Measles and Vitamin A: Mechanisms of Action

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Abstract

Measles virus (MV) infects an estimated 30 million individuals each year, leading to ~880,000 deaths. Measles related deaths are largely attributed to the potent immunosuppression that typically follows MV infection. The only "treatment" currently available for MV infection is high dose vitamin A, which can significantly reduce mortality. The mechanisms that underlie both MV-associated immunosuppression and the dramatic benefit of vitamin A remain poorly understood. Vitamin A supplements are also important for the eradication of vitamin A deficiency, which is implicated in the death of ~1 million children each year, due to blindness and severe infections. In recent years, there has been increasing pressure to include vitamin A supplements in "universal" childhood vaccination programs in the developing world (e.g.: EPI). In this thesis, three studies dealing with the relationship between measles virus, vitamin A and the immune system are presented. First, the induction of PBMC apoptosis by MV is described and correlated with viral output and proliferation of these cells. In this study, wild-type MV strains replicated more efficiently and induced more rapid apoptosis than vaccine strains ($p \leq 0.003$). These results suggest that immune cell apoptosis may be a key mechanism underlying the immunosuppression associated with measles. In the second study, the results from a large trial of vitamin A supplementation at the time of MV vaccination are presented. This trial was designed to evaluate the effect of vitamin A on cellular and humoral immune responses to MV vaccine. We were unable to confirm the previously reported negative impact of such supplementation on seroconversion rates and found no effect on the induction of cell-mediated immunity. Finally, as part of ongoing studies into the mechanism underlying the beneficial effect of vitamin A supplementation in MV infections, changes in the retinoid (vitamin A) signaling cascade during MV infection of a monocytic cell line (U937) were measured.

Résumé

Le virus de la rougeole (MV) infecte environ 30 millions d'individus chaque année, causant le décès d'environ 880,000 d'entre eux. Ces décès sont attribués en grande partie à l'immunosuppression qui suit typiquement l'infection du MV. Jusqu'à aujourd'hui, le seul "traitement" disponible contre la rougeole est un supplément de vitamine A qui réduit la mortalité des personnes infectées de manière significative. Les mécanismes responsables de l'immunosuppression associée avec la rougeole et à l'effet bénéfique de la supplémentation de vitamine A sont mal compris. Les suppléments de vitamine A sont également importants pour combattre la déficience de cette vitamine, qui joue un role dans le décès d'un million d'enfants chaque année, qui souffrent de cécité et d'infections graves. Durant ces dernières années, il y a une pression grandissante d'inclure des suppléments de vitamine A dans des programmes universels de vaccination d'enfance (par exemple: PEV) dans les pays en voie de développement. Dans cette mémoire, trois études traitant du rapport entre le MV, la vitamine A et le système immunitaire sont présentées. D'abord, l'induction de l'apoptose des cellules mononucléaires du sang periphéral causé par MV est décrite ainsi qu'une corrélation avec la prolifération cellulaire ainsi que la production virale. Les souches de MV infectueuses sont plus efficaces pour induire l'apoptose que les souches vaccinales ($p \leq 0.003$). Ces résultats suggèrent que l'apoptose des cellules immunitaires pourrait être un mécanisme important responsable de l'immunosuppression associée avec MV. En second lieu, les résultats d'une grande étude sur la supplémentation de la vitamine A au moment de la vaccination de la rougeole sont présentés. Cette étude avait l'intention d'évaluer l'effet de la vitamine A sur l'immunité humorale et cellulaire résultants de la vaccination de la rougeole. L'effet négatif de la vitamine A sur la séroconversion qui était décrit antérieurement n'a pas été reconfirmer, et aucun effet sur l'induction de l'immunité cellulaire n'a été constaté. Finalement, des études en cours ont préliminairement démontrés des changements dans la cascade du signalement rétinoïque pendant l'infection de MV, ce qui pourrait expliquer un des mécanismes responsible de l'effet bénéfique de la supplémentation de vitamine A comme traitement contre la rougeole.

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Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the **candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.**

Contribution of Authors

Manuscript I:

Stephanie Fox (myself) did the plaque assays, some TUNEL assays and co-wrote the manuscript.

Fred Bertley performed the majority of the TUNEL assays and FACS analysis and also co-wrote the manuscript.

Serign Ceesay worked on the lymphoproliferation assays.

Nathalie Martel is a technician in our lab who helped with the various techniques.

Manuscript II:

Stephanie Fox did statistical analysis on data from this study and wrote the manuscript.

Josh Jones performed the lymphoproliferation assays.

Felicity Cutts advised on the project design and was consulted as it progressed.

Rota Kanashiro in Abraham Vaisberg's laboratory performed the plaque neutralization reduction (PRN) assays.

Betzabe Butrón and Claudio Lanata were co-PI on this field project.

Mary Penny helped with planning and overseeing the field work, collecting the samples and compiling and analysing the data. She also provided editorial help.

Additionally, Ana Huapaya was involved in the data entry and background data analysis.

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Additional studies:
Stephanie Fox (myself) performed all experiments, with supervising by Brian Ward. Avi Chatterjee and Jennifer Manalo generated preliminary data used to guide this work.

Appendix I:
Norma Bautista-López performed most of the experiments and wrote this manuscript.
Stephanie Fox performed the cytokine ELISAs.
Marie-Christine Guiot performed some of the cytokine mRNA assays.
Elaine Mills helped collect the blood samples tested in this study.

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List of Abbreviations

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCG	Bacille Calmette Guerin
BILT	Bilthoven measles virus strain
CBMC	Cord Blood Mononuclear Cells
CDC	Center for Disease Control
CHD	Child Health and Development
CHI	Chicago measles virus strain
CMI	Cell Mediated Immunity
CMV	Cytomegalovirus
CN	Edmonston Enders measles virus (Connaught labs)
CPE	Cytopathic effect
CPM	Counts Per Minute
CRABP	Cellular Retinoic Acid Binding Protein
CRBP	Cellular Retinol Binding Protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
OPV	Oral Polio Vaccine
DPT	Diphtheria-Pertussis-Tetanus vaccine
DR	Direct Repeats
DTH	Delayed Type Hypersensitivity
dUTP	Deoxyuridine triphosphate
EDB	Edmonston B measles virus strain
EPI	Expanded Programme on Immunization
ER	Everted Repeats
EZ	Edmonston Zagreb measles virus strain

FBS	Fetal Bovine Serum
GAMB	Gambia measles virus strain
GAPDH	Glyceraldehyde phosphate dehydrogenase
HAW	Hawaii measles virus strain
HIV	Human Immunodeficiency Virus
HTLV-1	Human T-cell Leukemia Virus 1
IDV	Integrated Density Value
Ig	Immunoglobulin
IU	International Units
LPR	Lymphoproliferative response
MOI	Multiplicity Of Infection
MASS	Massachusetts measles virus strain
MINN	Minnesota measles virus strain
MMR	Measles-Mumps-Rubella vaccine
MRDR	Modified Relative Dose Response
mRNA	Messenger ribonucleic acid
MV	Measles virus
NIAID	National Institute of Allergy and Infectious Diseases
OMS	Organisation Mondiale de la Santé (WHO)
PBMC	Peripheral Blood Mononuclear Cells
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
PHA	Phytohemagglutinin
PPAR	Peroxisome proliferator receptor
PRN	Plaque Reduction Neutralization assay
PT	Whole pertussis antigen
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Element
RBP	Retinol Binding Protein

RNA	Ribonucleic acid
ROH	Retinol
RT	Reverse Transcription
RXR	9-cis retinoic acid receptor
RXRE	9-cis retinoic acid response element
SEM	Standard Error of Mean
SCZ	Schwarz measles virus strain
SI	Stimulation Index
SIV	Simian Immunodeficiency Virus
SSPE	Subacute sclerosing panencephalitis
T_3R	Thyroid hormone receptor
TAE	Tris-Acetate-EDTA
TdT	Terminal deoxytransferase
TT	Tetanus Toxoid
U	Unit
UNICEF	United Nations Children's Fund
UV/UVR	Ultraviolet radiation
VAD	Vitamin A Deficiency
VD_3R	Vitamin D3 receptor
VIT A	Vitamin A
WHO	World health organization

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LITERATURE REVIEW

Chapter 1: Introduction and rationale

The World Health Organization (WHO) has recently targeted measles virus (MV) for eradication on or before the year 2010. In the pre-vaccine era, virtually all children acquired natural measles before the age of 15 years. Today, despite the availability of an effective, live attenuated vaccine for over 30 years, there are an estimated 30 million measles cases each year, resulting in approximately 880,000 deaths (WHO/EPI 2000).

In order to achieve the eradication of MV, many aspects of the immunology and immunopathology of both natural MV infection and MV vaccination need to be better understood. For example, we do not yet understand fully the mechanisms by which lifelong immunity is achieved after natural infection or the potent and prolonged immunosuppression that complicates infection (Griffin D.E. *et al* 1994). The true duration of protection after vaccination and the mechanism(s) by which MV vaccines fail also remain unknown. Further, little is known about the correlates of immunity after either natural disease or vaccination (Ward B.J. *et al* 1995). If the protective immune response for measles can be more clearly defined, better vaccines can be developed that would surpass the 85-95% efficacy rates of the formulations currently in use (CDC 2000). Another area of interest is the role played by vitamin A in the outcome of MV infection. While mortality in children with MV infection can be reduced by 50% with high dose vitamin A supplements (Fawzi W.W. *et al* 1993), the mechanisms underlying the striking clinical observation remain uncharacterized. Also, the reasons for the acute and transient vitamin A deficiency (VAD) that often accompanies natural MV infection need to be discovered (Hussey G.D. *et al* 1992).

Our lab is involved in addressing many issues in measles research, with projects ranging from the characterization of antigen-specific cytokine profiles after vaccination to the development of a novel proteosome-based vaccine. In this particular thesis, three measles studies are presented. First, the induction of apoptosis in peripheral blood mononuclear cells (PBMC) by wild type and vaccine strains of MV is described and correlated with the growth of these different strains (manuscript I). The results support the induction of immune cell apoptosis as a key mechanism underlying the immunosuppression associated with measles. Second, results from a large vaccine trial

are presented. This trial was designed to evaluate the effect of simultaneous vitamin A supplementation and MV vaccination on cellular and humoral immune responses to the vaccine (manuscript II). Finally, as part of ongoing studies into the mechanism underlying the beneficial effect of vitamin A supplementation in measles, changes in the retinoid (vitamin A) signaling cascade during MV infection of a monocytic cell line (U937) were measured (additional studies). As an appendix to this thesis, a manuscript that addresses the antigen-specific cytokine response to MV vaccination is presented.

The introduction is divided into sections dealing with the epidemiology and clinical presentation of measles (chapter 2), a brief discussion of the history of the MV vaccine development as well as current vaccination programs (chapter 3) and the correlates of immunity after both natural MV infection and MV vaccination (chapter 4). Finally, the focus switches to vitamin A and discussions of the critical role this micronutrient plays in health and development (chapter 5), and the retinoid signaling cascade (chapter 6). A particular emphasis is placed on the association between vitamin A and MV infections.

Chapter 2: Natural measles infection

2.1: Causative agent – Measles virus

MV is a member of the family *Paramyxoviridiae*. The enveloped virion is spherical and 100-250nm in diameter (Griffin D.E. *et al* 1996). It contains a helical nucleocapsid that protects the 15,900nt linear, single-stranded, negative sense RNA genome (Griffin D.E. *et al* 1996). This RNA is composed of non-overlapping genes that encode at least six viral proteins. These include the phosphoprotein (P), large protein (L), and nucleoprotein (N) which are components of the nucleocapsid as well as the fusion (F), hemagglutinin (H), and matrix proteins (M), which are associated with the envelope of the virion (Griffin D.E *et al* 1996). The F protein is responsible for the formation of giant multi-nucleated cells that are typical of MV infection (Griffin D.E. *et al* 1994). The virus has a replication rate of approximately 24 hours. *In vivo*, MV can infect a wide range of tissues including endothelial cells, epithelial cells, and immune cells such as B cells, T cells, monocytes, and macrophages (Griffin D.E. *et al* 1994, Esolen L.M. *et al* 1993, Schneider-Schaulies S. *et al* 1999).

