aspects of the enhanced adjuvancy functioned in MyD88-dependent or -independent fashions, although the former was required for a balanced Th1/Th2 response (i.e.: activation without MyD88 signalling resulted in severe skewing towards Th2-type immunity) (76). Thus, it is evident that both OMP and LPS contribute to the adjuvancy of these nanoparticles.

It is currently unknown exactly how the viral antigens interact with Prl adjuvants. Electron microscopy analyses of immunogold-stained viral surface glycoprotein/Prl preparations have demonstrated co-localization of the two vaccine components (77;78), however information is lacking as to the exact nature of this interaction. It is hypothesized that the amphipathic nature of the

surface glycoproteins allows their intercalation within the nanoparticles, thus allowing for stable delivery to (and adjuvancy within) the immune compartments of the airway mucosa. Studies are currently underway to determine the exact nature of these interactions (76).

7. Overall project goal

The overall goal of the thesis project is to generate inactivated Prl-based vaccines against MuV for intranasal administration, using both native virion and recombinant protein approaches. Prl has shown great promise with closely related viruses, suggesting its possible use in MuV vaccine development. An inactivated nasal vaccine against MuV infection would be an appealing alternative to current vaccines. The nature of the vaccine and route of administration may plausibly circumvent or mitigate many of the problems associated with contemporary vaccines. Not only would intranasal vaccination avoid needles (which are painful and create waste), this route would also make the vaccine much easier to administer. Also, a nasal vaccine better mimics the route of natural infection and may avoid interference from maternal neutralizing antibodies. Finally, an inactivated vaccine may avoid some of the most serious side-effects documented to occur with live-attenuated MuV vaccines. Our initial hypothesis was that inactivated MuV-Prl vaccines administered intranasally would be safe and immunogenic in a mouse model.

MATERIALS AND METHODS

1. Viral growth

Vero green monkey kidney cells (ATCC# CCL-81) were seeded into a Corning 10-level CellSTACK culture chamber in EMEM (Wisent Corporations, St. Bruno, QC) supplemented with 5% fetal bovine serum (FBS), 1% HEPES, and 50 µg/mL gentamicin (Wisent Corporations, St. Bruno, QC) and allowed to grow to 90-95% confluency at 37°C, 5% CO_2. Upon reaching appropriate confluency, cells were infected with Jones strain MuV at a multiplicity of infection (MOI) of 0.0001, and grown in EMEM supplemented with 3% FBS, 1% HEPES and 50 µg/mL gentamicin at 33°C, 5% CO_2. Starting at day two post-infection (p.i.), supernatants were removed every 24 hours and stored at -80°C until further processing. Fresh culture media was added to the infected cells at each viral harvest and infection was terminated at day 10 p.i..

2. Viral titration

Viral concentrations were determined by $TCID_{50}$, defined as the tissue culture infectious dose at which 50% of cells in a monolayer are infected. Vero cells were seeded in a flat-bottomed 96-well MICROTEST™ tissue culture plate (Beckton Dickinson Labware, Franklin Lanes, NJ) at a concentration of 1.5×10^6 cells/mL. Ten-fold serially diluted MuVsamples were added to wells in quadruplicate and plates were incubated for 4 days at 37°C, 5% CO_2. Wells demonstrating cytopathic effect were recorded manually and titers were calculated using the Kärber method (79).

3. Preparation of inactivated MuV antigen based on native virions

Thawed supernatants from viral harvests were centrifuged at 2100 g for 10 minutes at 4°C to remove cellular debris. Clarified supernatants were transferred to sterile bottles and centrifuged at 25,000 g at 4°C for 7 hours. For crude antigen preparations, pellets were resuspended in sterile PBS (Wisent), aliquotted and stored at -80°C until further use. For vaccine-grade antigen, pellets were resuspended in cold sterile TNE buffer (25 mM TrisCl, 150 mM NaCl, 5 mM EDTA) and placed on a biphasic sucrose gradient (60% and 20%) and ultracentrifuged at 200,000 g at 4°C for 90 minutes. Viral antigen was collected at the sucrose interface, washed once in cold sterile TNE buffer and pelleted by ultracentrifugation (200,000 g, 4°C, 90 minutes). Pellets were resuspended in 1% detergent (Empigen®, EMD BioScience Inc., La Jolla, CA) and placed on ice for 1 hour. Detergent-killed virus was placed in dialysis cassettes of 10,000 molecular weight cut-off (Pierce Slide-A-Lyzer® Gamma Irradiated Dialysis Cassette, ThermoFisher Scientific, Rockford, IL) and dialyzed against sterile PBS at 4°C for seven days (dialysis buffer was changed daily). Dialyzed antigen was aliquotted and stored at -80°C until further use. Protein concentration was determined colorimetrically using the Pierce bicinchoninic acid (BCA) protein assay (ThermoFisher Scientific, Rockford, IL) and calculated based on a bovine gamma globulin (BGG) fraction II/bovine serum albumin (BSA) fraction V standard curve.

4. Generation of HN and F gene cloning vectors for future preparation of inactivated MuV antigen based on recombinant protein technology

pTM1 plasmids containing MuV HN and F gene open reading frames (ORFs) were generously donated by Kathy Carbone (Division of Viral Products, Food and Drug Administration, Bethesda, MD) (80). The HN and F ORFs corresponded to 1.73 and 1.6 kb fragments, respectively, and were amplified by RT-PCR using the following primers: HN-P1 (5'-ATGGAACCCTCAAAACTCTTCACAATATCAGACAATG-3') and HN-P2 (5'-CCAAATTCTACCTGTGCTAACCAGATTGACTATCACTTGA-3'); F-P1 (5'-ATGAAGGCTTTCTCAGTTATTTGCTTGG-3') and F-P2 (5'-CACAATATCAAGTAGTGTCGATGATCTCATCAGGTACTAA-3'). All primers were obtained from Invitrogen Corporations (Carlsbad, CA). PCR fragments were run on a 0.8% agarose gel to verify for correct amplification. Additional 3'- A-overhangs were added to each fragment post-amplification. 3'-A-modified PCR fragments were cloned into a pCR8®/GW/TOPO® vector using the pCR8®/GW/TOPO® TA Cloning Kit (Invitrogen Corporations, Carlsbad, CA). Plasmid DNA was purified using QIAprep® Miniprep kit (QIAGEN Incorporated, Valencia, CA). Restriction enzyme digestion (EcoRI) and sequence analysis were performed to ensure that sequences were inserted in the appropriate orientation and reading-frame.

5. Characterization of inactivated MuV antigen based on native virions

Purified inactivated viral preparations were separated by electrophoresis on a 7.5 % polyacrylamide gel under denaturing conditions and visualized by

staining overnight with Coomassie Brilliant Blue (American Chemicals Ltd). Individual protein amounts within the antigen preparation were estimated by quantitative densitometric analysis of stained gels using Image J software. Proteins were transferred to PVDF membranes (ImmunoBlot™ PVDF membrane, Bio Rad Laboratories, Hercules, CA) at a constant 350 mA for 1 hour at 4°C. Membranes were blocked with 5% milk in PBS containing 0.05% Tween (PBS-T) or 3% bovine serum albumin (BSA – Sigma-Aldrich Inc., St. Louis, MO) in PBS for 1 hour. Membranes were washed in PBS-T and MuV-specific antibodies diluted in PBS-T were added to membranes and incubated overnight at 4°C. MuV-specific antibodies included mouse anti-F monoclonal antibody (MAb) (Chemicon International, Temecula, CA), mouse anti-NP MAb (GeneTex® Inc., San Antonio, TX), and mouse anti-HN polyclonal antibody (donated by Dr. Kathy Carbone). Membranes were washed in PBS-T and rabbit anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Santa Cruz Laboratories, Santa Cruz, CA) was added for 1 hour at room temperature. Membranes were washed in PBS-T and proteins were detected by chemiluminescence using ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) and visualized by autoradiography.

6. Formulation of vaccine based on native inactivated MuV virions

Prl adjuvant was formulated as previously described (70) and obtained from ID Biomedicals of Canada doing business as GlaxoSmithKline Biologicals of North America (courtesy of Dr. David Burt). Immediately prior to vaccine administration, a constant amount of Prl (4 μg) was added to 2, 4, or 8 μg MuV

antigen immediately prior to vaccine administration. Final amounts of Prl and MuV antigen were based on LPS content for the Prl and MuV surfaces glycoprotein (F and HN) content for the MuV viral antigen components respectively.

7. Mouse immunization

All animal procedures were approved by the McGill University Animal Care and Use Committee (protocol #4481). Twelve groups of 6-8 week old BALB/c female mice (Charles River Laboratories, St. Constant, QC) were used for the study. Group numbers were either n=5 or n=10 (Table 1.) Three dose groups were vaccinated at day 0 and given booster doses on both day 14 and day 28, while two dose groups were vaccinated at day 0 and given only one booster on day 14. Prior to vaccinations, animals were bled from the lateral saphenous vein. During vaccination, animals were anaesthetized by isofluorane, and vaccines were administered intranasally by pipetting 12.5 μL of vaccine or control solution into each nare during quiet breathing. For intramuscular vaccination, 40 μL of vaccine solution was administered into the hind thigh muscle of anaesthetized mice.

8. Animal procedures and sample collection

Mice were monitored for three days immediately following each vaccination and bi-weekly throughout the experiment for weight loss and behavioural changes. On the terminal day of each study (day 28 for two-dose groups; day 42 for three-dose groups), mice were euthanized by CO_2 asphyxiation

Table 1. Description of experimental groups. Six to eight week-old female BALB/c mice were vaccinated at day 0 and given booster vaccines at day 14 (two and three-dose groups) and day 28 (three-dose groups). Animals were weighed regularly throughout the study and bled from lateral saphenous vein prior to vaccination. Study termination was performed two weeks after the last vaccination (two dose groups: day 28; three-dose groups: day 42).

Description	Amount of antigen (µg)	Amount of Prl (µg)	Route of administration	Number of Doses	Number of animals
4 µg MuV + Prl	4	4	intranasal	2	5
4 µg MuV + Prl	4	4	intranasal	3	10
4 µg MuV + Prl	4	4	intramuscular	3	5
4 µg MuV alone	4	n/a	intranasal	3	5
8 µg MuV + Prl	8	4	intranasal	2	5
8 µg MuV + Prl	8	4	intranasal	3	10
8 µg MuV + Prl	8	4	intramuscular	3	5
8 µg MuV alone	8	n/a	intranasal	3	5
Prl alone	n/a	4	intranasal	3	5
Vero + Prl	4	4	intranasal	3	5
PBS	n/a	n/a	intranasal	3	10

and exsanguinated by cardiac puncture. Sera were obtained from blood samples by centrifugation, aliquoted and stored at -20^{o}C until further use. Lung mucosal secretions were collected by bronchoalveolar lavage (BAL). BAL were performed by incising the trachea and inserting a 22 gauge catheter (BD Insyte, Beckton Dickinson & Co., Sandy, UT). Catheters were fixed into place with a suture loop and a syringe (Luer-Lok^{TM} Tip, Beckton Dickinson & Co., Franklin Lakes, NJ) containing 1 mL protease-inhibitor cocktail (AEBSF, EDTA, bestatin, E-64, leupeptin, apronitin; Sigma-Aldrich Inc., St. Louis, MO) diluted in cold sterile PBS (Wisent) containing 0.1% BSA was attached to the catheter. Wash fluid was administered into lung and slowly aspirated to obtain mucosal secretions. A total of 2 mL (2 syringes) was used for each animal, with an average fluid recovery of 80%. BAL samples were aliquoted and stored at -20^{o}C until further use. Spleens were collected and pooled by group. Spleens were homogenized using a syringe plunger and single-cell suspensions were prepared using sterile 70 μm nylon cell strainers (BD BioSciences, Bedford, MA). Splenocytes were washed in sterile PBS containing 50 μg/mL gentamicin and red blood cells were removed by cell lysis. Remaining splenocytes were resuspended in 1640 RPMI containing 5% FBS, 1% HEPES and 50 μg/mL gentamicin. Pooled cells were aliquoted and placed in cryogenic solution (90% FBS, 10% DMSO) and stored in liquid nitrogen until further use.

9. Antibody determination by ELISA

Serum total IgG, IgG1 and IgG2a and mucosal IgA levels were determined by enzyme-linked immunosorbant assay (ELISA). Crude MuV

antigen was diluted in carbonate/bicarbonate buffer, added to U-bottomed 96-well Greiner microplate (MJS BioLynx, Brockville, ON), and incubated overnight at 4^{o}C. Plates were washed with PBS-T and blocked with 2% milk in PBS-T for two hours at room temperature. Plates were washed with PBS-T and diluted sera/diluted BAL samples were added to wells in duplicate and incubated for two hours at room temperature. Plates were washed with PBS-T and secondary antibody was added to wells and incubated for 45 minutes at room temperature. Secondary antibodies used were as follows: goat anti-mouse IgG-HRP (Jackson Immunoresearch Laboratories, West Grove, PA), goat anti-mouse IgG1-HRP (Southern Biotechnologies Associates, Birmingham, AL) and goat anti-mouse IgA-HRP (Sigma-Aldrich, St. Louis, MD). Plates were washed with PBS-T and TM Blue substrate (Chemicon International, Temecula, CA) was added to wells. Reaction was terminated with 0.5 M sulfuric acid (Sigma-Aldrich, Germany) and absorbancy was read at 450 nm. All dilutions were made in PBS-T. Antibody titers were calculated based on a standard curve run on each plate using purified IgG (Sigma-Aldrich Canada, Oakville, ON), purified IgG1 (Pharmigen BD, San Diego CA), purified IgG2a (Pharmigen BD, San Diego, CA) or purified IgA (Bethyl Laboratories, Montgomery, TX). Goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and goat anti-mouse IgA (Sigma Aldrich, St. Louis, MO) were used as the standard coating antigen for the IgG/IgG1/IgG2a and IgA assays, respectively.

10. Cytokine secretion of re-stimulated splenocytes

Splenocytes (adjusted to 6 x 10^6 cells/mL in 1640 RPMI containing 5% FBS, 1% HEPES, and 50 μg/mL gentamicin) were added in quadruplicate to a flat-bottomed 96-well MICROTEST^{TM} tissue culture plate. Splenocytes were stimulated with 1 μg/mL crude MuV antigen or (total volume of 200 μL) and incubated at 37^{o}C, 5% CO_2 for 72 hours. Plates were spun for at 1,900 g at room temperature and supernatants were removed and stored at -80^{o}C until further analysis. Cytokine supernatants were diluted (1/2, 1/20) and all cytokine concentrations were determined by Q-PLEX^{TM}-Mouse Cytokine Screen 16-plex (Quansys BioSciences, Logan, UT).

11. Determining neutralizing antibody titers in animal sera

Sera from individual mice were diluted ten-fold in a flat-bottomed 96-well MICROTEST^{TM} tissue culture plate, and serially diluted two-fold. All dilutions were performed in quadruplicate in EMEM supplemented with 5% FBS, 1% HEPES and 50 μg/mL gentamicin. Concentrated viral stock (3.56 x 10^6 $TCID_{50}$ units/mL) was diluted to 2 x 10^3 $TCID_{50}$ units/mL and added in equal amounts to each well. Plates were incubated at 37^{o}C, 5% CO_2 for 30 minutes. Vero cells were diluted to 1 x 10^5 cells/mL and added to wells. Plates were incubated for four days at 37^{o}C, 5% CO_2. Wells demonstrating no cytopathic effect were recorded manually and titers were calculated using the Kärber method (79) to determine 50% end-point neutralization. The neutralizing titer is defined as 1/[serum dilution] that fully inhibits viral replication.

RESULTS

1. Growth under optimized conditions produces high viral titers

Viral titers obtained previously in the laboratory were sub-optimal for vaccine-antigen production purposes ($\leq 2 \times 10^5$ $TCID_{50}$ units/mL), and MuV growth therefore required further optimization. Infecting at a MOI of 0.0001, growing at 33^{o}C and performing growth in a cell factory (as opposed to the bioreactor) allowed us to achieve titers as high as 2×10^8 $TCID_{50}$ units/mL (Figure 4).

2. Whole-virus based antigen contains MuV surface glycoproteins

MuV-specific protective immune responses are usually directed against viral surface glycoproteins (HN and F) (81;82), therefore MuV antigen content in the vaccines was based on these two proteins. Coomassie-staining of antigen preparations demonstrated the presence of both MuV-specific proteins and residual Vero cell proteins in even the 'purified' antigen preparation (Figure 5A). Western blot analysis of antigen preparations revealed the presence of monomeric and dimeric forms of both HN and F proteins (Figure 5B; HN: 70 and 140 kDa; F: 60 and 120 kDa). Based on densitometric calculations, these two proteins represented approximately 15% of the total protein content of the MuV antigen preparations used for vaccination.

Figure 4. Kinetics of optimized MuV growth. A cell factory seeded with Vero cells at and grown to ~90% confluency was infected with MuV Jones strain (M.O.I.= 0.0001) and grown at 33°C. Viral supernatants were collected and titered at the days indicated. Titer was measured in $TCID_{50}$ units/mL.

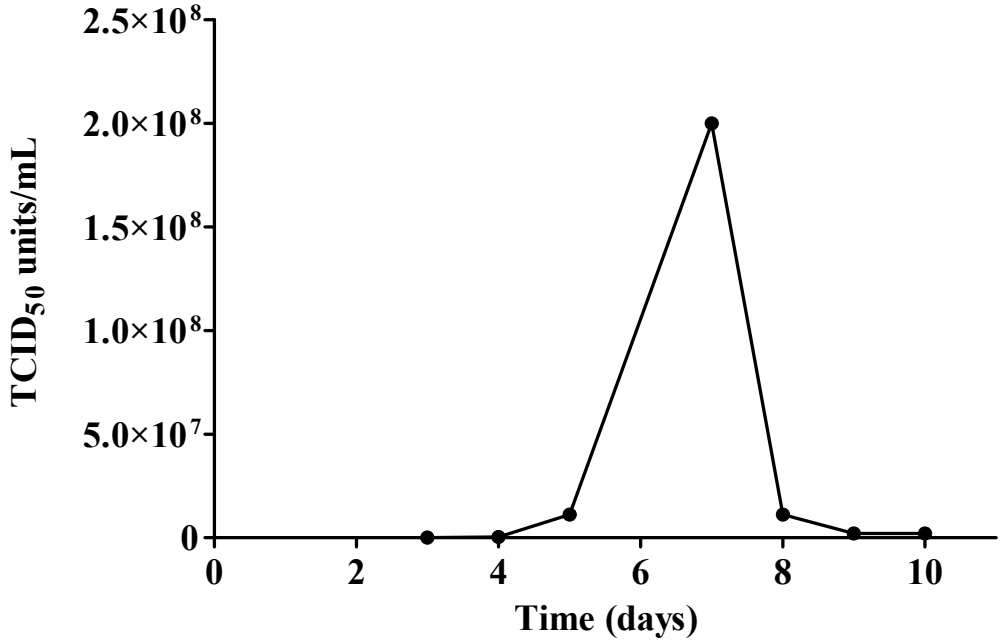
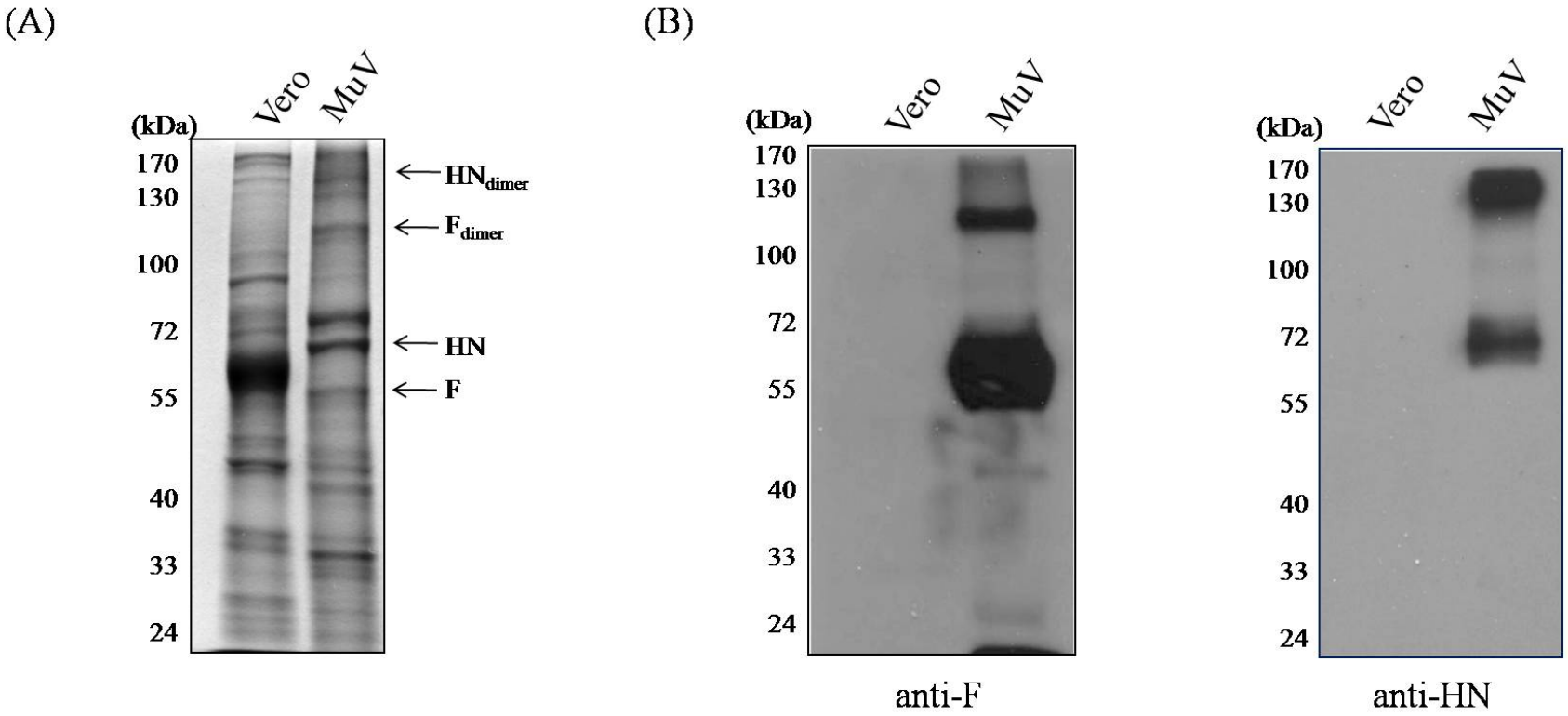


Figure 5. Characterization of MuV antigen preparation.

(A) Twenty-five (25) µg of MuV antigen and Vero cell extract preparations were run on a 7.5% polyacrylamide denaturing gel and stained overnight with Coomassie-Blue. A standard protein ladder (indicated in kDa) was run in parallel and used to interpolate molecular weights of individual proteins within antigen and cell-based preparations based on relative migration distance. (B) Western blot analyses were performed and demonstrated the presence of F and HN proteins within MuV antigen preparations.



3. Generation of recombinant HN and F genes to be placed into an expression system

MuV HN and F ORFs were cloned out of their native pTM1 plasmids. Agarose gel electrophoresis analysis of amplified sequences confirmed a successful amplification process (Figure 6). Amplification of the F-containing plasmid demonstrated the presence of a strong band at the predicted fragment length of ~1.6 kb. Multiple bands of variable intensity were seen upon amplification of the HN-containing plasmid. This was somewhat surprising as the HN primers were not specific for any other region within either the HN or pTM1 plasmid sequences, indicating that the primers were not 100% specific. Still, a strong band was observed at ~1.7 kb, in agreement with the predicted length of the HN fragment.

Amplified HN and F sequences were placed into the pCR®/GW/TOPO® vector (Figure 7A), a plasmid of ~2.8 bp in length. To confirm that the sequences had been inserted properly into the vector, EcoRI restriction digest was performed on both clones (Figure 7B). As demonstrated by the pCR®/GW/TOPO® vector map, two EcoRI sites exist within the plasmid, on either side of the gene insertion site. For the F gene, no internal EcoRI sites exist, therefore one would expect EcoRI digestion to yield two bands of ~2.8 kb and ~1.6 kb. These predictions were found to be true (Figure 7B). The HN gene contains one internal EcoRI site (at bp = 538), and so digestion should give three bands of ~2.8 kb, ~1.2 kb, and ~0.5 kb. Restriction digest of HN-containing pCR®/GW/TOPO® plasmids demonstrated the presence of a strong band at ~2.8 kb and a fairly faint band at ~1.2 kb (the 0.5 kb fragment ran off the gel and was therefore not visualized). To

Figure 6. Cloning MuV F and HN genes. F and HN ORF were removed from pTM1 plasmids and RT-PCR amplified to generate fragments of predicted length (F = ~1.6 kb; HN = ~1.7 kb). M denotes the HindλIII marker.

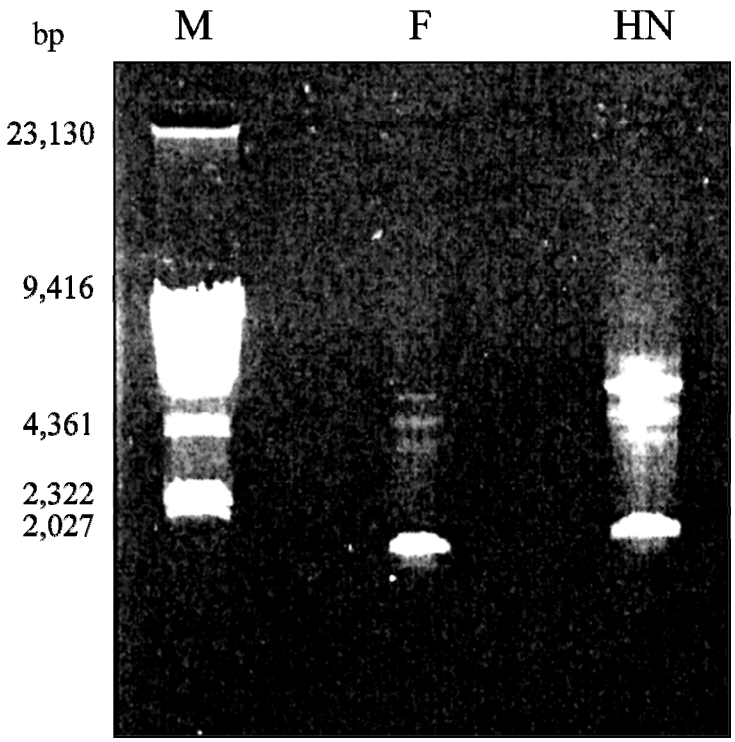
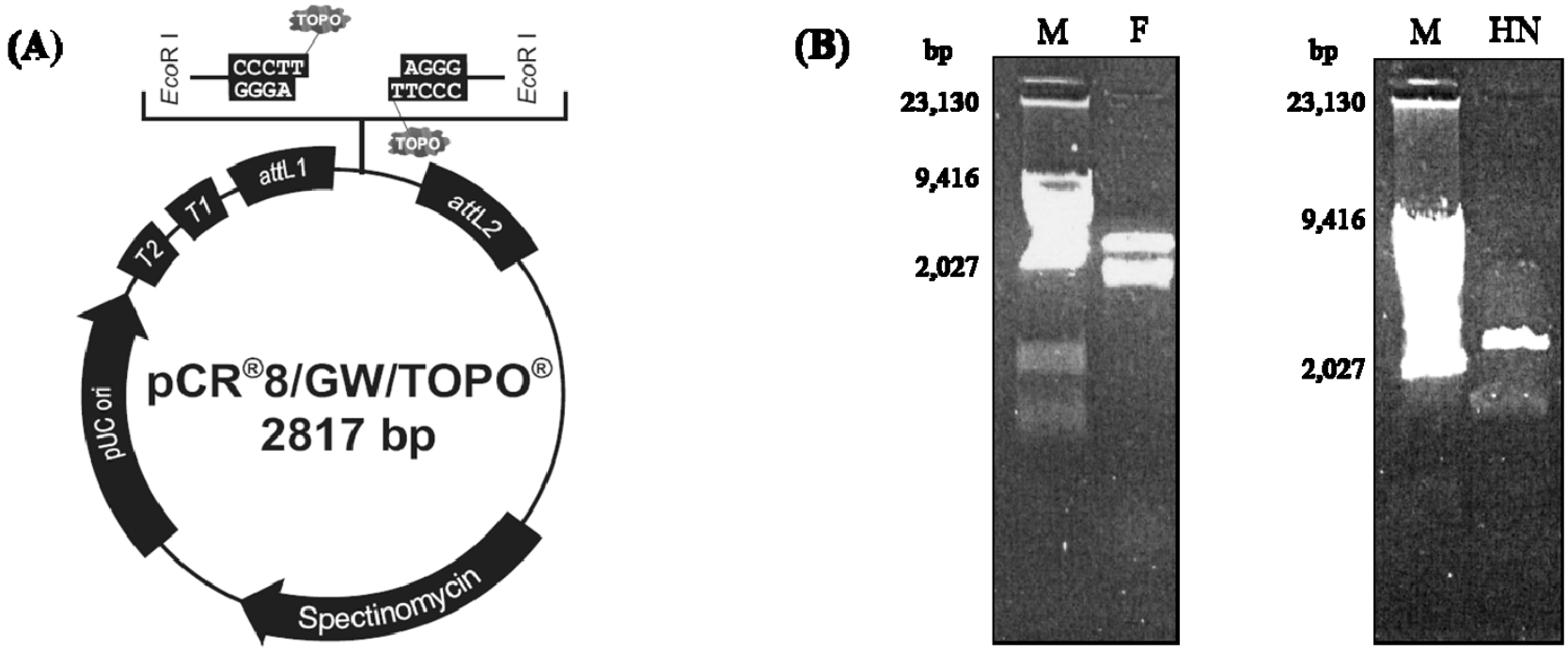


Figure 7. Restriction digest of cloning vector containing MuV F and HN genes.