2.2: Prevalence of the disease

Before the introduction of the live attenuated MV vaccine in 1963, virtually all children were infected with MV before they were 15 years old. At the current time, estimates of vaccine coverage worldwide are approximately 85% (CDC 2000) and the number of MV cases reported to the World Health Organization (WHO) has dropped from 1,356,992 cases in 1990 to 560,000 cases in 2000 (NIAID 2000, WHO 2000). However, the incidence of MV infection is substantially underreported and the actual global disease burden may be as high as 36 million cases and 880,000 deaths each year (WHO 2000), despite the availability of highly effective vaccines for more than 30 years.

2.3: Viral spread and disease

Measles is one of the most contagious diseases (CDC 1995). The virus is spread efficiently by aerosol and replicates initially in the respiratory tract before spreading to the local lymphatic tissue (Griffin D.E. *et al* 1994). A primary viremia then spreads MV

to reticuloendothelial tissues throughout the body as well as a wide range of other target tissues, including skin, kidney, gastrointestinal tract, and liver (Schneider-Schaulies S. *et al* 1999). Replication and spread of the virus continues during the 14- to 21- day latent period that spans the time from exposure to appearance of clinical symptoms (Griffin D.E. *et al* 1994). The first symptoms typically appear with the onset of the secondary viremia. These include fever, cough, conjunctivitis, and rapid development of a maculopapular rash that lasts for 3-5 days (CDC 1995). The rash usually begins on the face and spreads first to the trunk and then to the extremities (Griffin D.E *et al* 1996). In uncomplicated cases, recovery begins soon after the appearance of the rash (Griffin D.E *et al* 1996).

Despite rapid recovery from natural MV infections by the majority of individuals, there can be serious complications. Hospitalization can be required in up to 50% of children under the age of one (Baffin Island outbreak) and 30% of infected adults (USA outbreak) (MMWR 1991). Infections in pregnant women can result in miscarriages or premature labor (CDC 1995). Even in developed world settings, 10% of natural MV infections are complicated by ear infections that can result in permanent deafness in up to 0.1% of children (CDC 1995). Another serious complication of natural measles is encephalomyelitis, which occurs in 0.1% of natural infections (CDC 1995). In areas where vitamin A deficiency (VAD) is endemic, the acute reduction in vitamin A levels associated with MV infection can dramatically increase the risk of corneal damage leading to blindness. Indeed, MV infection in VAD children was one of the most common causes of blindness in developing world children (WHO/EPI 1994, Sommer A. 1994). Much effort has been devoted to developing vaccines in order to decrease the number of complicated measles cases.

Historically, the mortality rates following MV infection among naïve populations have been as high as 25-50% (Hussey G.D. *et al* 1992). Measles-associated mortality remains high in developing nations, with approximately 800,000 deaths annually. Pneumonia due to secondary bacterial infections is the most important cause of MV-associated deaths (Griffin D.E. *et al* 1994). These secondary infections are widely attributed to the prolonged and profound suppression of immunity that occurs following natural MV infections (Schneider-Schaulies S. *et al* 1993). MV has the capacity to infect

a wide range of immune cells, including monocytes/macrophages and B and T lymphocytes (Esolen L.M. *et al* 1993, Smedman L. *et al* 1994, Roscic-Mrkic B. *et al* 2001). The potent immunosuppression induced by natural MV infection is multifactorial but results in part from the direct infection and destruction of immune cells (Smedman L. *et al* 1994). This immunosuppression is further described in Chapter 4.

2.4: Treatment – Vitamin A

In 1987, the WHO "recommended that all children with MV in countries where the MV-associated fatality rate is ≥1% should receive 30-60 mg (100,000 – 200,000 IU) of vitamin A" (WHO 1987). Vitamin A is the only "therapy" available for MV infection and it costs only 0.06-0.18$ CDN, making it one of the most cost-effective medical interventions known (Shepard D.S. 1994). The rationale behind the recommended supplementation is described in detail in Chapter 5.

Chapter 3: Vaccination

3.1: Vaccine development

In 1963, the first live, attenuated MV vaccine that was called Edmonston B was developed by Enders *et al.* This vaccine induced excellent antibody responses and conferred immunity to the natural disease (Redd S.C. *et al* 1999). However, 50% of those who received this vaccine developed a rash, with high fever in 20-40% (CDC 1995). As a result, the Edmonston B vaccine was often administered with immunoglobulin (Ig) to reduce the frequency of side effects. Its use was discontinued in 1975 with the development of the further attenuated MV vaccines (Redd S.C. *et al* 1999). Between 1963 and 1967 a killed vaccine that induced satisfactory levels of neutralizing antibodies was also widely used (CDC 1995). Unfortunately, approximately 15% of subjects who received the killed product were susceptible to a new and severe form of measles, called atypical measles, upon subsequent exposure to wild type virus (Griffin D.E. *et al* 1994). Uncertainty about the precise mechanism that underlines atypical disease has complicated vaccine development. The original theory that imbalances in neutralizing antibody levels to hemagglutinin and fusion proteins somehow 'primed' the children for atypical measles (Norrby E. *et al* 1975a,b) has recently been contradicted by studies in rhesus macaques. These contemporary studies suggest that atypical measles results from immune complex deposition associated with systemic and pulmonary eosinophilia (Aueraerter P.G *et al* 1999, Polack F.P. *et al* 1999). A recently developed DNA vaccine containing only the hemagglutinin gene has been shown to protect monkeys without making them susceptible to atypical measles (Polack F.P. *et al* 2000). This novel DNA vaccine offers hope that the obstacles slowing new vaccine development (e.g.: maternal antibodies, atypical measles) can be overcome.

3.2: Present-day situation

Currently, several distinct further attenuated vaccines are in use throughout the world although most are derived from the original Edmonston strain isolated by Enders (e.g.: Schwarz, Moraten) (Griffin D.E. *et al* 1994). Whether all MV vaccine strains are equally immunogenic is a subject of current research but all can be used to effectively control MV (CDC 1995). MV vaccines can be administered alone but they are most

commonly administered in combination with mumps and rubella vaccines (MMR) in the developed world (CDC 1995). MV vaccine must be administered as early as possible since the highest mortality rates occur in children less than 1 year of age (Polack F.P. *et al* 2000). However, early vaccination is complicated by the presence of maternal antibodies that can neutralize the vaccine virus (Albrecht P. *et al* 1977, Gans H.A. *et al* 1998). As a result, MV vaccination is typically delayed until 9-12 months of age. In regions experiencing MV epidemics, children are typically vaccinated as early as 6 months of age (CDC 1995). Although high titered MV vaccines developed in the late 1980s could successfully induce seroconversion in children as early as 3-4 months of age, these vaccines also proved to be immunopathologic in as yet poorly understood ways (Holt E.A. *et al* 1993, León M.E. *et al* 1993). The use of these vaccines was discontinued in 1993 (Griffin D.E. *et al* 1994).

Standard MMR vaccine typically has a 90-95% efficacy rate in the developed world and the systematic use of these vaccines has been successful in decreasing MV-associated morbidity and mortality by ~95% (Ito M. *et al* 1997a,b). However, outbreaks of MV continue to occur in highly vaccinated populations, suggesting that the protection from the vaccine may decrease over time (Griffin D.E. *et al* 1994). A more thorough understanding of the correlates of immunity following both natural MV infection and MV vaccination is needed to develop vaccines that will provide more durable immunity (Griffin D.E. *et al* 1994).

3.3: Co-administration of vitamin A supplements

As discussed in more detail in chapter 5, VAD is associated with significant morbidity and mortality in young children in many parts of the world (WHO/OMS 2001). Since vitamin A supplements can reduce overall infant mortality by ~23% (Fawzi W.W. *et al* 1993), the WHO currently recommends that children ≥6 months old living in areas with VAD receive vitamin A supplements once every 4-6 months (WHO/EPI, 1994). At the 1987 WHO Expanded Programme on Immunization (EPI) global advisory meeting, the possibility of administering vitamin A supplements using delivery systems established for routine immunization programs was raised (WHO/EPI, 1987). The timing of the EPI MV vaccination (6-19 months of age) made it the most likely

"partner". However, vitamin A supplementation in even younger children could have been achieved by "partnering" with the DPT/OPV vaccines (6-14 weeks of age) (WHO/CHD 1998).

Vitamin A is a potent immunomodulator with the potential to influence (for good or ill) the efficacy of any vaccine, including the live, attenuated MV vaccine. In 1995, Semba *et al* reported reduced seroconversion in 6-month-olds who received vitamin A supplements at the same time as their MV vaccine. This effect was limited to children who had maternal antibodies at the time of vaccination (Semba R. *et al* 1995). Since then, several studies in 9- to 12-month-olds have shown either no changes (de Francisco A. *et al* 1996, Benn C.S. *et al* 1997, Semba R. *et al* 1997, Bahl R. *et al* 1999, Arya S. *et al* 2000) or increased seroconversion (Bhaskaram P. *et al* 1997) in children receiving similar doses of vitamin A at the same time as the MV vaccine. The presence or absence of maternal antibodies at the time of vaccination had no apparent effect in these later studies. To date, only one multi-center trial has examined the safety and potential benefit of early administration of vitamin A supplements partnered with the DPT/OPV vaccines. Although these investigators concluded that vitamin A supplements could be administered safely with these vaccines, little benefit was seen (WHO/CHD 1998). At the current time, administration of vitamin A within the EPI continues, with ongoing studies to look for any adverse effects.

Chapter 4: Correlates of immunity

Although the correlates of immunity for both natural MV infection and MV vaccination are poorly understood, there is both *in vivo* and *in vitro* evidence that natural disease initiates a strong cell-mediated response that rapidly switches to a predominantly humoral response (Ward B.J. *et al* 1991, 1995). In contrast, a limited amount of data suggests that MV vaccine induces a strong antibody response almost immediately (Ward B.J. *et al* 1993).

4.1: Humoral immune response

Both natural MV infection and MV vaccine generate strong antibody responses. The first antibodies detected at the time of the rash are predominantly IgM, followed by IgG1 and IgG4 (Griffin D.E. *et al* 1994). Although a large proportion of the humoral response targets the nucleoprotein, neutralizing antibodies are principally directed against the surface proteins, fusion and hemagglutinin (Griffin D.E *et al* 1996). Antibodies clear virus from the blood, induce lysis of infected cells, and can suppress the intracellular synthesis of viral protein and RNA *in vivo* (Griffin D.E. *et al* 1994). High titers of maternal or exogenous antibody (e.g. intravenous Ig) can neutralize both wild-type and vaccine strain viruses (CDC 1995), and there is a strong correlation between titer and protection from infection (Griffin D.E. 1995). However, it is the cell-mediated immune response that appears to play the crucial role in controlling MV infection since children with isolated agammaglobulinemia recover from measles uneventfully and are immune for life (Burnet F.M. 1968). This last observation is important because vaccine efficacy has typically been measured only in terms of antibody production.

4.2 Cell-mediated immune response

Unlike children with agammaglobulinemia, children and adults with defective cell-mediated immunity often suffer progressive and fatal MV infections (50-100%) (Kaplan L.J. *et al* 1992). The rash associated with MV infection is a marker for the cell-mediated immune response since individuals with T-cell defects typically die from measles without developing a rash (Kaplan L.J. *et al* 1992). The relatively underdeveloped or diminished capacity to mount cell-mediated immune responses in the

very young and in the elderly, respectively, may explain why MV-associated mortality is highest in these two groups (Griffin D.E. *et al* 1994).

During the course of a natural MV infection, there is evidence of extensive lymphocyte and monocyte activation during the rash (Ward B.J. *et al* 1990). It is believed that the $CD8^+$ cytotoxic T lymphocytes targeting cells expressing viral peptides play a critical role at this stage, although no direct evidence is available to date (Griffin D.E. *et al* 1993). There is also proliferation and pronounced activation of $CD4^+$ T cells during this phase. These cells provide B cell help and activate effector cells (e.g. $CD8^+$ T cells, LAK) which clear the virus from the tissues through the production of cytokines (Griffin D.E. *et al* 1993). Vaccination does not appear to induce such a strong cell-mediated response. Although there are measurable $CD4^+$ and $CD8^+$ cytolytic responses after vaccination, only ~50% of those vaccinated have detectable lymphoproliferative responses (Gans H.A *et al* 1999, Pabst H.F. *et al* 1999, Bautista-López N. *et al* 2000).