(A) Plasmid map of pCR®/GW/TOPO® vector. Genes destined for this vector are inserted into the plasmid at the TOPO sites indicated, mediated by the topoisomerase reaction. (B) Restriction digest analysis of pCR®/GW/TOPO® vector containing F and HN ORFs. M denotes the HindλIII marker.



support the restriction digest results, automated sequencing confirmed the appropriate size and orientation of the inserted genes, and demonstrated that no mutations had occurred within the gene sequences.

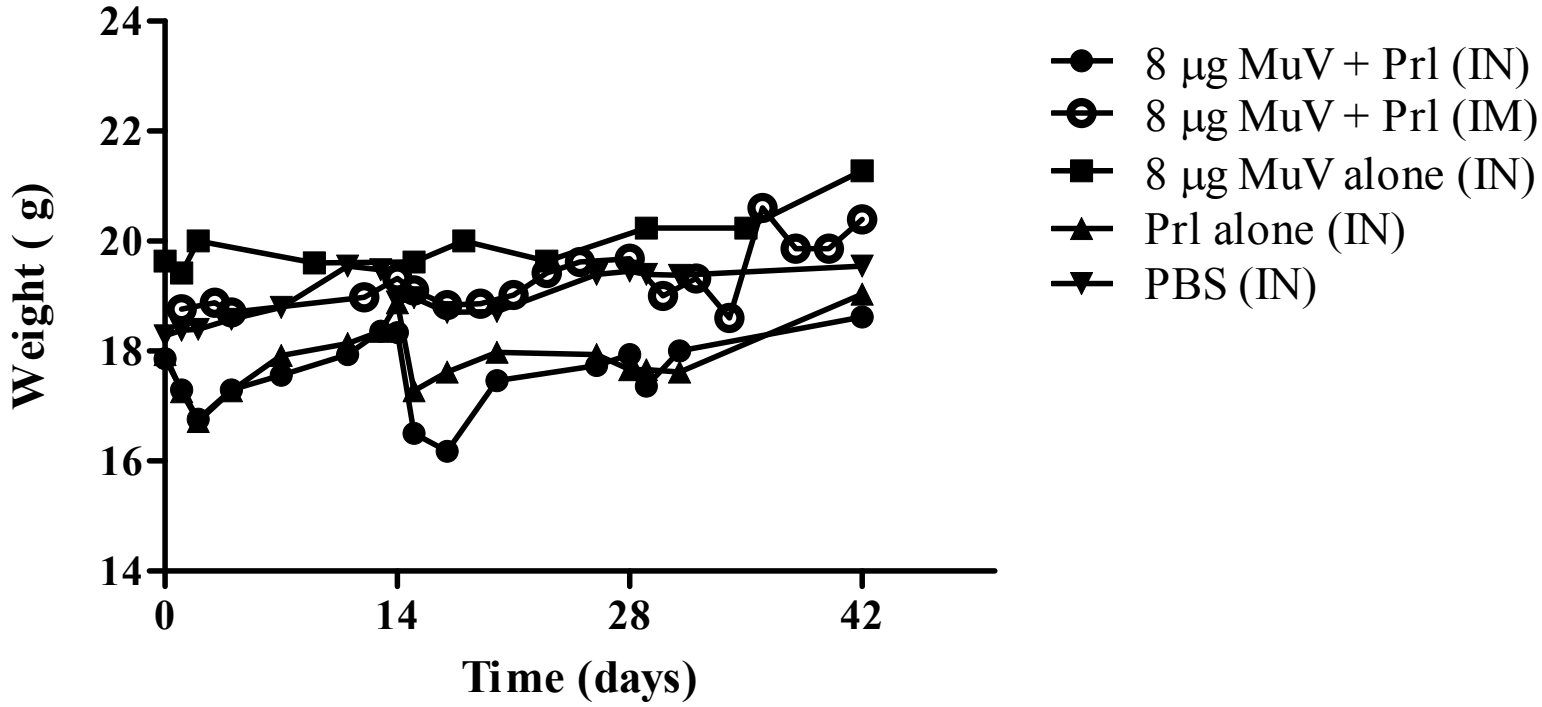
4. MuV-Prl vaccines were well-tolerated by animals

Overall, the vaccines were well-tolerated. Small behavioural changes (hunched posture, erect fur) and minor weight fluctuations were observed (≤ 15% total body weight) in animals receiving any MuV-Prl or Prl alone formulations (Figure 8). These effects lasted no longer than three days post-vaccination, at which point behaviour returned to normal and weight no longer fluctuated.

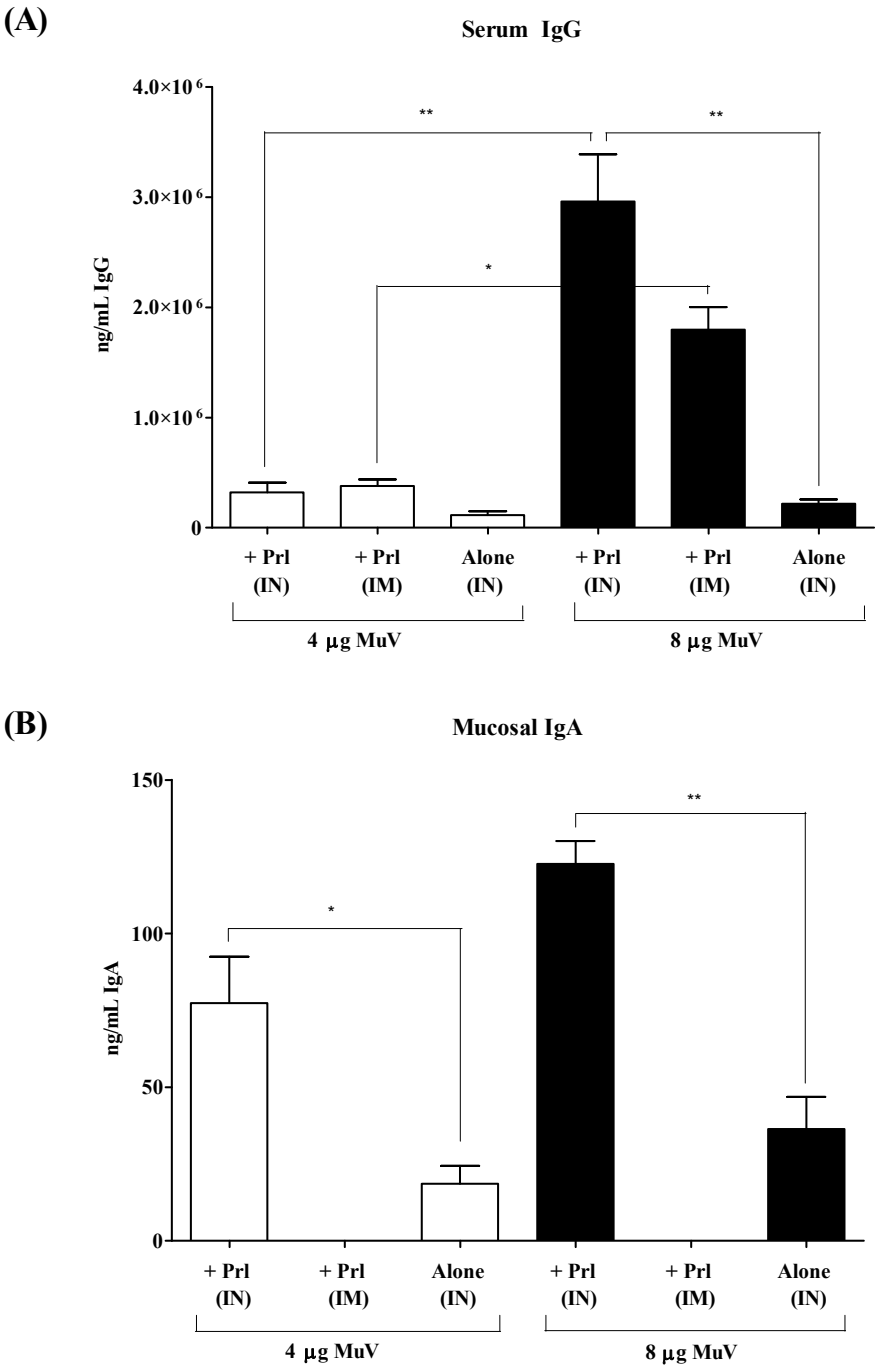
5. MuV-Prl vaccines elicit stronger serum IgG and mucosal IgA than MuV alone

MuV-specific serum IgG antibodies were detectable in animals after two doses of MuV-Prl, at both 4 μg and 8 μg MuV antigen (data not shown). In the three dose study, an additional dose of MuV-Prl increased IgG levels in the case of 8 μg MuV-Prl (by approximately two-fold), but not for 4 μg MuV-Prl as the levels remained constant at either two or three vaccine doses (data not shown). Three doses of 8 μg MuV-Prl formulations elicited a stronger serum MuV-specific IgG response than three doses of 4 μg MuV-Prl vaccines for both intranasal and intramuscular administration (intranasal: 2.96×10^6 ng/mL vs. 3.20×10^5 ng/mL, $p < 0.01$; intramuscular: 3.76×10^6 ng/mL vs. 1.80×10^5 ng/mL, $p < 0.05$), demonstrating the response to MuV-Prl formulations to be antigen-

Figure 8. MuV-Prl vaccine safety. Weights were monitored over course of study. Only groups receiving highest dose MuV-Prl vaccines are shown in graph. Data are reported as the mean weight.



<u>Figure 9.</u> MuV-specific serum and mucosal antibody responses in animals after three vaccine doses. Serum and BAL samples obtained at study termination were assayed for levels of (A) MuV-specific total IgG and (B) IgA , respectively, by ELISA. Control groups (Prl, Prl + Vero, PBS; data not shown) had low (≤ 1000 ng/mL) or no levels of serum IgG and no detectable mucosal IgA. Data are shown as the mean ± s.e.m.. Statistical analyses were performed by non-parametric student's *t*-test (Mann-Whitney test). ***p < 0.01, *p < 0.05*.



concentration-dependent (Figure 9A). However, this concentration-dependency was not seen in antigen alone groups, as 8 μg MuV alone elicited a similar level of serum IgG to that induced by 4 μg MuV alone (2.15×10^5 ng/mL vs. 1.12×10^5 ng/mL; no statistically significant difference). The presence of Prl in vaccine formulations greatly enhanced the IgG response for 8 μg MuV groups (8 μg MuV-Prl vs. 8 μg MuV alone; 2.96×10^6 ng/mL vs. 2.15×10^5 ng/mL, $p < 0.01$), but this was not the case for 4 μg MuV formulations (3.2×10^5 ng/mL vs. 1.12×10^5 ng/mL, no statistically significant difference). Similarly, with the 8 μg MuV formulations, intranasal administration seemed to enhance the MuV-specific IgG responses compared to intramuscular administration (2.96×10^6 ng/mL vs. 1.80×10^6 ng/mL, respectively), though this difference was not found to be statistically significant. This effect was not seen for 4 μg MuV intranasal formulations, as both vaccination routes generated similar IgG levels (3.20×10^5 ng/mL vs. 3.70×10^5 ng/mL, for intranasal and intramuscular administration respectively) (Figure 9A). Control groups (Prl, Prl-Vero, PBS, non-vaccinated animals) elicited very low or no serum MuV-specific IgG. These data demonstrate that three doses of the 8 μg MuV-Prl vaccines administered intranasally induce a greater serum MuV-specific IgG response than MuV antigen alone. This response was concentration-dependent and, surprisingly, was greater than the response induced by intramuscular administration.