4.3: Immunosuppression

MV is potently immunosuppressive. Much of the MV-associated morbidity and mortality is directly attributable to this immunosuppression (e.g. secondary bacterial infections are the most common cause of death) (Smedman L.A. *et al* 1994, Ward B.J. *et al* 1995). A general suppression of cell-mediated immunity follows MV infection (Smedman L.A. *et al* 1994). This manifests as profound lymphopenia during the rash phase of the disease (Griffin D.E. *et al* 1986), reduction in delayed-type hypersensitivity (DTH) (Smedman L.A. *et al* 1994) and natural killer activity (Griffin D.E. *et al* 1990b), and lowered *in vitro* proliferative response by peripheral blood mononuclear cells (PBMC) in response to mitogens and antigens (Yanagi Y. *et al* 1992). Although there is an overall decrease in T lymphocyte numbers, the ratio of $CD4^+$ to $CD8^+$ cells is unchanged (Griffin D.E. *et al* 1994). Acute disease is also associated with marked changes in the cytokine production pattern (Ito M. *et al* 1997a)

MV vaccination is also associated with a wide range of immunologic changes. These induce temporary loss of cutaneous reactivity (e.g. to tuberculin), leukopenia, atypical lymphocytosis, altered cytokine production and reductions in lymphoproliferation, DTH, and neutrophil function (Smedman L.A. *et al* 1994).

Factors that may contribute to MV-associated immunosuppression include altered cytokine production patterns (Karp C.L. *et al* 1996), soluble suppressors (Sun X. *et al* 1998), changes in co-stimulatory molecule expression (Ravanel K. *et al* 1997), cell-cycle arrest (Schnorr J.J. *et al* 1997b, Naniche D. *et al* 1999) and apoptosis of immune cells (Fugier-Vivier I. *et al* 1997). Another element that may plausibly contribute to measles-induced immunosuppression is the decreased levels of serum retinol, or vitamin A, induced by infection with MV (Hussey G.D. *et al* 1992). VAD in itself is associated with a general suppression of the immune system. Apoptosis and VAD related to MV infection will be discussed further in subsequent sections of chapters 4 and 5 respectively.

4.4: Apoptosis

Apoptosis, or programmed cell death (PCD), is an important process for normal embryological development, the maintenance of homeostasis, and the maturation and regulation of the immune system (McDonnell T.J. *et al* 1996, Hoffmann K. 1999). Dysregulation of apoptosis is associated with a wide range of human diseases, such as cancer, neurodegenerative disorders (e.g.: Alzheimer's and Parkinson's diseases), and the immunodeficiency syndrome associated with human immunodeficiency virus (HIV) (Ashkenazi A. *et al* 1998, Pignata C. *et al* 1998).

A number of viruses that cause persistent infections, such as adenovirus, Epstein-Barr virus, herpesvirus, poxvirus, and hepatitis B virus, have been shown to prevent or delay apoptosis (Rao L. *et al* 1992, Henderson S. *et al* 1993, Krilov L.R. *et al* 2000, Pan J. *et al* 2001). Recently, viruses such as HIV, influenza, varicella-zoster and MV have been shown to trigger apoptosis of human immune cells (Meyaard L. *et al* 1992, Takizawa T. *et al* 1993, Pignata C. *et al* 1998).

MV, as stated previously, causes marked immunosuppression in infected individuals. Both lymphopenia and transient loss of T cell memory (e.g. DTH) responses may plausibly be attributed at least in part to MV-induced apoptosis of both infected and uninfected immune cells (Wesley A. *et al* 1978, Tamashiro V.G. *et al* 1987). Spontaneous apoptosis of PBMC has been reported for weeks to months after natural MV infection, in both infected and non-infected CD4+ and CD8+ T cells, B cells,

neutrophils and monocytes (Pignata C. *et al* 1998, Okada H. *et al* 2000). MV-induced apoptosis has also been detected in the brain tissues of subacute sclerosing panencephalitis (SSPE) patients (Mcquaid S. *et al* 1997) and during intracerebral infections in CD46 transgenic mice (Evlashev A. *et al* 2000). MV infection in a HuSCID mouse model can cause massive apoptosis of thymocytes (Auwaerter P.G. *et al* 1996) and *in vitro* studies have documented MV-induced apoptosis in monocytes (Esolen L.M. *et al* 1995), follicular dendritic cells (Fugier-Vivier I. *et al* 1997, Bhardwaj N. 1997, Servet-Delprat C. *et al* 2000), and isolated T-lymphocytes (Addae M.M. *et al* 1995). Despite these observations, the role of PCD in the immunosuppression associated with MV infections remains controversial since only modest levels of MV-induced apoptosis have been reported in human lymphoid cell lines and peripheral blood lymphocytes (Ito M. *et al* 1997b, Schnorr J.J. *et al* 1997a,b).

Chapter 5: Vitamin A

5.1: Sources and functions

Fat soluble A, now called vitamin A, was first described by Davis and McCollum in 1913 (McCollum E.V. *et al* 1913). Vitamin A is found naturally in the diet, predominantly in the form of retinyl esters in liver, poultry, eggs, ham, and dairy products. It can also be obtained in the form of provitamin A carotenoids in green and leafy vegetables and colorful fruits, such as spinach, carrots, citrus fruits, mangos and cantaloupe (Olson J.A. 1994). There are over 300 naturally occurring retinoids in the human diet. Retinol (ROH), all-trans retinoic acid (atRA) and 9-cis retinoic acid (9cRA) are believed to be the most active non-synthetic retinoids in the human biological system (Napoli J.L. 1993).

Vitamin A is vital to the health and survival of humans and other animals. It plays a role in many essential functions including regulation of immunity, embryonic development (Ross A.C. *et al* 1994, Means A.L. *et al* 1995), growth and development of hematopoietic and bone cells (Sporn M.B. *et al* 1994), maintenance of epithelial surface integrity (Dawson M.I. 2000), and vision (WHO/CHD 1998, Sommer A. 1994). VAD is one of the leading causes of blindness in the developing world, causing eye damage in 2-3 million pre-school children each year (WHO/EPI 1994). Early VAD manifests as night blindness, reflecting the requirement for vitamin A in rhodopsin, the visual pigment needed for vision under low levels of illumination (Sommer A. 1994). If VAD is severe and prolonged, ocular deterioration and corneal ulceration can lead to blindness. All but the final stages of corneal degeneration can be rapidly and completely reversed with a single dose of vitamin A (Sommer A. 1994).

Vitamin A has also been shown to have anti-tumor activities. Retinoids can retard or reduce the incidence of epithelial tumors, including skin, lung, bladder, trachea, oral mucosa (Shalinsky D.R. *et al* 1997), and mammary gland (Gottardis M.M. *et al* 1996). Retinoids can also inhibit the growth of several transplantable tumors (Zhang L. *et al* 1996, Makishima M. *et al* 1999). For the past 15 years, 13-cis retinoic acid has been used as therapy for certain cancers (Smith M.A. *et al* 1992, Boehm M.F. *et al* 1995).

5.2: Vitamin A in immunity

Vitamin A plays an important role in developing mucosal, cell-mediated, and humoral immune responses to infections and has long been recognized as an "anti-infective" nutritional factor (Green H.N. *et al* 1928). Children with even mild VAD have a relatively depressed immune response, with fewer $CD4^+$ T cells and a lower CD4:CD8 ratio (Semba R.D. *et al* 1992, 1993). Mucosal immunity and secretory IgA levels also appear to be depressed in children with VAD (Semba R.D. 1994).

Results from animal studies clearly show that VAD animals have impaired immune responses, including reduced antibody responses (Kinoshita M. *et al* 1991, Wiederman U. *et al* 1993), a reduction in NK cell activity (Bowman T.A. *et al* 1990), decreased T cell proliferative responses (Sklan D. *et al* 1989), and impaired mucosal immunity (Wiedermann U. *et al* 1995). Replenishment of animals with vitamin A reverses these effects.

In vitro treatment of cord blood mononuclear cells (CBMC) and PBMC with vitamin A in the form of ROH or RA improves Ig synthesis through actions on T cells (Israel H. *et al* 1991, Yagi J. *et al* 1997). Pharmacological doses of vitamin A improve post-operative lymphocyte proliferation (Cohen B.E. *et al* 1979) and reverse post-burn immunosuppression (Fusi S. *et al* 1984). Several vitamin A supplementation trials, administering 100,000 to 200,000 IU of vitamin A to 4 month to 6 year old infants, corroborate the *in vitro* and animal model studies (Fawzi W.W. *et al* 1993, WHO/CHD 1998). Children whose serum ROH levels are adequate following supplementation have larger DTH responses (WHO/CHD 1998), an increase in total lymphocyte counts and Ig levels (Coutsoudis A. *et al* 1992), higher numbers of circulating CD4+ T cells and higher CD4:CD8 ratios (Semba R.D. *et al* 1993). These findings strongly suggest that vitamin A has considerable importance in the immune response to infection.

5.3: Vitamin A deficiency:

VAD is associated with the death of over 1 million children each year, due to blindness and increased severity of infections. It is a public health problem in at least 73 countries worldwide (WHO/EPI 1994). Although malnutrition is the leading cause of VAD, many diseases, including diarrhea and respiratory disease (Sommer A. *et al* 1983),

dysentery (Sommer A. *et al* 1987), measles (Hussey G.D. *et al* 1992), and parasitic infections (Friis T. *et al* 1996) induce either temporary or long-term VAD in infected individuals.

Overall, mortality rates of 6-month-old to 5-year-old children living in areas where VAD is a problem can be reduced by ~23% with vitamin A supplementation (Fawzi W.W. *et al* 1993). At this time, the WHO recommends that children ≥6 months old living in areas with VAD receive a vitamin A supplement once every 4-6 months (WHO/EPI 1994). Studies on the benefits and safety of vitamin A supplementation in children < 6 months old are currently underway. Preliminary results suggest that early supplementation is safe but has little impact on long-term vitamin A status or survival, at least in the short-term (WHO/CHD 1998).

5.4: Measles and vitamin A deficiency

Of particular interest to these studies, MV is now accepted as a risk factor for the development of VAD, with severe VAD occurring in as many as one-third of children with natural MV infections (Varavithya W. *et al* 1986). Measles patients have lower serum ROH and retinol binding protein (RBP) levels compared to healthy controls due to decreased absorption and increased utilization of vitamin A, which depletes liver stores (Coutsoudis A. *et al* 1991). This raises the possibility of a malignant synergism between the immunosuppression of MV infection itself and the immunologic changes associated with VAD.

5.5: Measles and vitamin A supplementation

One of the earliest trials to investigate the effect of vitamin A supplementation on MV-associated morbidity and mortality was conducted by Ellison in London, England in 1932. Because VAD had been shown to affect epithelial surfaces and the respiratory tract in experimental animals, Ellison reasoned that administration of vitamin A might be used to treat children with MV infection. In this hospital-based trial, vitamin A (300 IU) was given daily for 1-3 weeks and reduced MV-associated mortality by ~50%, from 8.7% in the untreated group to 3.7% in the treated group (Ellison J.B. 1932). This observation was essentially forgotten until 1983 when Sommer *et al* observed increased

mortality in Indonesian children with mild MV infection. This "rediscovery" led to several large clinical trials in developing countries around the world that investigated anew the benefits of vitamin A supplementation (West K.P. *et al* 1991, Herrera M.G. *et al* 1992, Rahmathullah L. *et al* 1990, Sommer A. *et al* 1986, Vijayaraghavan K. *et al* 1990, Daulaire N.M *et al* 1992, Muhilal *et al* 1988, and Kothari G. 1991). As stated previously, meta-analyses on these trials show a reduction in childhood mortality of ~23% in children living in areas where MV infection is a problem (Fawzi et al 1993). Several of these trials, involving >200,000 children, showed a significant decrease in MV mortality with vitamin A supplementation (Barclay A.J. *et al* 1987, Hussey G.D. *et al* 1990, Coutsoudis A. *et al* 1991). In 1987, Barclay *et al* essentially repeated Ellison's 1932 study and documented a 50% decrease in MV mortality in supplemented children. Even greater reductions in MV mortality upon supplementation with vitamin A (~80%) have been observed in at least one well designed study (Hussey G.D. *et al* 1990).