In general, mucosal MuV-specific IgA titers were much lower than serum IgG levels (Figure 9B). MuV-specific mucosal antibodies were detected after two of both 4 μg and 8 μg MuV-Prl doses, though increased by approximately two-fold with the addition of a third dose (data not shown). Three doses of MuV-Prl

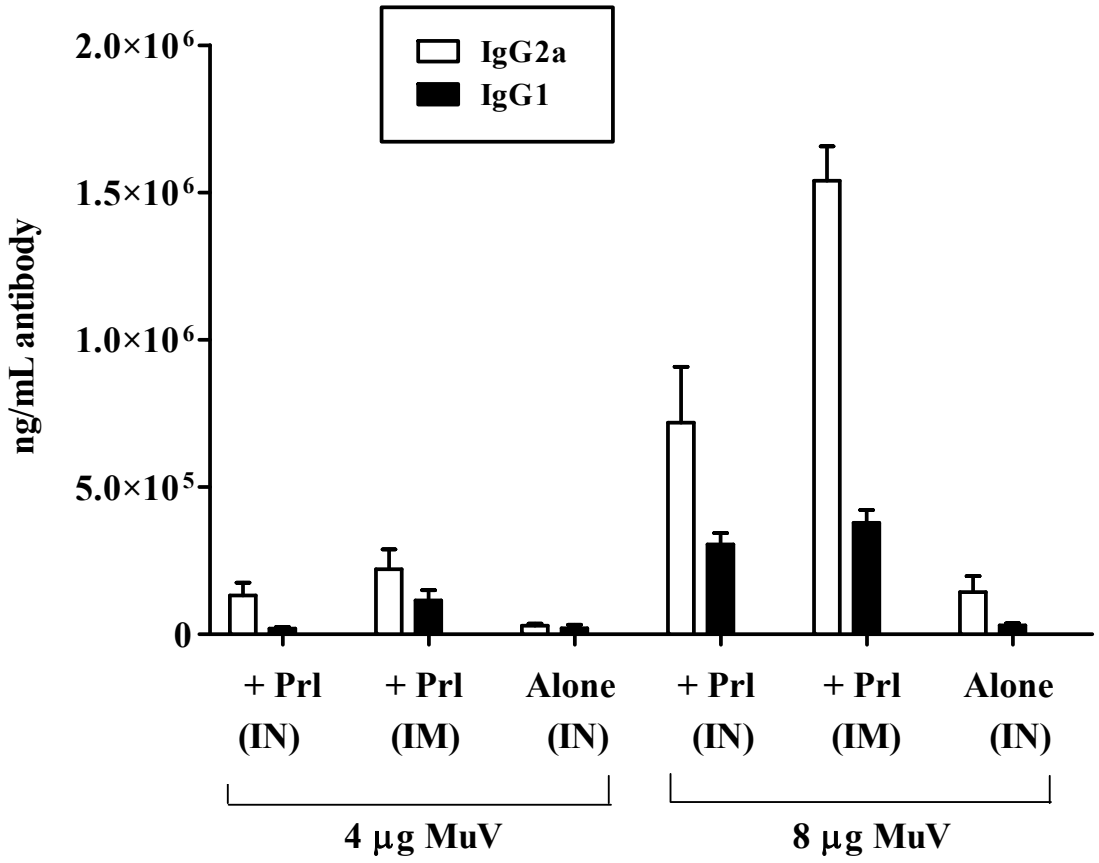
vaccines administered intranasally elicited significantly higher IgA levels than MuV alone, for both the 4 μg (77 ng/mL vs. 19 ng/mL, respectively; $p < 0.05$) and 8 μg antigen groups (123 ng/mL vs. 36 ng/mL, respectively; $p < 0.05$). Unlike serum IgG, mucosal IgA responses were not as strongly influenced by the amount of MuV antigen within the formulation, as the differing levels of IgA generated by 4 μg and 8 μg were not statistically significant (for both MuV-Prl and MuV alone). As expected, no mucosal IgA was detected in animals receiving intramuscular vaccination. Control groups (Prl, Prl-Vero, PBS, non-vaccinated animals) elicited very low or no MuV-specific mucosal IgA. These results demonstrate that intranasal administration of three doses MuV-Prl vaccines can elicit mucosal MuV-specific IgA greater than MuV antigen alone.

6. Immune environment generated by MuV-Prl vaccines

6.1. MuV-Prl vaccines favour IgG2a production

Typically, IgG2a isotypes have been associated with the Th1 arm of the immune response, while IgG1 antibodies tend to be indicative of a Th2 environment (83). Ig isotype analysis can therefore provide clues whether individual formulations tended to induce a biased immune microenvironment. At the lower MuV antigen concentration studied (4 μg MuV alone), and in the absence of Prl, IgG2a levels were slightly higher than IgG1 levels (2.95×10^4 ng/mL vs. $2.07 \times 10^4 \times 10^4$ ng/mL, respectively) (Figure 10). The addition of Prl to the vaccine tended to generate a greater bias towards induction of IgG2a following both intranasal and intramuscular administration (intranasal: 1.32×10^5 ng/mL vs. 2.00×10^4 ng/mL, for IgG2a and IgG1 respectively; intramuscular:

Figure 10. Serum IgG isotypic analysis in animals given three vaccine doses. Levels of IgG2a and IgG1 were determined by ELISA in sera obtained on termination day. Control groups (Prl, Prl + Vero, PBS; data not shown) had very low or undetectable levels of antibody. Data are reported as the mean ± s.e.m..



2.21 x 10^5 ng/mL vs. 1.15 x 10^5 for IgG2a and IgG1 respectively). A different trend was seen if the MuV content was increased. Higher levels of MuV antigen seemed to bias towards the generation of IgG2a antibodies over IgG1, even in the absence of Prl (8 μg MuV alone). The addition of Prl to high antigen formulations further enhanced this trend.

6.2. Splenocytes from MuV-Prl vaccinated animals generate a broad antigen-specific cytokine response

We measured cytokine levels in splenocyte supernatants in response to MuV antigen re-stimulation to determine the cytokine profiles generated by the different vaccines (Table 2). The presence of Prl in MuV vaccines generated a much different cytokine/chemokine profile than vaccines formulated only with MuV antigen. In this regard, the presence of Prl in the vaccines tended to favour high production of Th0/Th1-like and pro-inflammatory cytokines/chemokines, particularly IFN-γ, IL-2 and MIP-1α. Other characteristic cytokines involved in the inflammatory cascade (e.g.: TFN-α, IL-1α, IL-1β, GM-CSF) seemed to be favoured by MuV-Prl vaccines, though to much lower levels. Interestingly, IL-10 and IL-9, two cytokines important in anti-inflammatory and Th2-like responses, respectively, were both also favoured by the presence of Prl in the vaccines compared to MuV alone, though again to lower levels than those observed for the Th0/Th1-like and pro-inflammatory cytokines/chemokines.

The presence of MuV alone in formulations had a strikingly inhibitory effect on the production of certain cytokines/chemokines important for inflammatory processes (IL-6, MIP-1α, RANTES) and generation of Th1-type

Table 2. Cytokine secretion responses from splenocytes in the presence of MuV stimulation. Pooled splenocytes obtained at study termination from animals receiving three doses of vaccines (all intranasally) were stimulated with 1 µg/mL crude MuV antigen (antigen-specific stimulus). Supernatants were sampled at 72 hours post-stimulation and assayed for cytokine levels by multiplex ELISA. Cytokine values obtained for PBS groups have been subtracted from MuV + Prl, MuV alone and Prl alone groups (shown in table), and are shown in pg/mL. MuV-containing vaccines had 8 µg MuV content.

	MuV + Prl (pg/ml)	**MuV alone (pg/ml)**	**Prl (pg/ml)**
Th0/Th1-like and pro-inflammatory cytokines			
IFN-γ	280	67	44
TNF-α	16	0	2
IL-2	1628	113	-29
IL-1α	15	0	13
GM-CSF	69	9	16
IL-1β	25	15	17
IL-3	2	0	0
IL-12p40	-212	-450	120
IL-6	40	-44	-42
Th2-like/regulatory cytokines			
IL-4	4	6	0
IL-5	9	79	-14
IL-9	89	1	0
IL-10	21	-2	1
Chemokines			
MIP-1α	364	-92	326
MCP-1	29	33	5
RANTES	-236	-812	326

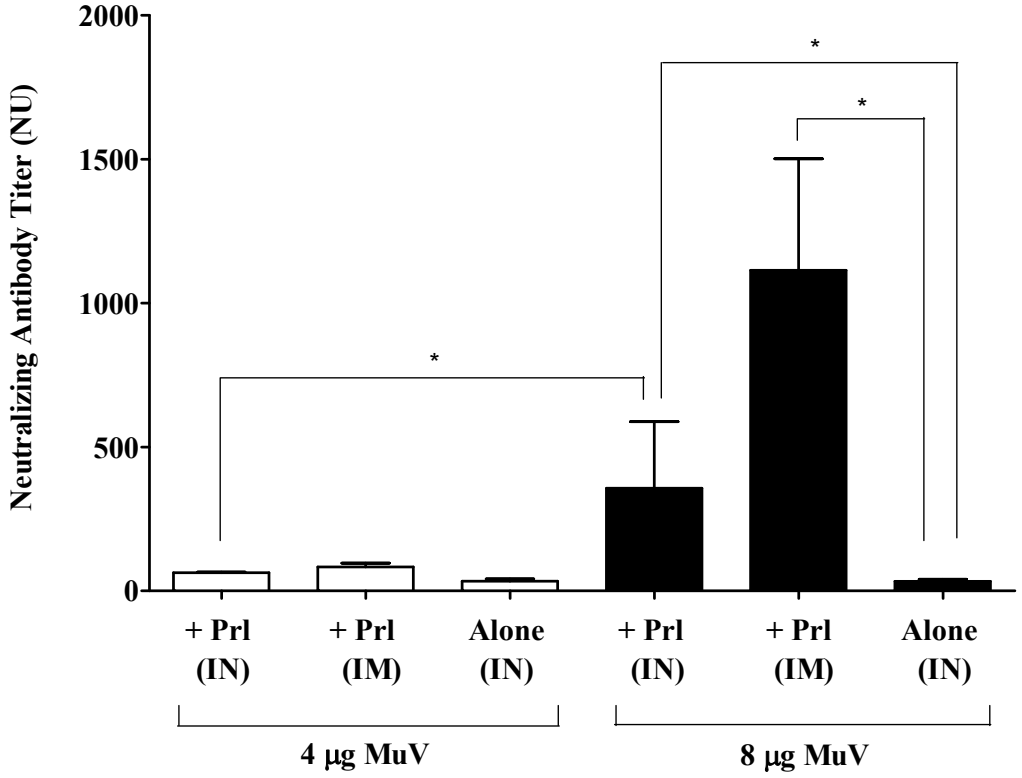
immunity (IL-12p40). In fact, the only cytokine that seemed to be up-regulated due to MuV-alone vaccination was IL-5, a cytokine that is particularly well-known for its involvement in Th2-type immune responses. Contrary to MuV-Prl vaccines, it would therefore seem that MuV alone formulations tended to favour a more Th2-skewed cytokine response.

7. MuV-Prl vaccines elicit greater serum neutralizing antibody levels than MuV alone

Three doses of 8 μg MuV-Prl vaccines, administered both intranasally and intramuscularly, generated significantly higher serum neutralizing antibody responses (measured in arbitrary neutralizing units, NU) than their three dose 4 μg MuV-Prl counterparts (intranasal: 357 NU vs. 63 NU, $p < 0.05$; intramuscular: 1115 NU vs. 83 NU, $p < 0.05$) (Figure 11). However, for MuV-alone formulations, both high and low antigen doses yielded similar responses (33 NU vs. 32 NU, respectively; no statistically significant difference). The 8 μg MuV-Prl formulations administered intranasally induced significantly higher levels of neutralizing antibody than 8 μg MuV alone (357 NU vs. 32 NU, respectively; $p < 0.05$), a result which was not observed with the 4 μg formulations (63 NU vs. 33 NU, respectively; no statistically significant difference). At the lower dose of MuV-Prl, the route of administration seemed to have no statistically significant effect on the neutralizing antibody response. This was not the case at higher dose MuV-Prl, at which intramuscular administration elicited a much higher serum neutralizing antibody response than intranasal, though high titers were still achieved following intranasal vaccination (1115 NU vs. 357 NU, for

intramuscular and intranasal administration, respectively). Two doses of MuV-Prl vaccines elicited MuV-specific serum neutralizing antibodies, but only at 8 μg MuV antigen level (72 NU; data not shown in Figure 11). No neutralizing antibodies were detected in the serum of animals receiving two doses of 4 μg MuV-Prl. No mucosal neutralizing antibodies were detected in lung secretions. These results demonstrate that the MuV-Prl formulations used in this study are able to elicit strong serum neutralizing antibody responses, both intranasally and intramuscularly, yet do not generate detectable levels of neutralizing antibodies in the respiratory secretions.

Figure 11. **MuV-specific neutralizing antibody titers in animals given three vaccine doses.** 2,000 $TCID_{50}$ units/mL of MuV were added to serial dilutions of sera obtained from animals on study termination and assayed for their levels of anti-MuV neutralizing antibody. Neutralizing antibody levels in control groups (Prl, Prl + Vero, PBS) were below the detection limit of the assay (titer < 14). The neutralizing titer (measured in neutralizing units; NU) is defined as 1/[serum dilution] that is fully capable of inhibiting virus replication. Data are represented as mean ± s.e.m.. Statistical analyses were performed by non-parametric student's *t*-test (Mann-Whitney test). *$p < 0.05$.



DISCUSSION

The recent outbreaks of mumps in a wide range of developed and developing world countries reveal the need for constant improvement in both vaccines and vaccine programs. Use of the current vaccines over the last 20 to 30 years has not been adequate to fully protect any population from the threat of MuV. Also, with the introduction of vaccines, the epidemiology of the disease has shifted to older age groups. This latter change is of some concern as the complications of infection are often more serious when the illness is contracted after childhood. Despite their overall stellar track-records to date, the currently licensed MuV-containing vaccines have several important limitations, and the development of better vaccines is still desirable. We felt that an inactivated MuV vaccine that could be administered intranasally would be a good candidate to circumvent many of the problems associated with current vaccines.

We wished to generate MuV vaccines based on both whole split virions and recombinant antigens. Split virus vaccine formulations require large numbers of virions which can be very expensive. It was therefore imperative to demonstrate that large-scale growth of MuV was possible. This was not at all trivial, as MuV is a 'finicky' virus that is notoriously difficult to grow to high titers (84). The optimizing of growth conditions for MuV was of paramount importance to meet scalability requirements. Previous work in our laboratory had focused on a 3.2 litre bioreactor for MuV growth, however, viral titers were generally poor ($\leq 2 \times 10^5$ $TCID_{50}$ units/mL) and titers rapidly decreased after three to four days, limiting yield. Furthermore, several of the subsequent purification steps used to produce viral antigen from virions were dependent on a threshold

concentration of virus particles, and our initial inability to achieve high enough concentrations of virions had a serious negative effect on downstream processing. The initial lack of suitable amounts of antigen was a serious limitation on the number of vaccine formulations we were able to test in animals.

Our search for alternate strategies led us to assess multi-level 'cell factories'. The CellStack 10-level flask had been used by the Ward laboratory for large-scale antigen production for other viruses (MeV, PIV3), therefore it seemed logical to test MuV growth kinetics in this system. Using this platform, and employing similar growth conditions to those used for other paramyxoviruses, we were eventually able to achieve virus titers as high as 2×10^8 $TCID_{50}$ units/mL, a value almost three logs higher than in the bioreactor. Furthermore, we were able to sustain the infection for ten days, with viral titers exceeding 1×10^6 $TCID_{50}$ units/mL for five out of ten collection days. This was an exciting accomplishment, considering MuV is notoriously difficult to grow to high titers. Success in optimizing this initially limiting step was pivotal to our ability to carry out the subsequent animal studies.