In 1987, in response to results from these clinical trials, the WHO/UNICEF recommended that "all children with measles in countries with measles fatality rates >1% should receive 30-60mg (100,000 – 200,000 IU) of vitamin A" (WHO/UNICEF 1987). This "therapy" costs ~0.06-0.18$ CND per dose (Shepard 1994). As such, vitamin A supplementation for acute MV infection is one of the most cost-effective therapeutic interventions known.

Chapter 6: Vitamin A signaling cascade

6.1: From food to cells

Following ingestion, retinoids and provitamin A carotenoids are converted to retinyl esters by the intestinal enterocytes. These retinyl esters are packaged into chylomicrons and transported via lymphatic circulation to the liver (Ong D.E. 1993). Over 80% of the body's retinoids are stored in the liver as retinyl esters (Napoli J.L 1996). Vitamin A is transported to various other organs and tissues in the form of ROH, although trace levels of other retinoids, such as RA, can be found in the serum (Napoli J.L. 1996). Upon release from the liver, ROH is transported in the plasma bound to RBP and transthyretin in a trimolecular complex (Ong D.E. 1993).

6.2: Uptake and conversion to active metabolites

Uptake into target cells is believed to be mediated through an as yet unidentified RBP cell surface receptor (Bavik C.-O. *et al* 1992). Upon entry into a cell, ROH is converted to various forms of the active RAs. The types of retinoids produced and the resultant signaling pathways used depend on numerous factors, including the cell type and the stage of development (Napoli J.L. 1996). For example, atRA and its isomer, 9cRA, are the most common retinoids produced, found in nearly every cell type. On the other hand, 11-cis retinal is a component of the chromophores found in photoreceptors in the eye. Therefore, RAs likely mediate global changes in the body whereas retinal is only involved in improving vision (Giguere V. 1994).

6.3: Cytoplasmic binding proteins

Because retinoids are hydrophobic molecules, they are transported through the aqueous cellular environment by a system of small retinoid binding proteins (Giguere V. 1994). Aside from RBP, which is involved in transportation of ROH through the plasma, there are two other families of retinoid binding proteins: two cellular retinol-binding proteins (CRBP-I and -II) and two cellular retinoic acid-binding proteins (CRABP-I and –II). These cytoplasmic chaperone molecules play an important role in the catalytic conversion of ROH into its metabolically active derivatives (Giguere V. 1994). Individually, these proteins are differentially expressed and mediate different processes

(Ong D.E. 1994). CRBP-I is widely expressed in adult tissues and serves as a substrate for a specific NADP-dependent dehydrogenase that converts ROH to retinal. Retinal can then be modified by cytosolic retinal dehydrogenase leading to production of atRA (Posch K.C. *et al* 1992). CRBP-II expression is restricted to the small intestine where it directs the esterification of ROH for storage via lecithin:retinol acetyltransferase (Ong D.E. 1994). CRABP-I functions as a carrier substrate for the catabolism of atRA to more polar metabolites by cytochrome p450 (Fiorella P.D. *et al* 1991). The function of CRABP-II is currently unknown. Neither CRABP-I nor CRABP-II bind 9cRA, suggesting that other as yet identified carrier proteins may be involved in a distinct metabolic pathway for the cis-retinoids (Ong D.E. 1994).

6.4: Nuclear retinoid receptors

Once at the nucleus, the RAs bind to one of six members of the steroid and thyroid hormone receptor superfamily. The retinoic acid receptors (RAR α, β, or γ) bind atRA at high affinity (Kd = 1-5nM) and 9cRA at a lower affinity (Kd = 0.2-0.7nM) while the retinoid X receptors (RXR α, β, or γ) bind strongly to 9cRA (Kd = 10nM) (Mangelsdorf D.J. 1994).

The retinoid receptors are ligand-activated transcription factors that bind DNA as dimers and modulate gene expression downstream of specific hormone response elements (Chambon P. 1996). They can be divided into 4 functional regions. The carboxy-terminal domain is the ligand-binding and dimerization domain. Following this region, there is a hinge domain which, based on studies of other hormone receptors, may be a nuclear localization signal. Next is the DNA-binding domain with its two zinc finger motifs. Finally, the amino terminal domain has transactivating capabilities and seems to work upon cell- and promoter-specific activation (Chambon P. 1996). Overall, the RARs and RXRs show greater than 91% homology in the DNA-binding domain and over 85% homology in the ligand-binding domain. Structurally, the RXR are very similar to the RARs and share high homology in the DNA (92%) and ligand (87%) binding domains (Chambon P. 1996).

The retinoid receptors may be expressed widely (e.g. RAR-α, RXR-β) or in particular tissues (e.g. RXR-α is found mainly in visceral tissues including lungs and

spleen), adding to the diversity of the retinoid signaling cascade (Leid M. *et al* 1992). Once bound to their ligands, these receptors form RAR-RXR heterodimers or RXR homodimers that initiate or regulate gene transcription by binding directly to retinoid receptor response elements on DNA (Chambon P. 1996). It is important to note that RXR appears to be a key element in signaling cascades for numerous hormones (Mangelsdorf D.J. *et al* 1995). RXR can heterodimerize with the thyroid receptor (T_3R), the vitamin D receptor (VD_3R), and many orphan receptors, including COUP-TF, and PPAR (Mangelsdorf D.J. *et al* 1995).

6.5: Retinoid response elements

Retinoid response elements (RARE and RXRE) are located within the promoter region of target genes. They are comprised of direct repeats (DR) with 1, 2, or 5 nucleotides in between the PuGGTCA half-site or, rarely, of everted repeats of this half-site with 8 spaces (ER8) (Chambon P. 1996). The RAR/RXR heterodimers bind specifically to DR-2 and DR-5 RARE to activate transcription of downstream genes, while the RXR/RXR homodimers bind primarily to DR-1 RXRE (Giguere V. 1994). Some of the genes known to contain such response elements include RAR-α2, RAR-β2, RAR-γ2, rCRBP-I, mCRBP-I, mCRABP-I, ADH_3, ApoA1, MCAD and CMV-IEP (Giguere V. 1994). RAR/RXR heterodimers also bind DR-1 RXRE but this results in repression of transcription of the downstream genes, including rCRBP-II, mCRABP-II, ApoA1 and other genes involved in vitamin A, lipid, and fatty acid metabolism (Giguere V. 1994). It is clear that there is a complex interplay between these two RA signaling cascades and that these signaling cascades involve feedback loops, leading to regulation of key elements (Giguere V. 1994).

6.6: Receptor cross talk

Frequently, there is cross talk between transcription factors, regardless of how they are activated. The retinoid receptors are no exception. The transcriptional enhancer AP-1 is a complex of the proto-oncogenes c-jun and c-fos that is activated by cell growth factors (Gronemeyer H. *et al* 1995). Retinoids can limit cell growth and block certain tumor promotors which is one reason they are so promising as anti-cancer drugs (van der

Saag P.T. 1996). It appears that one of the ways that retinoids repress cell growth is by interacting with AP-1. RAR, but not RXR, can bind c-jun to form nonproductive complexes, thereby decreasing signaling via AP-1 (Pfahl M. *et al* 1996). Some synthetic retinoids repress AP-1 signaling but cannot transactivate via RAREs (Pfahl M. *et al* 1996). AP-1 can also bind hormone response elements in some retinoid-responsive gene promoters, thereby competing for the binding site and decreasing retinoid signaling (Gronemeyer H. *et al* 1995, Schüle R. *et al* 1990).

6.7: Pleiomorphism of signaling cascade

This chapter clearly illustrates the multiple levels of diversity in the molecules involved in retinoid signaling. There are multiple biologically active retinoids, different cytoplasmic carrier proteins, different nuclear retinoid receptors, different dimers, and different DNA binding domains. All of this diversity means that a simple "vitamin" can ultimately trigger the transmission of many different signals in the body's different cells, allowing it to control and direct such vital and complex functions as embryogenesis or immunity.

SECTION II:

MANUSCRIPT I, MANUSCRIPT II, ADDITIONAL STUDIES

Connecting Statement I:

For the first manuscript in this thesis, the focus moves back to measles virus (MV) itself and, more specifically, to the immunosuppression associated with both natural MV infection and vaccination. The mechanism that underlies MV-associated immunosuppression is, as yet, poorly understood. In manuscript I, we have demonstrated that MV-induced apoptosis of PBMC likely contributes to this immunosuppression. We also use a panel of wild-type and vaccine strains to compare their ability to replicate and induce apoptosis in PBMC.

Chapter 7 – Manuscript I

Measles-induced Apoptosis in Human Peripheral Blood Mononuclear Cells

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Running head: Measles-induced apoptosis

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7.1: Abstract

Natural measles infection is associated with profound lymphopenia and immuno-suppression for weeks to months after infection. Spontaneous apoptosis in peripheral blood mononuclear cells (PBMC) has been noted for months after natural disease. We examined the kinetics and the role of viral strain on apoptosis in measles-infected PBMC using a panel of wild type and vaccine-strain viruses. Apoptosis induced by wild-type virus (Chicago-1) was first detectable 24 hours after infection, was substantial at 72 hours (67% ± 6%) and was massive by 96 hours (90 ± 11%). Replicating virus was required since little increase in cell death was seen in the absence of mitogen (PHA) or with inactivated virus. The induction of apoptosis was generally correlated with the rate of viral replication in PBMC ($r = 0.915$ for vaccine strains; $r = 0.981$ for wild-type viruses). However, several viral strains (vaccine and wild-type) induced high levels of apoptosis with little or no production of infectious viral particles. The induction of apoptosis was not directly related to the rate of PBMC proliferation and all MV strains completely inhibited mitogen-induced lymphoproliferation. Overall, wild-type strains were several-fold more potent than vaccine strains ($p \leq 0.003$) in the induction of apoptosis. These findings may help to explain both the lymphopenia and loss of effector cell function associated with natural measles infection.

7.2: Introduction

Measles virus (MV) causes massive disruption of the human immune system. Although MV can occasionally kill infected individuals outright (e.g.: giant cell encephalomyelitis, pneumonitis, subacute sclerosing panencephalitis (SSPE)) (Griffin D.E. *et al* 1996) most of the morbidity and mortality associated with this infection is thought to result from secondary infections that are facilitated by intense immune suppression (Beckford A.P. *et al* 1985, Griffin D.E. *et al* 1996). MV-induced immune suppression is characterized by massive lymphopenia, decreased natural killer (NK) cell function, poor mitogen responsiveness, loss of antigen-specific lymphoproliferative responses and disappearance of delayed type hypersensitivity (DTH) to recall antigens (Wesley A. *et al* 1978, Griffin D.E. *et al* 1990b, Ward B.J. *et al* 1991, Tamashiro V.G. *et al* 1987). The mechanisms that underlie these abnormalities have received considerable attention during the past decade. Factors that may contribute to MV-associated immune disruption include altered cytokine production patterns (Karp C.L. *et al* 1996, Bell A.F. *et al* 1997), soluble suppressors (Sun X. *et al* 1998), changes in co-stimulatory molecule expression and membrane receptor signaling (Ravanel K. *et al* 1997), altered Vβ T cell receptor usage (Auwaerter P.G. *et al* 1996a), as well as induced stasis (Schnorr J.J. *et al* 1997a, Naniche D. *et al* 1999) and apoptosis of immune cells (Fugier-Vivier I. *et al* 1997). The last mentioned mechanism is of particular interest because of its potential to explain both the massive lymphopenia associated with MV infection (Wesley A. *et al* 1978) and the transient loss of pre-existing immune responses such as DTH (Tamashiro V.G. *et al* 1987).

MV can productively infect a wide range of cells including macrophages, B and T lymphocytes (Smedman L. *et al* 1994). MV-induced apoptosis has been documented in monocytes (Esolen L.M. *et al* 1995), follicular dendritic cells (FDC) (Fugier-Vivier I. *et al* 1997), thymocytes in a HuSCID mouse model (Auwaerter P.G. *et al* 1996) and in brain tissue of SSPE patients (Mcquaid S. *et al* 1997). Furthermore, increased spontaneous PBMC apoptosis has been reported for weeks to months after natural MV infection (Pignata C. *et al* 1998). It is somewhat surprising therefore, that the only published attempt to demonstrate MV-induced apoptosis in isolated human PBMC found little or no effect (Ito M. *et al* 1997b). We describe the development of an *in vitro* model

for MV-induced apoptosis in PBMC. The role of viral strain on apoptosis was evaluated using a panel of wild-type and both partially (Edmonston B) and fully attenuated vaccine-strain viruses. The potential influences of viral replication rates and individual PBMC proliferation rates on the induction of apoptosis were also evaluated.