Commercial-scale production of virions using stirred tank bioreactors has been reported for various viruses, such as adenovirus and rabies virus (85;86). Indeed, several previous members of the Ward lab had successfully applied this technology to other viruses in the same genus as MuV, indicating the possibility of paramyxovirus growth in this system (78). It is therefore unclear at the present time why high-yield growth in the bioreactor could not be achieved for MuV. Perhaps MuV is intrinsically unsuited for growth on microcarrier beads in the stirred tank system, as the conditions are much different than in conventional

tissue culture flasks and scale-up is often difficult (87). However, it is noteworthy that the growth parameters chosen were based on both past experiences for large-scale culture of other paramyxoviruses as well as experience with MuV in small-scale tissue culture flasks. Even if MuV cannot be grown in larger-scale bioreactors, multi-level cell factories are widely used in commercial vaccine settings (e.g.: Sanofi Pasteur Inc.; personal communication with Dr. L. Barreto).

Although whole split virions are extensively used in vaccines directed against other pathogens (e.g.: influenza virus, hepatitis A virus, rabies virus), this approach has several unavoidable limitations. First, even when viral growth is fully optimized, the generation of large numbers of virions is both labour intensive and expensive. This approach can also be dangerous when wild-type pathogens are used (e.g.: poliovirus, rabies virus). Once the virions are produced, it can also be technically very difficult to eliminate all traces of the host cells and to precisely determine the virus-specific protein content within the eventual antigen preparation. In many cases with current, licensed vaccines, antigen 'purity' is determined using remarkably crude techniques (e.g.: Western blotting, densitometry). The use of recombinant protein technology for vaccine development would alleviate many of the problems associated with cell-based antigen production. Our primary goal in the work presented here was to establish 'proof of principle' for an inactivated mumps vaccine based on a split-virion antigen. However, a key long-term goal of the laboratory was to produce a more 'modern' MuV antigen through recombinant technology. In parallel with the split-virion work, we therefore sought to generate recombinant MuV antigens that could eventually be used in a recombinant MuV-Prl vaccine. After an exhaustive

search, we identified a group at the US Food and Drug Administration (led by Dr. Kathy Carbone) that had already generated plasmids containing each individual MuV gene, including the HN and F surface glycoproteins (note: at the time we contacted them, they had not published any of their work with MuV). We have successfully cloned these genes out of their native plasmids and into a holding vector with proper orientation and reading frames. Future work will place these genes into an appropriate expression vector suitable for large-scale production.

Producing large-scale quantities of recombinant MuV surface glycoproteins will not be trivial however, and will require the use of an appropriate expression system. MuV HN and F proteins are subject to a range of post-translational events, such as glycosylation, cleavage (in the case of F) and multimerization (1;88). These events are very likely to be important for the retention of antigenicity, and can vary greatly depending on the expression system used. It is reassuring that the limited number of expression studies performed to date targeting the MuV HN and F proteins suggest that it is possible to generate proteins that maintain critical immunologic and functional characteristics (e.g.: recognizable by Western blot, able to induce syncytium formation) (89;90). However, it is still not clear to what extent recombinant surface glycoproteins must resemble their native protein homologues to generate protective immune responses *in vivo*. Work performed with influenza A virus and MeV has demonstrated that proper glycosylation is not absolutely required to generate functional immunity in mice (91;92). Another challenge will be to demonstrate scalability of recombinant surface glycoprotein production, once they have been expressed successfully. To date, large-scale production of MuV proteins has only

been demonstrated for the NP protein. To our knowledge, no group has successfully produced large quantities of either recombinant MuV HN or F proteins (93;94). As a result, there are currently many uncertainties with regards to the generation of appropriately immunogenic MuV HN and F proteins using recombinant technology. Important consideration of all these factors will be required to choose an expression system capable of generating large-scale quantities of immunologically relevant proteins.

A recombinant vaccine consisting of only one or two viral components would likely limit the 'breadth' of the immune response elicited (i.e.: a reduced number of epitopes targeted). The resulting 'narrow' response would not necessarily be desirable, given the propensity of RNA viruses to mutate (95). It is generally accepted that protective antibody responses against MuV are generated against the HN protein, and to a lesser extent, the F protein (10-13;96;97), hence the choice of these proteins for our candidate recombinant MuV vaccine. However, neither the precise role nor the nature of cellular immunity in protection against MuV (or any paramyxovirus) has been established (1;5). If cellular immunity is absolutely necessary for protection, such protection may be based on epitopes that would perhaps be absent in a vaccine containing only recombinant HN/F proteins. Another concern in creating a vaccine that would be predicted to elicit a narrow immune response would be the possibility of escape mutations and genotype replacement, as has occurred with vaccines targeting *Streptococcus pneumonia* (for this pathogen, it was serotype replacement) (98;99). While such an 'escape' from vaccine-induced protection is theoretically possible, escape-mutants have not been observed for MuV with more than 40 years of use of the

current whole-virus based vaccines, which have been effective in preventing infection by heterologous strains. In the specific example of the recent US epidemics, the vaccine strain (belonging to genotype A) was effective in controlling infection by the epidemic-causing strain (belonging to genotype G) (31;42). Of course, it would be desirable to ensure that the proteins used in the recombinant vaccine generate protective immunity that is effective against a variety of heterologous MuV strains.

One major drawback with current MuV vaccination strategies is the use of needles for vaccine administration. There are many reasons to pursue alternative administration strategies over current needle-based vaccines: (a) needle-stick injuries have been extensively documented, prompting safety concerns for those administering vaccines (100); (b) improper re-use of single-use needles and syringes has become a problem in regions of the world where appropriate waste-disposal programs have not yet been implemented (101-103); (c) needle-based vaccines are harder to administer than some alternatives, and therefore require better trained personnel for safe and effective delivery (102;103); and (d) needle administration is painful to the recipient, and could be a cause for reduced vaccine acceptability/uptake due to this important physical and psychological effect (104-106). The need for alternative strategies, needle-free strategies has recently become a high-priority issue for various international health authorities and foundations, highlighted by the implementation of programs such as the Measles Aerosol Project (World Health Organization) and the Program for Appropriate Technology in Health (Bill and Melinda Gates Foundation) (107;108).

An appealing needle-free alternative is intranasal administration of vaccines. Nasal vaccination is quick and easy to administer, non-invasive, requires less expertise to administer properly than needle-based vaccines, and causes little distress to the recipient (102;109). Furthermore, vaccines administered to this compartment come in direct contact with a rich immunologic environment, capable of mounting robust immune responses to incoming antigen (109;110). Since MuV is a respiratory virus, it typically comes into first contact with cells of the upper respiratory tract. As previously discussed, the body's ability to fight MuV infection and prevent subsequent re-infection depends on the interplay between various cell-mediated and humoral immune responses, both at the site of infection and throughout the body. It is therefore logical to consider nasal vaccination for MuV-containing vaccines, as it would mimic the natural route of infection, and hopefully be more likely to generate relevant immune responses.

In our study, we sought to establish the use of intranasal vaccination as an alternative route of administration for MuV vaccination, using the Prl adjuvant-delivery system. Animals receiving intranasal formulations tolerated all vaccinations quite well, although both minor weight loss and small behavioural changes were observed in some animals in the days immediately following dosing. In future studies, it would be of interest to perform histopathological analyses to see if any damage is caused within the nasal compartment of the vaccinated animals. Our study has demonstrated that intranasal vaccination generates significant IgG and IgA responses in the blood and lung mucosa, respectively; in fact, in the case of serum IgG, higher levels of antibody were

generated by intranasal administration than by intramuscular administration. Vaccines administered intranasally were also able to generate strong cytokine responses by splenocytes in the presence of MuV antigen. Most importantly perhaps, intranasal administration generated a strong systemic neutralizing antibody response in mice. Overall, intranasal administration of MuV-containing vaccines in this study resulted in the production of strong humoral and cell-mediated immune responses, both locally and systemically, suggesting that intranasal administration is a promising route for MuV vaccination.

All currently available MuV vaccines use live-attenuated viruses, which are very effective at eliciting protective virus-specific immune responses without causing disease. Mentioned briefly in the introduction, however, these highly successful vaccines still have important drawbacks. These living vaccines cannot be used in immunocompromised individuals, due to the elevated risk of uncontrolled replication and severe morbidity, even death. Vaccination using live-attenuated viruses can also be less effective in very young individuals due to the persistence of maternal antibodies. The current compromise used to address this problem is to delay vaccination until maternal antibodies disappear (typically 9-12 months of age). The unavoidable result of this strategy is that virtually all young infants are susceptible to mumps for several months prior to 'routine' immunization. As the timing of the disappearance of these antibodies varies widely, the 'window of susceptibility' for each child is also highly variable. Also, the adverse events associated with the live-attenuated MuV-containing vaccines can be severe (42). Though rare, these side effects are usually similar to natural MuV symptoms (e.g.: aseptic meningitis, post-infectious encephalomyelitis,

sensorineural deafness, etc.), and are due to the live-attenuated nature of the vaccines (42;67). Switching to an inactivated MuV component would therefore be of great interest.

A switch from a live-attenuated vaccine to an inactivated formulation for reasons of safety would not be unprecedented for vaccine programs. This was precisely what occurred in the poliovirus vaccination campaign in virtually all developed world countries in the 1990's. It had long been known that the live-attenuated vaccine caused a number of vaccine associated paralytic poliomyelitis (VAPP) cases. As wild-type poliovirus slowly disappeared and vaccine coverage in the range of 90-95% was achieved in many wealthy countries, the safety concerns associated with the live-attenuated vaccine grew in prominence and vaccine health authorities recommended a switch from the live-attenuated oral poliovirus vaccine (OPV) to the inactivated poliovirus vaccine (IPV) (111). It seems very likely that an inactivated MuV vaccine will be needed in the final stages of mumps eradication for precisely the same reasons. Though MuV is not currently a priority for eradication programs due to the need to focus on other more life-threatening diseases (e.g.: measles), MuV eradication is theoretically possible using currently available vaccines (112). To achieve this, however, it is essential that vaccine uptake remain high (>90%). Currently, the risk of contracting disease outweighs the rare occurrence of developing a vaccine-induced severe adverse event, so the risk-benefit ratio still falls in favour of the vaccine. However, in a situation of lower disease incidence (e.g.: final phases of disease eradication), the chances of developing vaccine-associated adverse events might one day become greater than the risk of contracting the disease. Vaccine

acceptability would likely plummet in this scenario, causing vaccine coverage to fall into dangerously low levels. The availability of an inactivated MuV vaccine would help to ensure that high vaccine uptake is maintained.

The decision to use a non-living antigen posed new challenges however, namely that such antigens have the inherent characteristic of being poorly immunogenic, and thus require an adjuvant to enhance immunogenicity (113). Very few adjuvants have been licensed for use in humans to date and regulatory authorities, conservative at the best of times, have reason to be particularly careful with adjuvants that will be applied via the nose. The risk of such vaccines was highlighted by the recent removal from the market of a nasally administered inactivated influenza vaccine that was found to have an increased risk of causing Bell's palsy compared to the placebo. It was later discovered that the facial paralysis was most likely due to the use of *Escherichia coli*-derived lymphotoxin as an adjuvant (102;114). Such concerns made it imperative for us to determine if the Prl-adjuvanted nasal MuV vaccine candidate caused any serious adverse events in our model. Animals receiving formulations containing Prl showed signs of hunched posture, minor weight loss and erect fur, indicating a systemic reaction to this vaccine component. Such observations may warrant further investigation to determine the overall safety of Prl-containing vaccines. Overall, however, the vaccines were well-tolerated, which supports the safety observations for other Prl-adjuvanted virus vaccines in mice (71;77;115;116). In fact, many vaccines adjuvanted with proteosome-based adjuvants (proteosome, Prl) have been well-tolerated in various animal and human trials (117-120). Both the Proteosome and Prl particles have been tested in humans (in the context of

vaccination against of influenza and *S. flexneri*, respectively). Similar side effects were observed following both types of vaccine, and were limited to minor adverse events such as rhinorrhea, nasal congestion, low-grade fever, and mild headache (121-123). Our study therefore supports the notion of Prl as a well-tolerated adjuvant for nasal vaccine delivery.

Once we had established that the MuV-Prl vaccine was well-tolerated in our model, we then needed to determine whether or not it enhanced the immunogenicity to our inactivated antigen. As previously mentioned, the principal role of an adjuvant is to increase the potency of immune responses to the target antigen(s). In fact, the word 'adjuvant' derives from the Greek word '*ajuvare*', meaning 'to help' (113). Many different experimental adjuvants have been developed, though not many have made it as far as the clinical trial stage, and even fewer have been accepted for use in human. Much work has been done to determine the mechanisms by which experimental adjuvants exert their immune-potentiating effect. These studies, however, have mainly focused on intramuscular and subcutaneous routes of vaccination, as these are the most commonly-used immunization routes for current vaccines (124). There is in fact a great lack of information with regards to adjuvant biology within the nasal compartment. Given that the nasal delivery route would not necessarily be suitable for all adjuvants, we therefore needed to ensure that the adjuvant chosen for nasal administration in our study would be capable of enhancing MuV-specific immunogenicity.

Recently, the capabilities of Prl as a nasal adjuvant have been studied in various pre-clinical models (mouse, non-human primate) for vaccines targeting a

range of respiratory pathogens (71;77;78;116;125). In all models tested, Prl-based vaccines administered intranasally elicited potent pathogen-specific immune responses. The immunopotentiating effect of Prl as a nasal adjuvant in humans has not yet been studied, though work in rhesus macaques has demonstrated intranasal MeV-Prl vaccines to provide full protection against wild-type MeV challenge (126). Also, influenza vaccines adjuvanted with another Proteosome-based adjuvant administered nasally to humans in phase 1 clinical trials have demonstrated enhanced immunogenicity (increased hemagglutination inhibition and sIgA titers) compared to antigen alone (127;128). Given the success of Prl as a nasal adjuvant for other related viruses, it seemed logical that we would be able to generate enhanced MuV-specific immune responses by formulating our MuV antigen with the Prl adjuvant. Our study has demonstrated that, at higher antigen dose (8 μg), the presence of Prl within MuV vaccines elicited a much greater serum and mucosal antibody response than MuV antigen alone (as measured by ELISA). Unexpectedly, we found that, at high doses, MuV antigen was powerfully immunogenic even in the absence of adjuvant (although antibody titers were still lower than in the presence of Prl). The reasons for this striking immunogenicity of the MuV antigen at high dose are not yet fully clear. It is possible that other viral components contributed to this unexpected immunogenicity. For example, genomic material and viral replicative intermediates (double-stranded RNA) remaining within the preparation may have conferred immunogenicity to the antigen preparations by signalling through TLR-7 or TLR-3, respectively.