7.3: Methods:

Measles Strains. A total of 12 virus strains were used in this study: 3 fully attenuated vaccine strains; Edmonston Enders (CN) (gift from R Wittes, Connaught Laboratories LTD, Willowdale, ON), Edmonston-Zagreb (EZ) and Schwarz (SCZ) (gifts from P Rota, Atlanta, GA), one partially attenuated vaccine strain; Edmonston B (EdB) (gift from M Hilleman, Merck Vaccines), and 8 wild type strains; Bilthoven (Bilt) (gift from A Osterhaus, Rotterdam, Holland), Chicago-1 (Chi-1), Massachussetts (Mass), Minnesota (Minn), Utah, Hawaii (Haw), Gambia (Gamb), and Guam (gifts from W Bellini and P Rota, Atlanta, GA). Viruses were plaque purified using African green monkey Vero cells, and re-seeded on Vero monolayers in MEM (Life Technologies, Grand Island, NY) + 5% heat inactivated fetal bovine serum (FBS) (Life Technologies) for 48-72 hours. For each strain, the supernatant was replaced with a minimal amount of fresh media at 50% cytopathic effect (CPE) and monolayers were freeze-thawed at 90-95% CPE. Harvests were pooled, centrifuged at 500 x g for 10 minutes and supernatants were filtered (0.22μm; Corning-Costar, Cambridge, MA). Viral titers were determined by plaque assay. Although the precise passage history was not available for all viral strains, all of the vaccine strains and most of the wild type strains grew well in Vero cells, forming readily visible plaques in only 4-5 days. Guam and Utah wild-type viruses grew least well in Vero cells, but still formed obvious plaques within 6-7 days. Viral stocks were aliquoted and frozen at –76°C until used in assays. For some assays, virus was inactivated by heat (56°C for 30 minutes) or UV-irradiation (120 mJ/cm^2 for 4 minutes; Spectrolinker XL-1000, Spectronics Corporation, New York, NY).

Surface Expression of Measles Proteins. Cell surface expression of MV proteins was detected as previously described (Ward B.J. *et al* 1990). Briefly, immunoperoxidase staining was carried out on cultured PBMC with or without MV infection at 24, 36, 48, 72, and 96 hours using a polyclonal rabbit anti-measles serum. Two- to three- hundred cells were counted on each slide and results are reported as the percentage of PBMC expressing measles proteins on the cytoplasmic membrane.

PBMC isolation and infection. Heparinized blood samples were collected from healthy volunteers and processed within 8 hours of collection. PBMC were isolated by density gradient centrifugation at 300 x g for 30 minutes with Ficoll-Paque (Pharmacia LKB, Piscataway, NJ) as previously described (Ward B.J. *et al* 1995) and resuspended in freezing media (92.5% FBS + 7.5% dimethyl sulfoxide (DMSO; American Chemicals, Montreal, QC)) for cryopreservation in liquid nitrogen. PBMC were thawed and washed twice in HBSS (Wisent, St. Bruno, QC) and viable cells were stained with trypan blue and counted by hemocytometer. 1 x 10^6 PBMC in MEM were infected with vaccine strain or wild-type virus at an m.o.i. of 1.0. Virus particles were permitted to attach for 1.5 hours at 37°C with 5% CO_2. Infected cells were then washed once in HBSS, stimulated with phytohemagglutinin (PHA: 2.5μg/mL; Sigma Fine Chemicals, Oakville, ON) and cultured in 1mL of RPMI with 10mM HEPES, 2mM glutamine, 50μg/mL gentamicin (all Wisent) and 5% heat-inactivated FBS at 37°C and 5% CO_2 for up to 5 days. PBMC were tested for apoptosis and supernatants were collected to quantify viral titers for vaccine and wild-type viruses at various times after infection. Mock-infected PBMC served as negative controls.

Flow cytometry and TUNEL Assay. PBMC were harvested, washed twice in PBS, resuspended in PBS containing 5% bovine serum albumin (BSA; Sigma, St. Louis, MO) and stained with FITC or PerCP-conjugated monoclonal antibodies to CD4, CD8, CD14, CD19 (Becton Dickinson, San Jose, CA) and CD95 (Dako, Toronto, ON) according to the manufacturer's instructions. Labeled cells were washed with ice cold PBS and resuspended in PBS containing 1% paraformaldehyde and 0.05% sodium azide (American Chemicals). The TUNEL assay was used to detect cells undergoing apoptosis. Briefly, control and infected cells were washed, permeabilized with 0.1% Triton X-100 (ACP Chemicals Inc., Montreal, QC) in 0.1% sodium citrate (Fisher Scientific, Oakville, ON) for 2 minutes on ice. Cells were then washed twice in PBS and resuspended in 50μl of TUNEL reaction mixture: terminal deoxytransferase (TdT, 0.3U/ml Pharmingen, Mississauga, ON); dUTP-biotin (0.01nmol/ml Boerhinger Mannheim, Toronto, ON); TUNEL reaction buffer (200 mM potassium cacodylate, 25mM Tris-HCL, 1mM $CoCl_2$ Pharmingen, Mississauga, ON) and incubated for 1 hour

at 37°C. After washing, PBMC were blocked with 2% BSA for 15 minutes at 37°C. PBMC were then washes and incubated in the dark with 5μL of streptavidin-PE (Dako Diagnostics) for 30 minutes at 37°C. After washing dUTP incorporation was measured by flow cytometry within 2 hours of staining and data was analyzed using CellQuest software (Becton Dickinson, Mississauga, ON). PBMC exposed to ultraviolet radiation (UVR; 120 mJ/cm^2 for 4 minutes, using the Spectrolinker XL-1000) and processed as above were used as positive controls for apoptosis induction throughout. Results are expressed as the percent of total PBMC or individual PBMC subsets undergoing apoptosis.

Plaque Assay. Viral titers in PBMC cultures and stock virus preparations were measured by plaque assay. Briefly, 85% confluent Vero cell monolayers in 24-well plates (Corning-Costar) were washed with HBSS and infected with 100 μL of serial 10-fold dilutions of culture supernatant for 90 minutes at 37°C in 5% CO_2 (duplicate wells, 100μL/well diluted in HBSS). A 16% methylcellulose (ICN, Aurora, Ohio) overlay was applied to each well (1 mL) and plates were incubated at 37°C in 5% CO_2 for a further 4-5 days. The Vero monolayers were stained for 24 hours with 4% neutral red (ICN) and then fixed with 3.7% formalin (American Chemicals). Visible plaques in duplicate wells were counted and results are expressed as plaque forming units per mL (pfu/mL).

Lymphoproliferation Assay (LPA):

Lymphoproliferation of infected and control PBMC was evaluated as previously described (Ward B.J. *et al* 1995), although a new harvester (Tomtec harvester 96, Tomtec Inc., Hamden, CT) and beta-counter (Microbeta 1450, Wallec, Turku, Finland) were used. PBMC were stimulated with PHA (2.5μg/mL) and infected at an m.o.i. of 1 with different MV viral strains (Chi-1, Bilt, CN, E-Z or no MV). The incorporation of ^{3}H-thymidine was measured after a 48-hour infection. Uninfected and unstimulated cells served as controls. Results are reported as counts per minute (cpm) in MV-infected cells.

Statistical analysis:

Comparisons between and amongst viral strains were made using Student's t test (two-tailed). Correlations were evaluated using the Pearson test. Unless otherwise indicated, results are expressed as mean ± S.E.M.. Values were considered significant at $p \leq 0.05$.

7.4: Results:

Measles-induced apoptosis in PBMC

In the absence of PHA stimulation, relatively few PBMC underwent apoptosis during the first 72 hours in culture either with (12 ± 1%) or without (10 ± 1%) MV infection. Background apoptosis at 72 hours after mitogen stimulation was also low in mock-infected cultures (7.8 ± 0.9%; range 1 – 12%), but increased dramatically with MV infection (e.g. CHI-1 mean 54.5% ± 4%; range 39 – 96%, $p \leq 0.003$) (Figure 1). Viral replication was required to induce programmed cell death as neither heat inactivated nor UV-irradiated virus increased apoptosis above background levels (Figure 2). Very few cells (< 5%; range 0 – 5.1%) were found to express MV proteins in the absence of mitogen stimulation. With PHA, however, intense staining of PBMC was evident by 36 hours after infection (15 ± 4%; range 8 – 24%) and by 72 – 96 hours virtually 100% of PBMC were found to express MV proteins (97 ± 2%). Although all PBMC subsets were susceptible to MV-induced apoptosis, T cells ($CD4^+$ and $CD8^+$) and B cells ($CD19^+$) incorporated almost 3-fold more dUTP than monocytes ($CD14^+$) at 24 hours post-infection (33 ± 5%, 32 ± 5% and 24 ± 5% vs. 10 ± 3.5%; $p \leq 0.001$, $p \leq 0.001$, $p \leq 0.03$, respectively) (Table 1). At 72 hours post infection, almost all T and B cells were incorporating dUTP while monocytes lagged significantly behind (Table 1). In many cultures, the only cells remaining alive at 96 hours after infection were monocytes.

Kinetics of measles-induced apoptosis

In CHI-1 infected PBMC, MV-induced apoptosis was first detectable at 24 hours after infection (Figure 3a-f). Incorporation of dUTP increased progressively thereafter to peak between 72 and 96 hours after infection. The vast majority of infected, PHA-stimulated PBMC were dead by 96 hours (Figure 3f) as determined by trypan blue exclusion assay (data not shown). The pattern of induction of apoptosis closely paralleled viral output, which was minimal at 24 hours after infection (e.g.: 1000 ± 200 pfu Chi strain/mL) and peaked at 72 hours (e.g.: 33,000 ± 1000 pfu Chi strain/mL). The dramatic decrease in viral output at 96 hours after infection likely reflects a lack of living cells in which the virus can replicate.

Surface expression of Fas

Expression of CD95 on MVi/Chicago.USA/1-infected cells rose from a baseline level of 22 - 38% at the time of infection to almost 100% by 48 - 72 hours (Figure 4). Although CD95 expression on PBMC also rose following mock infection, the intensity of staining in mock-infected cultures was significantly lower than expression on infected cells throughout the four-day period of observation (Figure 4).

Effect of measles strain on apoptosis

The degree of MV-induced apoptosis varied considerably between the strains tested. For example, one of the wild type strains (Hawaii) consistently induced only modest amounts of apoptosis (16% ± 4%) compared with the Bilthoven (67 ± 4%; $p \leq 0.001$) and Guam strains (81 ± 3%; $p \leq 0.001$) (Figure 5). Overall, the wild-type strains induced ~2 – 3 fold higher rates of apoptosis in PBMC than the vaccine strains (40.5 ± 6% vs. 15.5 ± 2% at 72 hours in culture; $p \leq 0.003$). It is interesting that the partially attenuated vaccine strain, EdB, induced intermediate levels of apoptosis (26 ± 4%) (Figure 5).

Effect of measles strain on viral output and PBMC proliferation

The level of viral output from MV-infected PBMC also varied considerably between the strains tested and correlated roughly with the induction of apoptosis for both wild-type ($r = 0.915$) and vaccine strains ($r = 0.981$) (Figure 6). However, several viral strains (vaccine and wild-type) induced high levels of apoptosis with little or no production of infectious viral particles.

Proliferation of PBMC in the absence of PHA stimulation was negligible (80 ± 19 cpm) when compared to PHA-stimulated cells (30183 ± 1600 cpm). Infection of PBMC with MV significantly reduced cell proliferation for any of the strains tested (avg. 2094 ± 206, $p < 0.0001$, student's t test). However, the Chi-1 wild-type strain consistently induced the greatest reduction in proliferation (Chi-1 118 ± 10 cpm *vs.* Bilt 2034 ± 407 cpm; CN 2570 ± 502 cpm; E-Z 3335 ± 775 cpm). There is no overall correlation between proliferation rate and apoptosis or viral output.