Interestingly, although antigen alone was immunogenic at high doses, it appeared as though the presence of adjuvant was required to generate high levels of serum neutralizing antibody. MuV antigen alone demonstrated quite poor capabilities in this respect. Unfortunately, the data obtained in this study do not allow us to determine whether or not the neutralizing antibody levels generated by our MuV-Prl formulations would be sufficient to protect against natural infection, as the correlates of protection against MuV are presently unknown and the mouse model does not allow us to perform challenge studies. However, it has been established that a neutralizing titer of ≥120 in humans is sufficient to provide full protection against MeV (129). Considering MeV is closely related to MuV, this raises the possibility that the neutralizing antibody levels achieved in this study (71-1280 NU (intranasally), 452-2560 NU (intramuscularly)) are sufficient to provide protection against wild type MuV infection. Overall, these results demonstrate the capacity of Prl to enhance MuV-specific immune responses when MuV-Prl formulations are administered intranasally in mice, indicating this adjuvant to be immunologically suitable for the nasal administration of a MuV-specific vaccine.

The ultimate goal of vaccination is to induce an immune response that is 100% protective, with minimal vaccine-associated adverse events; an ambitious target that is rarely achieved. Live-virus vaccines tend to generate immunity that is similar to natural infection, as the vaccine consists of replicating virus (61). In the case of paramyxoviruses, this protection is generally thought to be mediated by both cellular and humoral branches of the immune response (though antibodies alone have been shown to be sufficient for protection, and cell-mediated

immunity is thought to be absolutely required in a primary response) (5). Vaccines made of dead antigens with or without adjuvants, are unlikely to generate a balanced response, however. Indeed, killed vaccines for paramyxoviruses have had a particularly bad history(130). Recent reports have suggested a role for formalin-modified viral antigens in Th2-skewing (131;132) , and recent publications have confirmed that RSV antigen alone in the nose yields Th2-type responses (78). Most recently, the induction of low-avidity antibodies by inactivated antigens has been implicated in elevated risk for aberrant immune responses (133). As a result, careful attention must be paid to the Th1/Th2 balance as well as the functional characteristics of antibodies induced in the context of paramyxovirus vaccine development. We have not yet assessed antibody avidity in our MuV-Prl vaccination model.

Previously, Prl-based vaccines generated against MeV and RSV have demonstrated a balanced Th1/Th2 response, as assessed by IgG1/IgG2a isotype analysis and cytokine profiling in response to antigen-specific stimulation (76-78). Similar types of analyses were performed in our study to ascertain the type of immune environment created by MuV-Prl vaccines. In terms of IgG isotypes, surprising differences were discovered. In the MeV model, lower doses of antigen within MeV-Prl formulations tended towards the production of IgG1 over that of IgG2a, and increasing levels of antigen served to balance the IgG1:IgG2a response (77), indicating that lower levels of MeV antigen (≤ 3 μg) favoured a Th2-biased response. In our study, both MuV antigen levels tested tended to favour the production of IgG2a over IgG1. This trend was seen regardless of the presence of the adjuvant. However, presence of Prl within the formulations

served to further skew towards an IgG2a response, at both low and high antigen levels. It is possible that the antigen levels chosen were too high to observe the IgG1 bias seen with other, closely-related antigens. However, it is also possible that MuV antigen has an inherent tendency to favour IgG2a production over that of IgG1.

The reasons why MuV might tend bias towards IgG2a production are unclear. Increased IgG2a production at higher antigen levels might have been driven by the presence of greater amounts of F protein, as this protein has been demonstrated to favour IgG2a isotype production. However, it should also be noted that HN has been observed to favour IgG1 production (134). Considering the antigen preparations appeared to contain more HN than F protein (based on rough densitometric estimation of Coomassie stained gels), it is unlikely that high F protein content was the sole reason for this IgG2a skewing. It is also possible that other MuV components may have driven this skewed isotype response.

The observation that high levels of MuV antigen are capable of driving aTh1 response (at least in terms of antibody response) is of particular interest, since this is not what would be expected based on the observations of Bretscher *et al.* (135). This group found that high doses of antigen in the context of *Leishmania* infection tended to drive the generation of Th2-type immunity: exactly the opposite of what was found in the present study. It therefore seems likely that the 'direction' of immune deviation is not only driven by the amount of antigen, but also by the nature of the antigen.

We were also able to assess how the various vaccine formulations affected the 'profile' of the immune response generated by measuring the patter of

cytokines generated by splenocytes in response to MuV antigen. Similar analyses had been performed with Prl-based vaccines targeting other paramyxoviruses (RSV, MeV), using conventional ELISA that assessed only a small number of cytokines, including IFN-γ, IL-5, IL-2, TNF-α, and IL-10 (77;78). In the case of MeV-Prl vaccination (where only IFN-γ was analyzed), strong IFN- γ responses were elicited upon exposure to MeV (77). A panel of cytokines was assessed in RSV-Prl vaccinated animals, in which strong IFN-γ, IL-2, TNF-α and IL-10 responses were observed following RSV-Prl vaccination (78). Unlike the earlier Prl studies, we used a multiplex ELISA kit developed by Quansys Biosciences, to screen for 16 different cytokines simultaneously. These cytokines can be roughly placed into three groups: Th0/Th1-like and pro-inflammatory cytokines, Th2-like/regulatory cytokines, and chemokines. In general, the presence of Prl in MuV-containing vaccines tended to increase cytokine secretion response to MuV antigen re-stimulation, compared to MuV alone. The cytokine group that seemed to be preferentially induced by splenocytes isolated from MuV-Prl vaccinated animals were the Th0/Th1-like and pro-inflammatory cytokines, many of which are known to be important for viral clearance (e.g.: IFN-γ). However, we also observed that exposure to MuV-Prl vaccines primed the animals for the production of Th2-like and regulatory cytokines such as IL-9 and IL-10, the latter of which plays an important role in the regulation and direction of immune responses (136). It is particularly interesting that a similar secretion trend of IL-10 was observed in the case of RSV-Prl vaccines (though the overall levels were higher for the RSV vaccine) (78), raising the possibility that this is a common feature of Prl-adjuvanted vaccines.

Overall, the cytokine pattern observed indicates that MuV-Prl vaccines can elicit a broad range of cytokines in response to MuV antigen. These results therefore tend to favour the idea that MuV-Prl vaccines are capable of generating a balanced immune environment upon re-exposure to MuV (compared with MuV antigen alone). This may be important in the context of natural MuV infection, to which various branches of the immune response are required for viral clearance. However, it is important to point out the limitations of the cytokine analysis. First, these data were obtained by analysis of pooled splenocytes from each group (hence no statistical analysis was possible). Furthermore, we sampled culture supernatants at a single time-point and antigen-driven cytokine production kinetics are known to be quite varied, and different cytokines are secreted at different times upon exposure to an antigen-specific stimulus (137;138). As a result, a more detailed analysis involving more mice and sampling at various time-points post-stimulation would give a more complete picture of the cytokine production profile following MuV vaccination and would be of great interest.

The immunologic data obtained in this study demonstrate that Prl would be a suitable adjuvant for a MuV-containing vaccine that targets the nasal mucosa in mice. However, though it has been accepted that nasal mucosal immune compartment between rodents and humans are functionally analogous (NALT vs. Waldeyer's Ring) (139-144), there are still important differences between these two immune environments . There may therefore be differences in terms of how nasal MuV-Prl vaccines generate immune responses within these two species. Further work will be necessary to determine if MuV-Prl vaccines are as promising in the mucosa of non-human primates/humans as they appear to be in mice.

Proteosome- and Prl-based adjuvants have demonstrated good immunologic track-records in several species (mice, rats, non-human primates, humans) for several other viruses, indicating these adjuvants to be an effective universal vaccine platform. However, to truly assess the possibility of a nasal MuV-Prl vaccine as a MuV vaccine candidate, a challenge study will be necessary. Unfortunately, challenge with MuV is impossible in a mouse model, as MuV cannot replicate in mice. Further work to determine the protective capabilities of MuV-Prl vaccines will therefore require an animal model in which MuV infection can be sustained (possibly hamster or non-human primates). Continued study of MuV-Prl vaccines for intranasal administration in other animal models is therefore warranted.

Our study has demonstrated the proof of concept that an inactivated nasal vaccine based on whole split virions and adjuvanted with Prl is safe and immunogenic in a mouse model. This vaccine was capable of generating both cell-mediated and humoral immunity, and elicited a strong neutralizing antibody response. We have also set the stage for the 'next generation' MuV vaccine by acquiring and performing initial work with recombinant MuV antigens. Naturally, a monovalent MuV vaccine would not survive as a marketable product, and so new MuV vaccine development needs to encompass the possibility of combining MuV antigens with inactivated measles and rubella virus (RV) antigens (at least) in the hopes of eventually generating new inactivated trivalent MMR formulations. Previous work has demonstrated MeV-Prl vaccines to be safe and immunogenic in mice and to provide protection in rhesus macaques challenged with wild-type MeV (145). Preliminary work in our laboratory has shown that a

divalent MeV/MuV-Prl vaccine can elicit MeV-and MuV-specific immune responses with no significant interference between the respective viral components (unpublished data). It will therefore be interesting to further characterize the immune responses to this combination vaccine, and to eventually add the RV component. In addition, we are presently at an early stage in generating recombinant MuV surface glycoproteins. Once we have these reagents, we plan to combine recombinant MuV surface glycoproteins with Prl and assess safety and immunogenicity in mice. Recombinant surface glycoproteins are available or under development for both MeV (e.g.: Dr. Veronika von Messling at Université de Québec à Montréal, Dr. Diane Griffin at Johns Hopkins University) and RV (commercially available). The ultimate goal will be to generate a trivalent MMR-Prl based solely on recombinant protein technology. We believe that such an inactivated combination MMR vaccine will be of great interest to the global health community.

Mumps-containing vaccines have had massive impact on reducing disease incidence over the last four decades. However, this is no time to become complacent, and it would be unwise to assume that these are the 'only' MuV-containing vaccines we will need to achieve eventual eradication. The worldwide MuV epidemics are continued reminders of the limitations of current MuV-containing vaccines. Though many reasons exist for these epidemics, the fact that a good proportion of individuals receiving both recommended doses of the vaccine succumbed to MuV infection indicates that vaccine efficacy is less than ideal and that new strategies for MuV vaccine development must be employed in the hopes of generating more efficacious vaccines. Furthermore, one must

consider the entirely desirable situation in which the incidence of all three diseases targeted by the MMR vaccine becomes so low that the benefit/risk ratio is tipped against receiving the vaccine. In other words, as these diseases are driven towards eradication, the risk of adverse events associated with the live-attenuated vaccines will eventually become greater than the risk of contracting the diseases. Even as these diseases approach eradication, it will be essential to maintain high vaccination coverage for a substantial period of time (as is currently the case for polio, for example). A vaccine such as the MMR that has adverse effects like thrombocytopenia and encephalitis may no longer be accepted by the public at a time when the risk of disease is effectively zero or close to zero. In this situation, a safer, inactivated MMR may be of vital importance to ensure that vaccine uptake is maintained and that the ultimate goal of disease eradication will be met.

REFERENCE LIST

(1) Carbone KM, Rubin S. Mumps Virus. In: Howley PM, Knipe DM, editors. Fields' Virology. 5 ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2007. p. 1527-50.

(2) Johnson CD, Goodpasture EW. An investigation of the etiology of mumps. The Journal of Experimental Medicine 1934 Jan 1;59(1):1-19.

(3) Johnson CD, Goodpasture EW. The etiology of mumps. American Journal of Hygiene 1935;21:46-57.

(4) Büchen-Osmond C. ICTVdB Management. 01.048.1.03.001. Mumps virus. In: Columbia University, editor. ICTVdB - The Universal Virus Database. 4 ed. New York: 2006.

(5) Lamb RA, Parks GD. Paramyxoviridae: the viruses and their replication. In: Howley PM, Knipe DM, editors. Fields' Virology. 5 ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2007. p. 1449-95.

(6) Horne RW, Waterson AP, Wildy P, Farnham AE. The structure and composition of the myxoviruses. I. Electron microscope studies of the structure of myxovirus particles by negative staining techniques. Virology 1960 May;11:79-98.

(7) Hosaka Y, Shimizu K. Lengths of the nucleocapsids of Newcastle disease and mumps viruses. J Mol Biol 1968 Jul 28;35(2):369-73.

(8) Jensik SC, Silver S. Polypeptides of mumps virus. J Virol 1976 Feb;17(2):363-73.

(9) Orvell C. Structural polypeptides of mumps virus. J Gen Virol 1978 Dec;41(3):527-39.

(10) Orvell C. Immunological properties of purified mumps virus glycoproteins. J Gen Virol 1978 Dec;41(3):517-26.

(11) Orvell C. The reactions of monoclonal antibodies with structural proteins of mumps virus. J Immunol 1984 May;132(5):2622-9.

(12) Server AC, Merz DC, Waxham MN, Wolinsky JS. Differentiation of mumps virus strains with monoclonal antibody to the HN glycoprotein. Infect Immun 1982 Jan;35(1):179-86.

(13) Merz DC, Scheid A, Choppin PW. Importance of antibodies to the fusion glycoprotein of paramyxoviruses in the prevention of spread of infection. J Exp Med 1980 Feb 1;151(2):275-88.

(14) Merz DC, Scheid A, Choppin PW. Immunological studies of the functions of paramyxovirus glycoproteins. Virology 1981 Feb;109(1):94-105.

(15) Merz DC, Server AC, Waxham MN, Wolinsky JS. Biosynthesis of mumps virus F glycoprotein: non-fusing strains efficiently cleave the F glycoprotein precursor. J Gen Virol 1983 Jul;64 (Pt 7):1457-67.

(16) Scheid A, Choppin PW. Two disulfide-linked polypeptide chains constitute the active F protein of paramyxoviruses. Virology 1977 Jul 1;80(1):54-66.

(17) Malur AG, Gupta NK, De BP, Banerjee AK. Analysis of the mutations in the active site of the RNA-dependent RNA polymerase of human parainfluenza virus type 3 (HPIV3). Gene Expr 2002;10(3):93-100.

(18) Poch O, Blumberg BM, Bougueleret L, Tordo N. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. J Gen Virol 1990 May;71 (Pt 5):1153-62.

(19) Svenda M, Berg M, Moreno-Lopez J, Linne T. Analysis of the large (L) protein gene of the porcine rubulavirus LPMV: identification of possible functional domains. Virus Res 1997 Apr;48(1):57-70.

(20) Paterson RG, Lamb RA. RNA editing by G-nucleotide insertion in mumps virus P-gene mRNA transcripts. J Virol 1990 Sep;64(9):4137-45.

(21) Fujii N, Yokosawa N, Shirakawa S. Suppression of interferon response gene expression in cells persistently infected with mumps virus, and restoration from its suppression by treatment with ribavirin. Virus Res 1999 Dec 15;65(2):175-85.

(22) Hamaguchi M, Yoshida T, Nishikawa K, Naruse H, Nagai Y. Transcriptive complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. Virology 1983 Jul 15;128(1):105-17.

(23) Elliott GD, Yeo RP, Afzal MA, Simpson EJ, Curran JA, Rima BK. Strain-variable editing during transcription of the P gene of mumps virus may lead to the generation of non-structural proteins NS1 (V) and NS2. J Gen Virol 1990 Jul;71 (Pt 7):1555-60.

(24) Wilson RL, Fuentes SM, Wang P, Taddeo EC, Klatt A, Henderson AJ, et al. Function of small hydrophobic proteins of paramyxovirus. J Virol 2006 Feb;80(4):1700-9.

(25) Elango N, Kovamees J, Varsanyi TM, Norrby E. mRNA sequence and deduced amino acid sequence of the mumps virus small hydrophobic protein gene. J Virol 1989 Mar;63(3):1413-5.

(26) Takeuchi K, Tanabayashi K, Hishiyama M, Yamada A. The mumps virus SH protein is a membrane protein and not essential for virus growth. Virology 1996 Nov 1;225(1):156-62.

(27) Takeuchi K, Tanabayashi K, Hishiyama M, Yamada A, Sugiura A. Variations of nucleotide sequences and transcription of the SH gene among mumps virus strains. Virology 1991 Mar;181(1):364-6.

(28) Nagai Y, Ogura H, Klenk H. Studies on the assembly of the envelope of Newcastle disease virus. Virology 1976 Feb;69(2):523-38.

(29) Sanderson CM, Wu HH, Nayak DP. Sendai virus M protein binds independently to either the F or the HN glycoprotein in vivo. J Virol 1994 Jan;68(1):69-76.

(30) Santos CL, Ishida MA, Foster PG, Sallum MA, Benega MA, Borges DB, et al. Detection of a new mumps virus genotype during parotitis epidemic of 2006-2007 in the state of Sao Paulo, Brazil. J Med Virol 2008 Feb;80(2):323-9.

(31) Kaaijk P, van der ZB, Boog M, Hoitink C. Increased mumps incidence in the Netherlands: review on the possible role of vaccine strain and genotype. Euro Surveill 2008 Jun 26;13(26).

(32) Muhlemann K. The molecular epidemiology of mumps virus. Infect Genet Evol 2004 Sep;4(3):215-9.

(33) Ebata SN, Cote MJ, Kang CY, Dimock K. The fusion and hemagglutinin-neuraminidase glycoproteins of human parainfluenza virus 3 are both required for fusion. Virology 1991 Jul;183(1):437-41.

(34) Wild TF, Malvoisin E, Buckland R. Measles virus: both the haemagglutinin and fusion glycoproteins are required for fusion. J Gen Virol 1991 Feb;72 (Pt 2):439-42.

(35) Collins PL, Hill MG, Cristina J, Grosfeld H. Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative-strand RNA virus. Proc Natl Acad Sci U S A 1996 Jan 9;93(1):81-5.

(36) Hardy RW, Harmon SB, Wertz GW. Diverse gene junctions of respiratory syncytial virus modulate the efficiency of transcription termination and respond differently to M2-mediated antitermination. J Virol 1999 Jan;73(1):170-6.

(37) Gubbay O, Curran J, Kolakofsky D. Sendai virus genome synthesis and assembly are coupled: a possible mechanism to promote viral RNA polymerase processivity. J Gen Virol 2001 Dec;82(Pt 12):2895-903.

(38) Schmitt AP, Leser GP, Waning DL, Lamb RA. Requirements for budding of paramyxovirus simian virus 5 virus-like particles. J Virol 2002 Apr;76(8):3952-64.

(39) Herrler G, Compans RW. Synthesis of mumps virus polypeptides in infected Vero cells. Virology 1982 Jun;119(2):430-8.

(40) Takimoto T, Murti KG, Bousse T, Scroggs RA, Portner A. Role of matrix and fusion proteins in budding of Sendai virus. J Virol 2001 Dec;75(23):11384-91.

(41) Waning DL, Schmitt AP, Leser GP, Lamb RA. Roles for the cytoplasmic tails of the fusion and hemagglutinin-neuraminidase proteins in budding of the paramyxovirus simian virus 5. J Virol 2002 Sep;76(18):9284-97.

(42) Mumps virus vaccines. Wkly Epidemiol Rec 2007 Feb 16;82(7):51-60.

(43) Rubin SA, Pletnikov M, Carbone KM. Comparison of the neurovirulence of a vaccine and a wild-type mumps virus strain in the developing rat brain. J Virol 1998 Oct;72(10):8037-42.

(44) Kurtz JB, Tomlinson AH, Pearson J. Mumps virus isolated from a fetus. Br Med J (Clin Res Ed) 1982 Feb 13;284(6314):471.

(45) Overman JR, Peers JH, Kilham L. Pathology of mumps virus meningoencephalitis in mice and hamsters. AMA Arch Pathol 1953 Jun;55(6):457-65.

(46) Rozina EE, Kaptsova TI, Sharova OK, Nikolaeva MA, Nesterova TP. Study of mumps virus invasiveness in monkeys. Acta Virol 1984 Mar;28(2):107-13.

(47) Gordon JE. The epidemiology of mumps. Am J Med Sci 1940;200:412-28.

(48) Philip RN, Reinhard KR, Lackman DB. Observations on a mumps epidemic in a virgin population. Am J Hyg 1959 Mar;69(2):91-111.

(49) Maris EP, Enders JF, Stokes J, Kane LW. Immunity in Mumps: IV The correlation of the presence of complement-fixing antibody and resistance to Mumps in Human Beings. J Exp Med 1946 Sep 30;84(4):323.

(50) Frankova V, Sixtova E. Specific IgM antibodies in the saliva of mumps patients. Acta Virol 1987 Aug;31(4):357-64.

(51) Bjorvatn B. Incidence and persistence of mumps-specific IgM and IgA in the sera of mumps patients. Scand J Infect Dis 1974;6(2):125-9.

(52) Sarnesto A, Julkunen I, Makela O. Proportions of Ig classes and subclasses in mumps antibodies. Scand J Immunol 1985 Sep;22(3):345-50.

(53) Ukkonen P, Granstrom ML, Penttinen K. Mumps-specific immunoglobulin M and G antibodies in natural mumps infection as measured by enzyme-linked immunosorbent assay. J Med Virol 1981;8(2):131-42.

(54) Chiba Y, Horino K, Umetsu M, Wataya Y, Chiba S. Virus excretion and antibody response in saliva in natural mumps. Tohoku J Exp Med 1973 Nov;111(3):229-38.

(55) Gut JP, Lablache C, Behr S, Kirn A. Symptomatic mumps virus reinfections. J Med Virol 1995 Jan;45(1):17-23.

(56) Ito Y, Tsurudome M, Hishiyama M, Yamada A. Immunological interrelationships among human and non-human paramyxoviruses revealed by immunoprecipitation. J Gen Virol 1987 May;68 (Pt 5):1289-97.

(57) Tsurudome M, Nishio M, Komada H, Bando H, Ito Y. Extensive antigenic diversity among human parainfluenza type 2 virus isolates and immunological relationships among paramyxoviruses revealed by monoclonal antibodies. Virology 1989 Jul;171(1):38-48.

(58) Bertotto A, Spinozzi F, Gerli R, Bassotti G, Forenza N, Pazzelli P, et al. Gamma delta T lymphocytes in mumps meningitis patients. Acta Paediatr 1995 Nov;84(11):1268-70.

(59) Kreth HW, Kress L, Kress HG, Ott HF, Eckert G. Demonstration of primary cytotoxic T cells in venous blood and cerebrospinal fluid of children with mumps meningitis. J Immunol 1982 Jun;128(6):2411-5.

(60) Hanna-Wakim R, Yasukawa LL, Sung P, Arvin AM, Gans HA. Immune responses to mumps vaccine in adults who were vaccinated in childhood. J Infect Dis 2008 Jun 15;197(12):1669-75.

(61) National Advisory Committee on Immunization. Canadian Immunization Guide. 7 ed. 2006.

(62) Statement on Mumps Vaccine. An Advisory Committee Statement (ACS). Can Commun Dis Rep 2007 Aug 1;33(ACS-8):1-10.

(63) Karagiannis I, van LA, van BR, Ruijs H, Ruijs H, Fanoy E, et al. Mumps in a community with low vaccination coverage in the Netherlands. Euro Surveill 2008 Jun 12;13(24).

(64) Anderson LJ, Seward JF. Mumps epidemiology and immunity: the anatomy of a modern epidemic. Pediatr Infect Dis J 2008 Oct;27(10 Suppl):S75-S79.

(65) Mumps epidemic--United kingdom, 2004-2005. MMWR Morb Mortal Wkly Rep 2006 Feb 24;55(7):173-5.

(66) Peltola H, Kulkarni PS, Kapre SV, Paunio M, Jadhav SS, Dhere RM. Mumps outbreaks in Canada and the United States: time for new thinking on mumps vaccines. Clin Infect Dis 2007 Aug 15;45(4):459-66.

(67) Huynh W, Cordato DJ, Kehdi E, Masters LT, Dedousis C. Post-vaccination encephalomyelitis: Literature review and illustrative case. J Clin Neurosci 2008 Dec;15(12):1315-22.

(68) Global Advisory Committee on Vaccine Safety, 29-30 November 2006. Wkly Epidemiol Rec 2007 Jan 19;82(3):18-24.

(69) Lowell GH, Burt D, White G, Fries L. Proteosome™ technology for vaccines and adjuvants. In: Levine MM KJRRLMGM, editor. New generation vaccines. 3 ed. New York: Marcel Dekker; 2003.

(70) Orr N, Robin G, Cohen D, Arnon R, Lowell GH. Immunogenicity and efficacy of oral or intranasal Shigella flexneri 2a and Shigella sonnei proteosome-lipopolysaccharide vaccines in animal models. Infect Immun 1993 Jun;61(6):2390-5.

(71) Jones T, Cyr S, Allard F, Bellerose N, Lowell GH, Burt DS. Protollin: a novel adjuvant for intranasal vaccines. Vaccine 2004 Sep 9;22(27-28):3691-7.

(72) Massari P, Ho Y, Wetzler LM. Neisseria meningitidis porin PorB interacts with mitochondria and protects cells from apoptosis. Proc Natl Acad Sci U S A 2000 Aug 1;97(16):9070-5.

(73) Muller A, Rassow J, Grimm J, Machuy N, Meyer TF, Rudel T. VDAC and the bacterial porin PorB of Neisseria gonorrhoeae share mitochondrial import pathways. EMBO J 2002 Apr 15;21(8):1916-29.

(74) Bramwell VW, Perrie Y. Particulate delivery systems for vaccines: what can we expect? J Pharm Pharmacol 2006 Jun;58(6):717-28.

(75) Chabot SM, Chernin TS, Shawi M, Wagner J, Farrant S, Burt DS, et al. TLR2 activation by proteosomes promotes uptake of particulate vaccines at mucosal surfaces. Vaccine 2007 Jul 20;25(29):5348-58.

(76) Cyr SL, Angers I, Guillot L, Stoica-Popescu I, Lussier M, Qureshi S, et al. TLR4 and MyD88 control protection and pulmonary granulocytic recruitment in a murine intranasal RSV immunization and challenge model. Vaccine 2008 Nov 14.

(77) Chabot S, Brewer A, Lowell G, Plante M, Cyr S, Burt DS, et al. A novel intranasal Protollin-based measles vaccine induces mucosal and systemic neutralizing antibody responses and cell-mediated immunity in mice. Vaccine 2005 Feb 3;23(11):1374-83.

(78) Cyr SL, Jones T, Stoica-Popescu I, Brewer A, Chabot S, Lussier M, et al. Intranasal proteosome-based respiratory syncytial virus (RSV) vaccines protect BALB/c mice against challenge without eosinophilia or enhanced pathology. Vaccine 2007 Jul 20;25(29):5378-89.

(79) Cornell RG. Kärber Method. Encyclopedia of Statistical Sciences. John Wiley & Sons, Inc.; 2006.

(80) Malik T, Wolbert C, Mauldin J, Sauder C, Carbone KM, Rubin SA. Functional consequences of attenuating mutations in the haemagglutinin neuraminidase, fusion and polymerase proteins of a wild-type mumps virus strain. J Gen Virol 2007 Sep;88(Pt 9):2533-41.

(81) Love A, Rydbeck R, Utter G, Orvell C, Kristensson K, Norrby E. Monoclonal antibodies against the fusion protein are protective in necrotizing mumps meningoencephalitis. J Virol 1986 Apr;58(1):220-2.

(82) Server AC, Merz DC, Waxham MN, Wolinsky JS. Differentiation of mumps virus strains with monoclonal antibody to the HN glycoprotein. Infect Immun 1982 Jan;35(1):179-86.

(83) Maassen CB, Boersma WJ, van Holten-Neelen C, Claassen E, Laman JD. Growth phase of orally administered Lactobacillus strains differentially affects IgG1/IgG2a ratio for soluble antigens: implications for vaccine development. Vaccine 2003 Jun 20;21(21-22):2751-7.