Effect of PBMC origin on apoptosis, viral output and proliferation

All of the outputs described in this paper varied somewhat with the origin of the PBMC used in any given experiment; however, the rate of MV-induced apoptosis in each donor's PBMC remained stable over time (Figure 7). For both apoptosis and viral output, a rank order of potency between virus strains was generally observed in different samples within an experiment and between experiments using samples from the same individual performed on different days. The rank orders for viral output and apoptosis correlated closely. For example, E-Z virus consistently had higher viral output and induced more apoptosis than CN, regardless of the PBMC origin. Conversely, individuals with the highest levels of viral output and apoptosis for one virus had the highest readouts for the other virus strains as well (data not shown). The proliferation of PBMC also varied somewhat between individuals, with a general rank order maintained for the different virus strains. However, no clear relation between the rank order for proliferation and those for viral output and apoptosis was detected.

7.5: Discussion:

The multifactorial nature of the immunosuppression that occurs in natural MV infection is well established, including the reduction of IL-12, the production of inhibitory factors and the induction of cell cycle arrest (Karp C.L. *et al* 1996, Schnorr J.J. *et al* 1997a,b, Sun X.M. *et al* 1998). Elevated levels of spontaneous apoptosis have been reported after natural disease (Pignata C. *et al* 1998) and MV-induced apoptosis has been demonstrated in monocytoid cell lines, thymocytes in HuSCID mice and human dendritic cell cultures (Esolen L.M. *et al* 1995, Auwaerter P.G. *et al* 1996b, Fugier-Vivier I. *et al* 1997). Despite these observations, the role of PCD in the immunosuppression associated with MV infection is controversial (Schnorr J.J. *et al* 1997a) since only modest levels of MV-induced apoptosis have been reported in human lymphoid cell lines and peripheral blood lymphocytes (Ito M. *et al* 1997b, Schnorr J.J. *et al* 1997a).

In the current study, we observed striking levels of apoptosis induced by many wild-type strains of MV. Although vaccine strain viruses were generally less able to initiate PCD in PBMC, there was considerable variability. One of the vaccine strains (the partially attenuated Edmonston B) consistently induced more apoptosis than two of the wild-type strains (Hawaii and Massachusetts). The relative resistance to MV-induced apoptosis that we observed in monocytes suggests that these cells may act as viral reservoirs *in vivo*. In this context, it is interesting that Esolen *et al* found MV nucleic acid only in the monocyte fraction of PBMC isolated from children with natural disease (Esolen L.M. *et al* 1993).

The virus strains we studied also differed in their ability to grow in PHA-stimulated human PBMC. This did not appear to be correlated to differences in PBMC proliferation in the presence of PHA stimulation. The wild-type strains inducing the highest levels of apoptosis (Bilthoven, Chicago-1) were among the viruses that replicated most effectively, while those virus strains inducing low levels of apoptosis (Gambia, Massachusetts) had barely detectable viral outputs. Although some of these variation may be explained by the relative adaptation of the various wild-type and vaccine strain viruses to tissue culture, there was no simple relationship between passage history and induction of PBMC apoptosis. The highest viral output observed was for a

vaccine strain (Edmonston-Zagreb) that induced much lower levels of apoptosis than most of the wild-type viruses. Other studies have recently shown that different strains of MV differ in their pathogenicity in Rhesus Macaques (Auwaerter P.G. *et al* 1999) and in their ability to replicate in and damage human thymocytes in a HuSCID mouse model (Valsamakis A. *et al* 1999, 2001).

Another source of variability in this study was the source of the PBMC used in individual experiments. Both apoptosis and viral output varied considerably in the PBMC obtained from different individuals. The differences between individuals were independent of viral strain (i.e. the relative potency of the various strains was maintained) and appeared to be stable characteristics of the individuals whose blood cells were used over a period of 10 - 12 months. PBMC proliferation in response to PHA also varied widely between individuals but was not correlated with differences in either viral output or apoptosis. It is interesting that Tishon and Oldstone have recently reported the relative superiority of cord blood lymphocytes to support viral growth (Tishon A. *et al* 1996) supporting a role for a "host" or "age" factor. We do not yet know if the individual differences in viral-induced apoptosis reflect permissiveness of the host PBMC (i.e. higher viral output/cell) or are adaptive (i.e. lower viral output/cell) but these studies are currently in progress. At the very least, our observations of host and virus strain variability in the induction of PCD in PBMC help to explain the differences in MV-induced apoptosis reported in literature (Ito M. *et al* 1997b, Schnorr J.J. *et al* 1997a, Fugier-Vivier I. *et al* 1997). Identification of the host factors involved in susceptibility to apoptosis induction may also give insight into the well known variability of natural disease (i.e. clinical presentation, immunosuppression, complications) (Griffin D.E. *et al* 1996) and possibly the increased mortality associated with high titer MV vaccines (Halsey N.A. 1993, Knudsen K.M. *et al* 1996).

The mechanism of MV-induced apoptosis remains unknown. Unlike HIV, SIV and HTLV-1 (Adamson D. *et al* 1996, Yamada T. *et al* 1994, Li C.J. *et al* 1995), receptor cross-linking alone is not sufficient stimulus to trigger the cell death cascade, as UV- and heat-inactivated MV were incapable of inducing apoptosis. In the case of MV, viral protein expression and replication seem to be requisite events before the apoptotic machinery is activated, as has been previously suggested (Fugier-Vivier I. *et al* 1997,

Schnorr J.J. *et al* 1997b). The upregulation of CD95 in our model suggests a possible mechanism by which measles signals the cellular death cascade. Although elevated CD95 expression was not found on PBMC by Pignata *et al* in their study of natural measles infection, it is unclear whether or not the cells undergoing spontaneous apoptosis in their study subjects were infected by measles virus (Pignata C. *et al* 1998). Other surface molecules, such as TNF, TNF receptor, CD95L, and TRAIL as well as the family of cytoplasmic proteins, (caspase 1-9), will need to be examined to fully understand the cascade of events leading to measles-induced apoptosis (Griffin D.E. *et al* 1994).

The findings of the current study further emphasize the extraordinary breadth of the attack initiated by MV against the human immune system. It is very likely that MV-induced apoptosis of PBMC contributes significantly to the lymphopenia and loss of memory cell functions associated with this disease (Wesley A. *et al* 1978, Tamashiro V.G. *et al* 1987). The potential influence of variable apoptosis (either host or virus-mediated) on the generation of immune responses (Grosjean I. *et al* 1997, Fugier-Vivier I. *et al* 1997, Ito M. *et al* 1997b, Schnorr J.J. *et al* 1997a) and the clinical presentation of the disease remain to be determined. Finally, our observations using a modest number of strains raise the possibility that MV-induced apoptosis might be a relatively simple surrogate marker for virulence.

FIGURE 1: Representative histogram of PBMC apoptosis in TUNEL assay 72 hours after infection with CHI-1 (wild-type) measles virus (87.9%) or mock-infection (3.8%) .

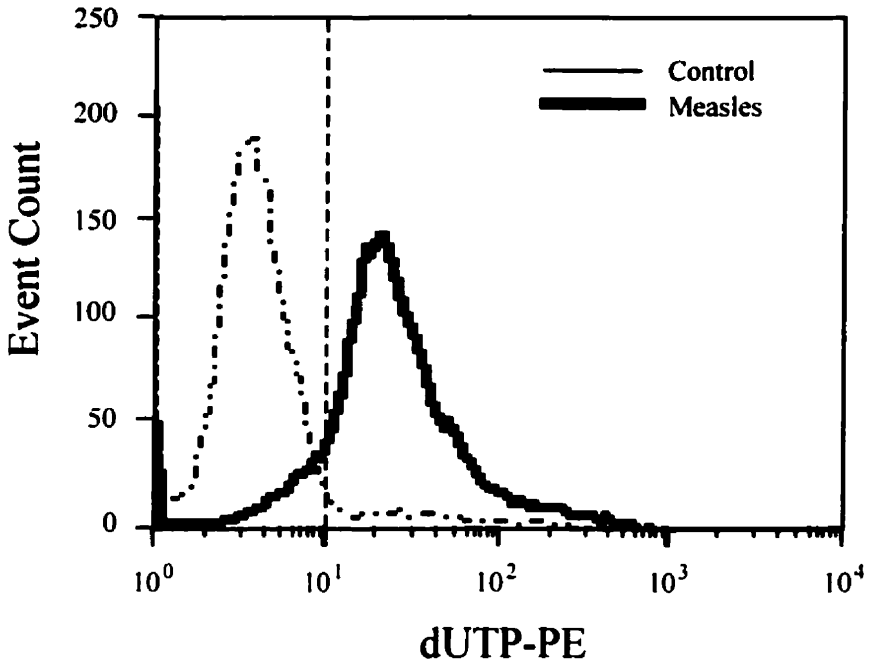


FIGURE 2: Apotosis in PBMC at 48 hours after mock infection (Control: 3.3%), infection with CHI-1 wild-type measles virus inactivated by heat (H-MV: 1.2%) or UV irradiation (UV-MV: 6.6%) or infection with virulent CHI-1 virus (53.3%). A multiplicity of infection (m.o.i.) of 1 was used for all infections. Histograms reflect incorporation of dUTP in the TUNEL assay.

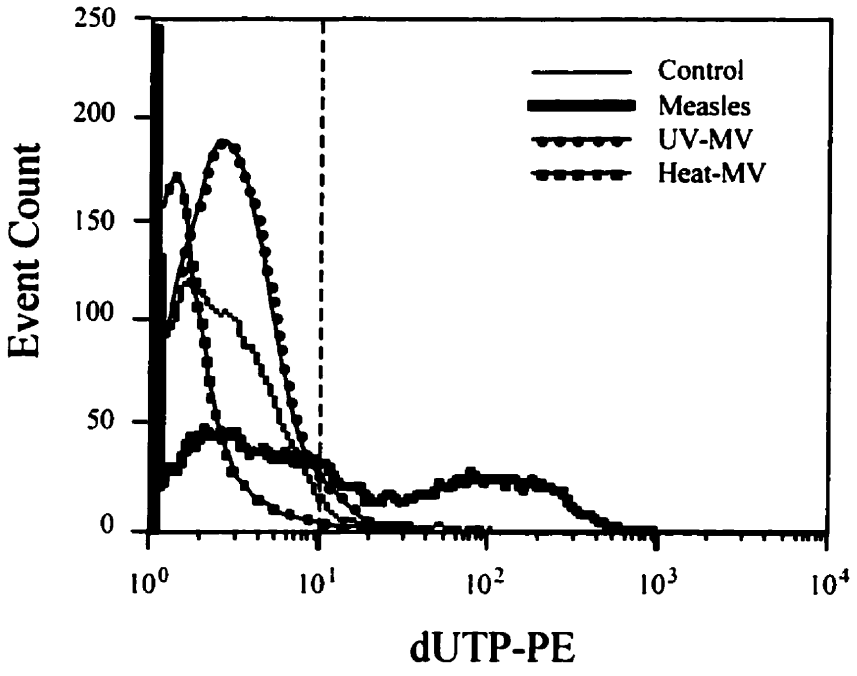


TABLE 1: Apoptosis of PBMC subsets (CD4+ and CD8+ T cells, CD19+ B cells and CD14+ monocytes) at 24 and 72 hours after infection with a wild-type measles virus (CHI-1, m.o.i. = 1.0) as determined by the TUNEL assay. Results are expressed as the percent of subsets undergoing apoptosis, (*$p < 0.001$, †$p < 0.01$ vs. monocytes).

	Percent Apoptosis	
Cell type	24 Hours	72 Hours
$CD4^+$	33.2 ± 4.8*	74.7 ± 1.2†
$CD8^+$	31.7 ± 5.1*	88.3 ± 9.8†
$CD19^+$	23.7 ± 4.9	64.8 ± 7.2
$CD14^+$	9.8 ± 3.5	31.1 ± 6.2

FIGURE 3: Kinetics of apoptosis in PBMC infected with a wild-type measles virus (CHI-1; m.o.i. = 1) as measured by the TUNEL assay at 12, 24, 48 72 and 96 hours (b – f respectively) after infection compared with mock-infected PBMC at 96 hours (a).