(84) McCarthy M, Lazzarini RA. Intracellular nucleocapsid RNA of mumps virus. J Gen Virol 1982 Jan;58 Pt 1:205-9.

(85) Green M, Loewenstein PM. Human adenoviruses: propagation, purification, quantification, and storage. Curr Protoc Microbiol 2006 Jan;Chapter 14:Unit.

(86) Rourou S, van der AA, van d, V, Kallel H. A microcarrier cell culture process for propagating rabies virus in Vero cells grown in a stirred bioreactor under fully animal component free conditions. Vaccine 2007 May 10;25(19):3879-89.

(87) Hu WS, Aunins JG. Large-scale mammalian cell culture. Curr Opin Biotechnol 1997 Apr;8(2):148-53.

(88) Liu Y, Zhu J, Feng MG, Tien P, Gao GF. Six-helix bundle assembly and analysis of the central core of mumps virus fusion protein. Arch Biochem Biophys 2004 Jan 1;421(1):143-8.

(89) McAleer B, Rima B. Cloning and secreted expression of the extracellular domain of the mumps virus fusion protein in Pichia pastoris. Virus Genes 2000;20(2):127-33.

(90) Somboonthum P, Yoshii H, Okamoto S, Koike M, Gomi Y, Uchiyama Y, et al. Generation of a recombinant Oka varicella vaccine expressing mumps virus hemagglutinin-neuraminidase protein as a polyvalent live vaccine. Vaccine 2007 Dec 17;25(52):8741-55.

(91) Bright RA, Ross TM, Subbarao K, Robinson HL, Katz JM. Impact of glycosylation on the immunogenicity of a DNA-based influenza H5 HA vaccine. Virology 2003 Apr 10;308(2):270-8.

(92) Marquet-Blouin E, Bouche FB, Steinmetz A, Muller CP. Neutralizing immunogenicity of transgenic carrot (Daucus carota L.)-derived measles virus hemagglutinin. Plant Mol Biol 2003 Mar;51(4):459-69.

(93) Samuel D, Sasnauskas K, Jin L, Beard S, Zvirbliene A, Gedvilaite A, et al. High level expression of recombinant mumps nucleoprotein in Saccharomyces cerevisiae and its evaluation in mumps IgM serology. J Med Virol 2002 Jan;66(1):123-30.

(94) Slibinskas R, Zvirbliene A, Gedvilaite A, Samuel D, Jin L, Beard S, et al. Synthesis of mumps virus nucleocapsid protein in yeast Pichia pastoris. J Biotechnol 2003 Jun 12;103(1):43-9.

(95) Duffy S, Shackelton LA, Holmes EC. Rates of evolutionary change in viruses: patterns and determinants. Nat Rev Genet 2008 Apr;9(4):267-76.

(96) Houard S, Varsanyi TM, Milican F, Norrby E, Bollen A. Protection of hamsters against experimental mumps virus (MuV) infection by antibodies raised against the MuV surface glycoproteins expressed from recombinant vaccinia virus vectors. J Gen Virol 1995 Feb;76 (Pt 2):421-3.

(97) Love A, Rydbeck R, Utter G, Orvell C, Kristensson K, Norrby E. Monoclonal antibodies against the fusion protein are protective in necrotizing mumps meningoencephalitis. J Virol 1986 Apr;58(1):220-2.

(98) Dunais B, Bruno P, Carsenti-Dellamonica H, Touboul P, Dellamonica P, Pradier C. Trends in nasopharyngeal carriage of Streptococcus pneumoniae among children attending daycare centers in southeastern France from 1999 to 2006. Pediatr Infect Dis J 2008 Nov;27(11):1033-5.

(99) Aguiar SI, Serrano I, Pinto FR, Melo-Cristino J, Ramirez M. Changes in Streptococcus pneumoniae serotypes causing invasive disease with non-universal vaccination coverage of the seven-valent conjugate vaccine. Clin Microbiol Infect 2008 Sep;14(9):835-43.

(100) Pruss-Ustun A, Rapiti E, Hutin Y. Estimation of the global burden of disease attributable to contaminated sharps injuries among health-care workers. Am J Ind Med 2005 Dec;48(6):482-90.

(101) Ekwueme DU, Weniger BG, Chen RT. Model-based estimates of risks of disease transmission and economic costs of seven injection devices in sub-Saharan Africa. Bull World Health Organ 2002;80(11):859-70.

(102) Giudice EL, Campbell JD. Needle-free vaccine delivery. Adv Drug Deliv Rev 2006 Apr 20;58(1):68-89.

(103) Levine MM, Campbell JD. Mucosal Immunization and needle-free injection devices. In: Levine MM KJRRLMGM, editor. New Generation Vaccines.New York: Marcel Dekker; 2004. p. 393-9.

(104) Jacobson RM, Swan A, Adegbenro A, Ludington SL, Wollan PC, Poland GA. Making vaccines more acceptable--methods to prevent and minimize pain and other common adverse events associated with vaccines. Vaccine 2001 Mar 21;19(17-19):2418-27.

(105) Nir Y, Paz A, Sabo E, Potasman I. Fear of injections in young adults: prevalence and associations. Am J Trop Med Hyg 2003 Mar;68(3):341-4.

(106) Tickner S, Leman PJ, Woodcock A. Factors underlying suboptimal childhood immunisation. Vaccine 2006 Nov 30;24(49-50):7030-6.

(107) Alternative needle-free delivery routes. World Health Organization 2008Available from: URL: http://www.who.int/immunization_safety/ispp/ispp_final_report_alternative_needle_free_routes/en/

(108) Program for Appropriate Technology in Health (PATH). Bill and Melinda Gates Foundation 2008Available from: URL: http://www.gatesfoundation.org/Grants-2003/Pages/Grant-30451.aspx

(109) Slutter B, Hagenaars N, Jiskoot W. Rational design of nasal vaccines. J Drug Target 2008 Jan;16(1):1-17.

(110) Zuercher AW, Coffin SE, Thurnheer MC, Fundova P, Cebra JJ. Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. J Immunol 2002 Feb 15;168(4):1796-803.

(111) Introduction of inactivated poliovirus vaccine into oral poliovirus vaccine-using countries. Wkly Epidemiol Rec 2003 Jul 11;78(28):241-50.

(112) Hinman A. Eradication of vaccine-preventable diseases. Annu Rev Public Health 1999;20:211-29.

(113) Ott G, Van Nest G. Development of Vaccine Adjuvants: A Historical Perspective. In: Singh M, editor. Vaccine Adjuvants and Delivery Systems. John Wiley & Sons, Inc.; 2007. p. 1-31.

(114) Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 2004 Feb 26;350(9):896-903.

(115) Cyr SL, Jones T, Stoica-Popescu I, Burt D, Ward BJ. C57Bl/6 mice are protected from respiratory syncytial virus (RSV) challenge and IL-5 associated pulmonary eosinophilic infiltrates following intranasal immunization with Protollin-eRSV vaccine. Vaccine 2007 Apr 20;25(16):3228-32.

(116) Hu MC, Jones T, Kenney RT, Barnard DL, Burt DS, Lowell GH. Intranasal Protollin-formulated recombinant SARS S-protein elicits respiratory and serum neutralizing antibodies and protection in mice. Vaccine 2007 Aug 21;25(34):6334-40.

(117) Fries LF, Montemarano AD, Mallett CP, Taylor DN, Hale TL, Lowell GH. Safety and immunogenicity of a proteosome-Shigella flexneri 2a lipopolysaccharide vaccine administered intranasally to healthy adults. Infect Immun 2001 Jul;69(7):4545-53.

(118) Langley JM, Halperin SA, McNeil S, Smith B, Jones T, Burt D, et al. Safety and immunogenicity of a Proteosome -trivalent inactivated influenza vaccine, given nasally to healthy adults. Vaccine 2006 Mar 6;24(10):1601-8.

(119) Pasetti MF, Resendiz-Albor A, Ramirez K, Stout R, Papania M, Adams RJ, et al. Heterologous prime-boost strategy to immunize very young infants against measles: pre-clinical studies in rhesus macaques. Clin Pharmacol Ther 2007 Dec;82(6):672-85.

(120) Treanor J, Nolan C, O'Brien D, Burt D, Lowell G, Linden J, et al. Intranasal administration of a proteosome-influenza vaccine is well-tolerated and induces serum and nasal secretion influenza antibodies in healthy human subjects. Vaccine 2006 Jan 16;24(3):254-62.

(121) Fries LF, Montemarano AD, Mallett CP, Taylor DN, Hale TL, Lowell GH. Safety and immunogenicity of a proteosome-Shigella flexneri 2a lipopolysaccharide vaccine administered intranasally to healthy adults. Infect Immun 2001 Jul;69(7):4545-53.

(122) Langley JM, Halperin SA, McNeil S, Smith B, Jones T, Burt D, et al. Safety and immunogenicity of a Proteosome -trivalent inactivated influenza vaccine, given nasally to healthy adults. Vaccine 2006 Mar 6;24(10):1601-8.

(123) Treanor J, Nolan C, O'Brien D, Burt D, Lowell G, Linden J, et al. Intranasal administration of a proteosome-influenza vaccine is well-tolerated and induces serum and nasal secretion influenza antibodies in healthy human subjects. Vaccine 2006 Jan 16;24(3):254-62.

(124) Lindlbad EB. Safety Evaluation of Vaccine Adjuvants. In: Singh M, editor. Vaccine Adjuvants and Delivery Systems. John Wiley & Sons, Inc.; 2007. p. 421-44.

(125) Pasetti MF, Resendiz-Albor A, Ramirez K, Stout R, Papania M, Adams RJ, et al. Heterologous prime-boost strategy to immunize very young infants against measles: pre-clinical studies in rhesus macaques. Clin Pharmacol Ther 2007 Dec;82(6):672-85.

(126) Pasetti MF, Resendiz-Albor A, Ramirez K, Stout R, Papania M, Adams RJ, et al. Heterologous prime-boost strategy to immunize very young infants against measles: pre-clinical studies in rhesus macaques. Clin Pharmacol Ther 2007 Dec;82(6):672-85.

(127) Langley JM, Halperin SA, McNeil S, Smith B, Jones T, Burt D, et al. Safety and immunogenicity of a Proteosome -trivalent inactivated influenza vaccine, given nasally to healthy adults. Vaccine 2006 Mar 6;24(10):1601-8.

(128) Treanor J, Nolan C, O'Brien D, Burt D, Lowell G, Linden J, et al. Intranasal administration of a proteosome-influenza vaccine is well-tolerated and induces serum and nasal secretion influenza antibodies in healthy human subjects. Vaccine 2006 Jan 16;24(3):254-62.

(129) Chen RT, Markowitz LE, Albrecht P, Stewart JA, Mofenson LM, Preblud SR, et al. Measles antibody: reevaluation of protective titers. J Infect Dis 1990 Nov;162(5):1036-42.

(130) Polack FP. Atypical measles and enhanced respiratory syncytial virus disease (ERD) made simple. Pediatr Res 2007 Jul;62(1):111-5.

(131) Moghaddam A, Olszewska W, Wang B, Tregoning JS, Helson R, Sattentau QJ, et al. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. Nat Med 2006 Aug;12(8):905-7.

(132) Polack FP. Atypical measles and enhanced respiratory syncytial virus disease (ERD) made simple. Pediatr Res 2007 Jul;62(1):111-5.

(133) Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med 2009 Jan;15(1):34-41.

(134) Cusi MG, Zurbriggen R, Valassina M, Bianchi S, Durrer P, Valensin PE, et al. Intranasal immunization with mumps virus DNA vaccine delivered by influenza virosomes elicits mucosal and systemic immunity. Virology 2000 Nov 10;277(1):111-8.

(135) Menon JN, Bretscher PA. Parasite dose determines the Th1/Th2 nature of the response to Leishmania major independently of infection route and strain of host or parasite. Eur J Immunol 1998 Dec;28(12):4020-8.

(136) Bettelli E, Nicholson LB, Kuchroo VK. IL-10, a key effector regulatory cytokine in experimental autoimmune encephalomyelitis. J Autoimmun 2003 Jun;20(4):265-7.

(137) Mocellin S, Wang E, Marincola FM. Cytokines and immune response in the tumor microenvironment. J Immunother 2001 Sep;24(5):392-407.

(138) Chen SJ, Liao CL, Hsieh SL, Chu ML, Fang MC, Sytwu HK, et al. Kinetics of adaptive immunity to Haemophilus influenzae type b meningitis in transgenic mice: evidence from diverse expression of double T1/T2 transgenes and Th1/Th2-related cytokines. Immunol Lett 2006 May 15;105(1):6-15.

(139) Bienenstock J, McDermott MR. Bronchus- and nasal-associated lymphoid tissues. Immunol Rev 2005 Aug;206:22-31.

(140) Cleary PP, Zhang Y, Park HS. Nasal associated lymphoid tissue & M cells, a window to persistent streptococcal infections. Indian J Med Res 2004 May;119 Suppl:57-60.

(141) Fujimura Y, Takeda M, Ikai H, Haruma K, Akisada T, Harada T, et al. The role of M cells of human nasopharyngeal lymphoid tissue in influenza virus sampling. Virchows Arch 2004 Jan;444(1):36-42.

(142) Jeong KI, Suzuki H, Nakayama H, Doi K. Ultrastructural study on the follicle-associated epithelium of nasal-associated lymphoid tissue in specific pathogen-free (SPF) and conventional environment-adapted (SPF-CV) rats. J Anat 2000 Apr;196 (Pt 3):443-51.

(143) Kuper CF, Hameleers DM, Bruijntjes JP, van dV, I, Biewenga J, Sminia T. Lymphoid and non-lymphoid cells in nasal-associated lymphoid tissue (NALT) in the rat. An immuno- and enzyme-histochemical study. Cell Tissue Res 1990 Feb;259(2):371-7.

(144) Kuper CF, Koornstra PJ, Hameleers DM, Biewenga J, Spit BJ, Duijvestijn AM, et al. The role of nasopharyngeal lymphoid tissue. Immunol Today 1992 Jun;13(6):219-24.

(145) Pasetti MF, Resendiz-Albor A, Ramirez K, Stout R, Papania M, Adams RJ, et al. Heterologous prime-boost strategy to immunize very young infants against measles: pre-clinical studies in rhesus macaques. Clin Pharmacol Ther 2007 Dec;82(6):672-85.

APPENDICES

A. McGill University Animal Use Protocol

B. McGill University Animal Care Committee Mouse Workshop Certification