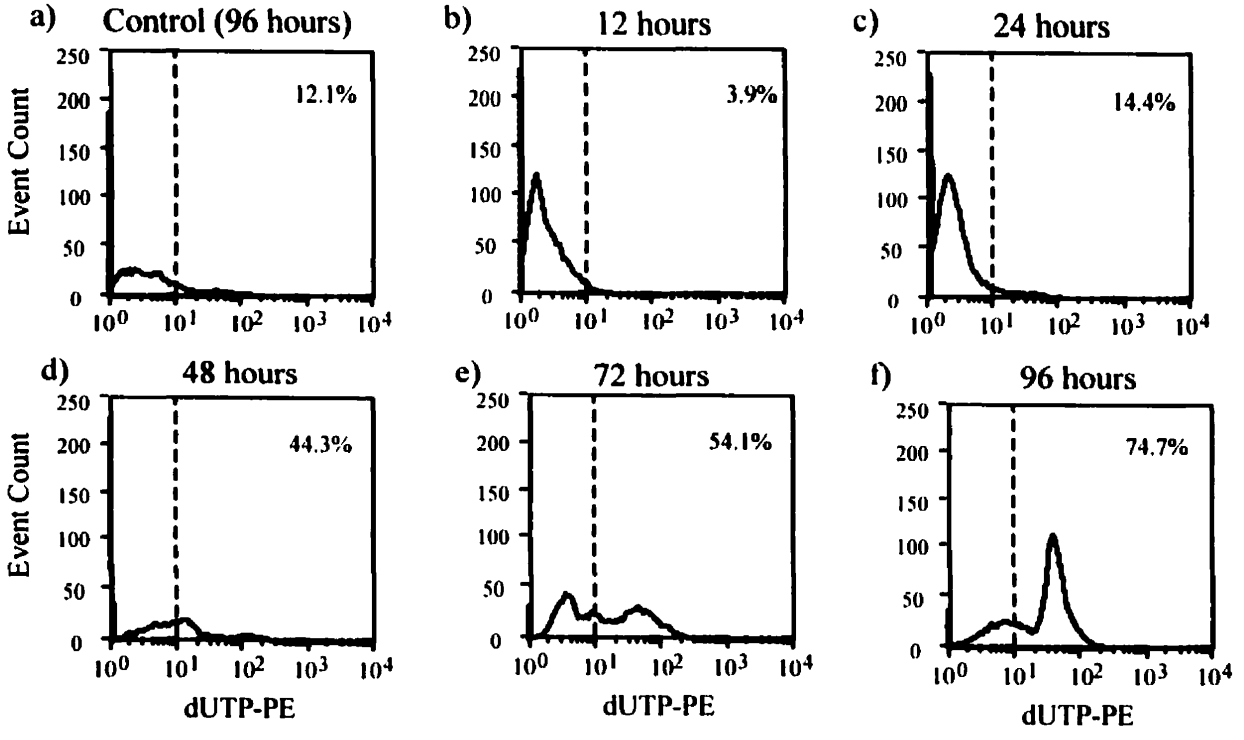


FIGURE 4: CD95 expression in PBMC infected with wild-type virus (CHI-1; m.o.i = 1.0) at 72 after infection. a) Dot plot of gated population b) Representative histogram comparing CD95 expression on measles infected PBMC (MV: 94.6%) with mock-infected cells (Control: 34.5%).

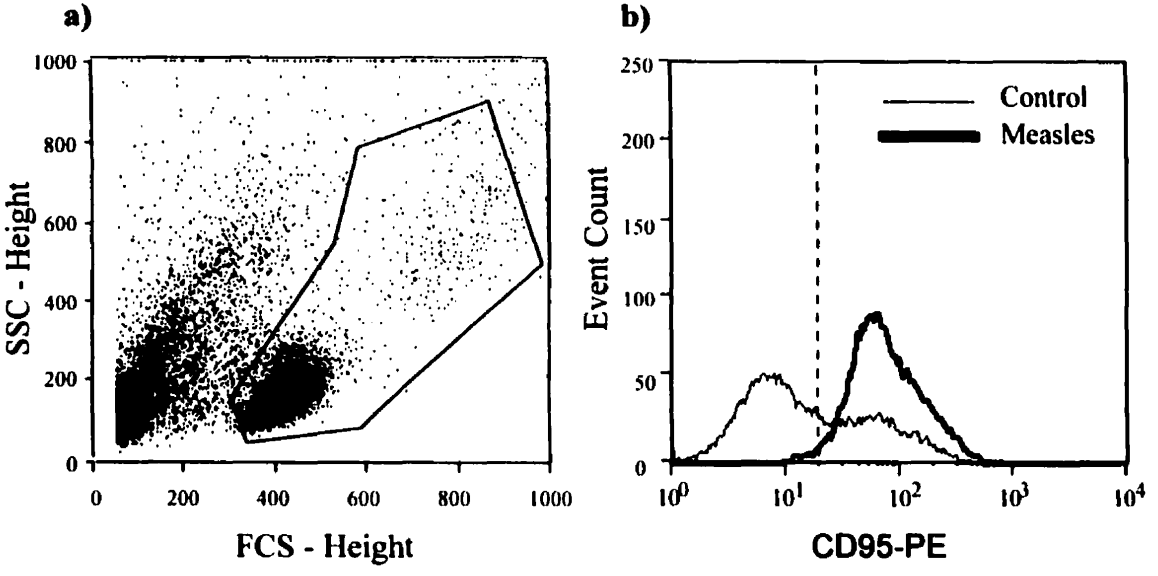


FIGURE 5: Apoptosis in mock infected PBMC (Control), PBMC infected (m.o.i.=1) with fully attenuated vaccine strain viruses: Connaught Moraten (MOR), Schwarz (SVW), Edmonston Zagreb (EZ); a partially attentuated vaccine strain: Edmonston B (EdB); or wild-type measles viruses: Chicago-1MVi USA- 89.1 (CHI-1), Bilthoven- Holland (BILT), Massachussetts-USA (MASS), Minnesota-USA (MINN), Utah-USA (UTAH), Hawaii.USA (HAW), Gambia (GAMB) and Guam (GUAM). Bars represent mean (± SEM) at 72 hours after infection as determined by TUNEL assay.

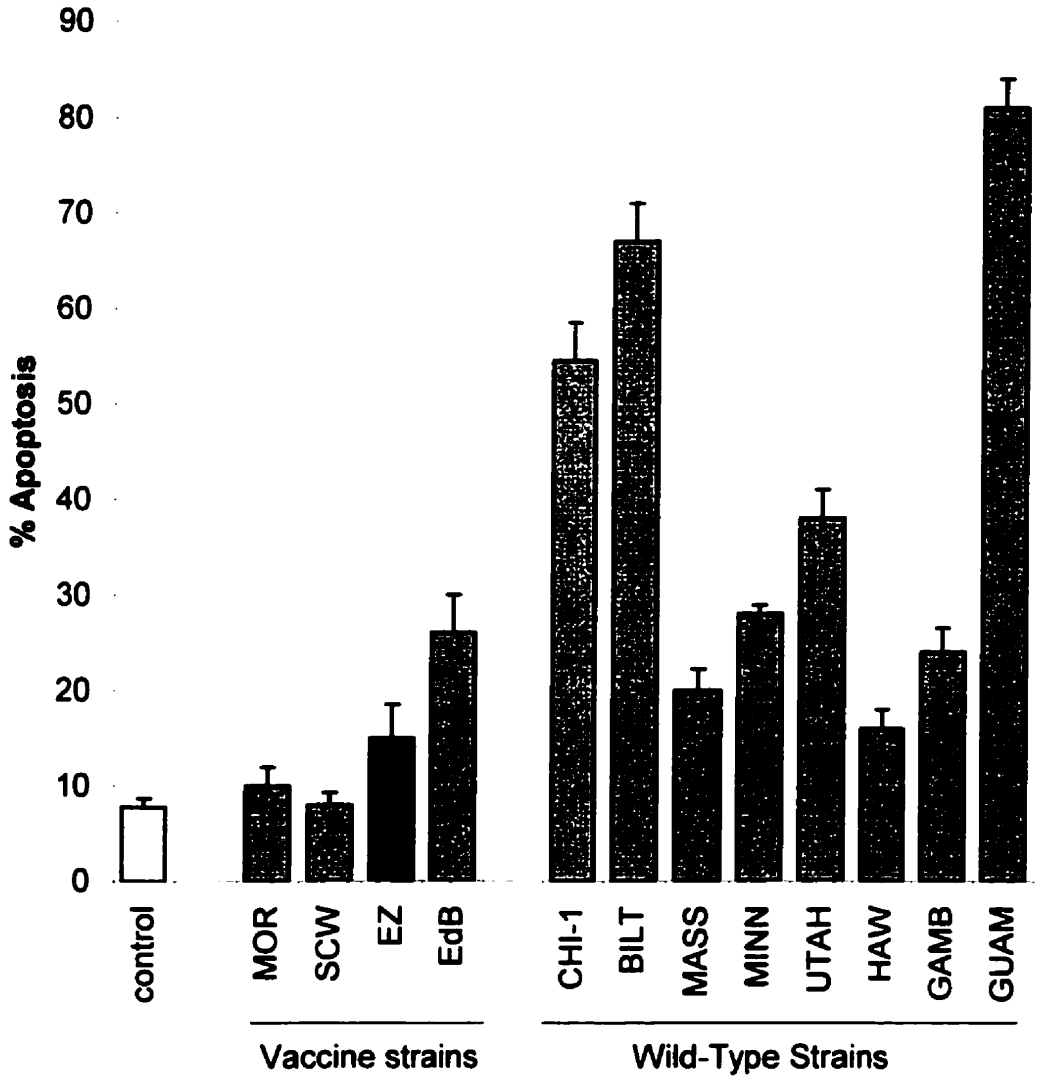


Figure 6: Induction of apoptosis (TUNEL assay) correlated positively with viral replication (plaque assay) in PBMC (72 hour infection; m.o.i. = 1) for both wild-type (r = 0.915) and vaccine strains (r = 0.981). Results are expressed as mean % apoptosis (line) and mean pfu/mL +/- S.E.M (bars).

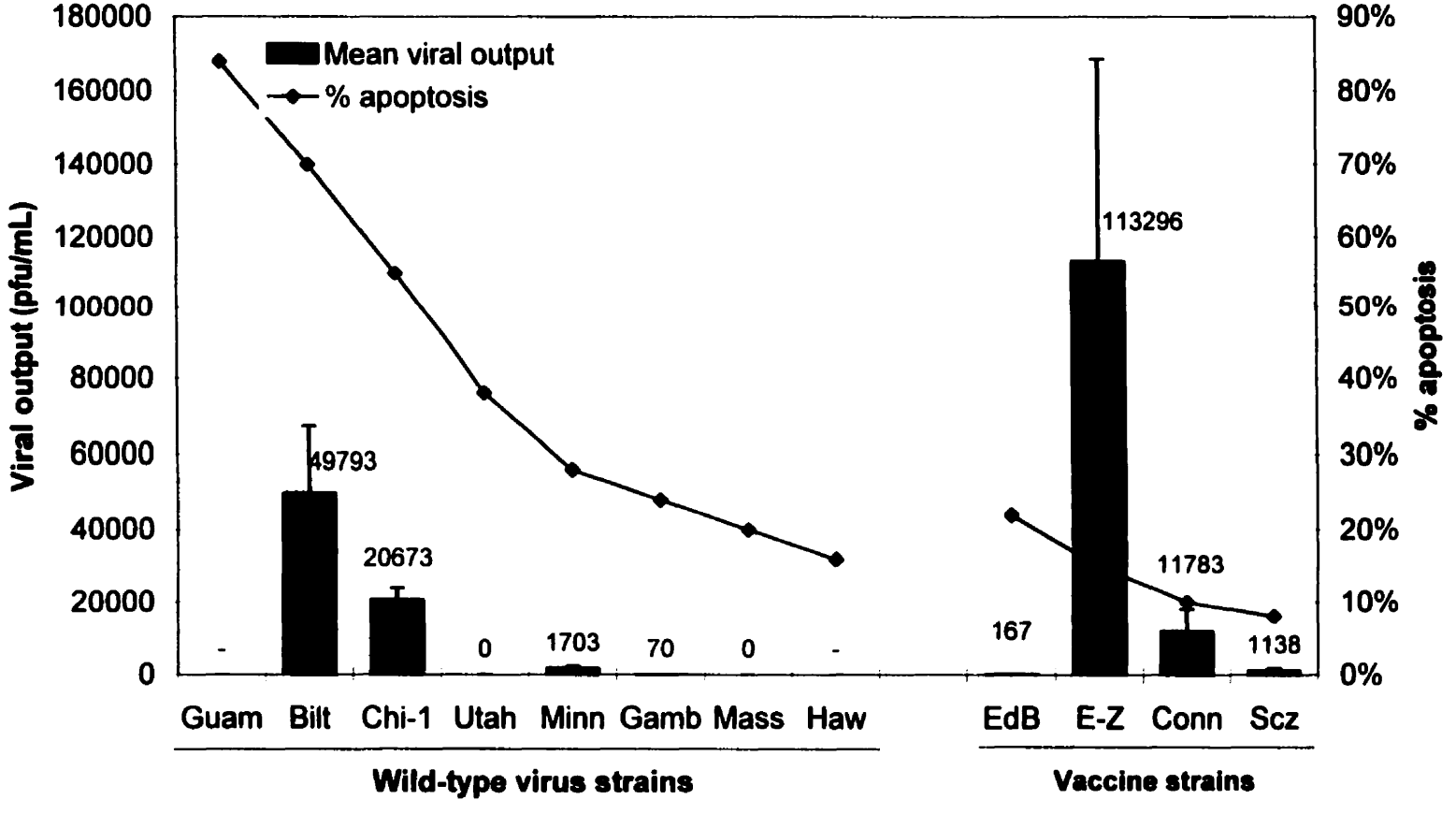
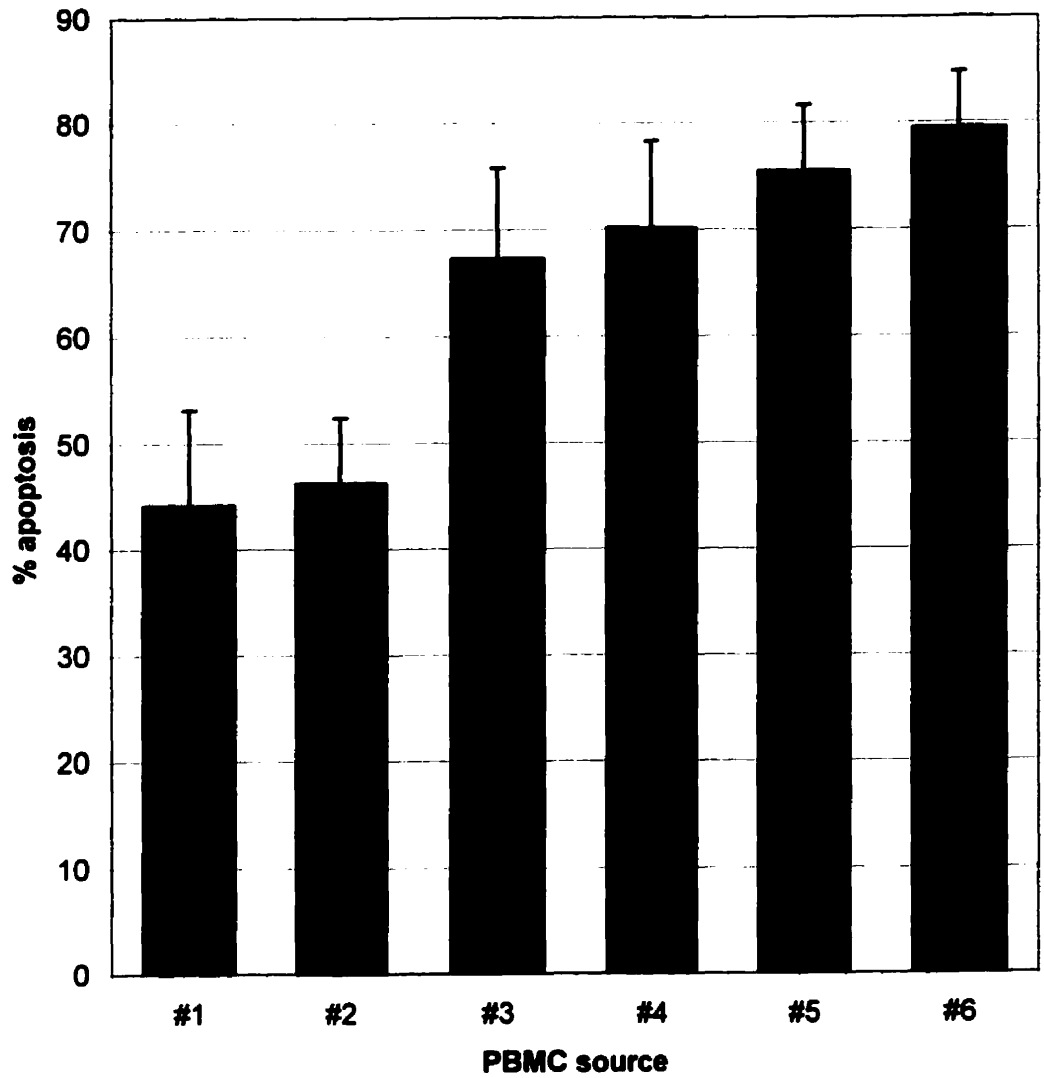


Figure 7: Effect of PBMC source on MV-induced apoptosis. Bars represent mean % apoptosis (+/- S.E.M.) after 48 hour infection of PBMC from different donors (#1-7) with Chi-1 virus.



Connecting Statement II:

In the following two chapters the focus shifts from MV-induced immunosuppression toward the striking association between vitamin A and measles. This second manuscript describes the outcome of a trial in Peru that examined the effect of vitamin A supplementation at the time of MV vaccination on both the humoral and cellular immune responses to MV antigens. Vitamin A supplements administered in an effort to combat blindness due to vitamin A deficiency are frequently delivered at the same time as the MV vaccine. However, vitamin A is a potent immunomodulator and, given the importance of MV vaccination, any possibility that concurrent medication could interfere with vaccine efficacy is of considerable concern. The conclusion drawn from this trial is that administration of vitamin A in high or low doses at the same time as MV vaccine does not affect the outcome of vaccination in 9-month-old Peruvian children.

Chapter 8 - Manuscript II

(Short Communication)

CELLULAR AND HUMORAL RESPONSES TO MEASLES VACCINATION GIVEN CONCURRENTLY WITH TWO DIFFERENT DOSES OF VITAMIN A COMPARED WITH NO VITAMIN A

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Running head: Vitamin A supplements and measles vaccination.

Key Words: Vaccine trial, Measles vaccine, Vitamin A supplements, Seroconversion, Lymphoproliferation

Vitamin A supplements and measles vaccination.

8.1: Abstract

Routine immunization programs have been recommended as delivery systems for vitamin A supplementation in countries with vitamin A deficiency. However, the effects of co-administration on vaccine efficacy are unclear. This randomized, double-blinded study evaluates both humoral and cellular immune responses to measles vaccine in 9-month-old Peruvian children (n=352) receiving either 25,000 or 100,000 IU of vitamin A at the time of vaccination. These results are compared to responses from a parallel, unblinded trial in which children received only measles vaccine (control n = 141). Measles-specific antibody titers and lymphoproliferative responses were measured at baseline and three months after routine vaccination at 9.5 ± 0.1 months of age. *Results:* Of the 392 children followed to 3-months post-vaccination, 95% seroconverted and 93% developed protective antibody titers. Detectable maternal antibodies were present in 48% of children at vaccination. Lymphoproliferative responses (LPR) to at least measles and control antigens were available in 111 children both pre- and post-vaccination. Positive LPR were detected in 7% of children pre-vaccine and 28% 3-months after vaccination. No significant differences in either humoral or cellular responses were observed between the control group and either of the vitamin A groups. No significant correlation between antibody and lymphoproliferative responses was observed. None of the children who failed to seroconvert developed LPR in any of the treatment groups (0/18). *Conclusion:* The administration of vitamin A in high or low doses at the same time as MV vaccine does not affect the outcome of vaccination in 9 month old Peruvian children.

Vitamin A supplements and measles vaccination.

8.2: Introduction

Vitamin A deficiency (VAD) is a public health problem in at least 73 countries worldwide (WHO/EPI 1994). It is one of the most important causes of preventable blindness in children worldwide and has been estimated to contribute to the death of over 1 million children annually (WHO/EPI 1994). In particular, VAD increases the mortality associated with measles (MV) infection (WHO/EPI 1994). Conversely, acute MV infection itself can cause temporary but profound VAD (Hussey G.D. *et al* 1992).

Mortality rates of 6-month to 5-year-old children living in areas where VAD is common can be reduced by ~23% with vitamin A supplementation (Fawzi W.W. *et al* 1993). Currently, the World Health Organization (WHO) recommends that children ≥6 months old living in areas with VAD receive a vitamin A supplement every 4-6 months (WHO/EPI 1994). Studies on the benefits and safety of vitamin A supplementation in children < 6 months old are currently underway. Although preliminary results suggest that such early supplementation is safe, there appears to be little beneficial effect on either short-term mortality or long-term vitamin A status (WHO/CHD 1998).

In 1987, the WHO/Expanded Programme on Immunization (EPI) global advisory group raised the possibility of administering vitamin A supplements using delivery systems already in place for routine immunization (e.g.: EPI). The timing of the MV vaccination (6-15 months) made this vaccine the most likely EPI partner to reach the targeted age group, although vitamin A supplementation in younger children could also have been linked to the DPT/OPV vaccines (6-14 weeks old) (WHO/CHD 1998). Any manipulation of the successful EPI program must be approached with the greatest caution. The measles component of the EPI is particularly important since only a single dose is given and even a small decrease in vaccine efficacy could lead to a substantial increase in the number of MV-related deaths worldwide (CDC 1995).

Vaccine-induced antibody titers are highly correlated with protection from natural disease and seroconversion rates (i.e. 4-fold rise in antibody titer) have been widely used as surrogate markers for MV vaccine efficacy. In 1995, Semba *et al* reported reduced seroconversion in 6-month-olds who were given vitamin A with MV vaccine. Although the effect of vitamin A supplements was observed only in children with detectable anti-MV maternal antibodies at the time of vaccination, this observation

raised considerable concern about partnering vitamin A with EPI vaccines. Subsequent studies have reported either no change or increased seroconversion in 9-month old children receiving vitamin A supplements with MV vaccine (Arya S. *et al* 2000, Bahl R. *et al* 1999, Benn C.S. *et al* 1997, Bhaskaram P. *et al* 1997, Semba R. *et al* 1997, de Francisco A. *et al* 1996).

None of these studies examined the potential impact of vitamin A supplements on vaccine-induced cellular responses. Although the correlates of immunity to MV are not yet fully understood, there is increasing evidence that both humoral and cellular responses to vaccine need to be considered (Ward B.J. *et al* 1995, Bautista-López N. *et al* 2000). In the current study, we examined the potential impact of the simultaneous administration of high and low dose vitamin A supplements and MV vaccine upon both humoral and cellular responses in 9-month old Peruvian children.

Vitamin A supplements and measles vaccination.

Patients and Methods

Study Site:

The study was conducted from January to November 1996 in the district of San Juan de Lurigancho, a shantytown outside Lima, Peru and one of the centers participating in the EPI-linked vitamin A supplementation trial (WHO/CHD 1998). Previous studies in this area have shown low rates of acute malnutrition but 24% of children under 4 years old have serum retinol levels below 20ug/dl (Bahl R. and the WHO/CHD Immunization-linked vitamin A Supplementation Study Group – personal communication). Natural MV cases are extremely rare but diarrheal illness rates are high with 8-10 episodes of diarrhea per child year (Lanata C.F. *et al* 1991, Penny M.E. *et al* 1999). The rates of respiratory tract infections are even higher (Lanata C.F. *et al* 1997).

Enrollment:

This study recruited children from two sources. First, 352 infants already enrolled in a double-blind, placebo-controlled, EPI-linked vitamin A supplementation trial were recruited. In this EPI-linked trial, mother-infant pairs were enrolled at 18-28 days postpartum and were randomized to receive vitamin A in one of two patterns. One group received a maternal dose of 200,000 IU (Tishcom Corporation, Westbury, NY) followed by four doses of 25,000 IU given to the infant with each DPT/OPV vaccine at 2, 3 and 4 months and with MV vaccine at 9 months of age. In the second group, placebo (Tishcom Corporation) replaced the maternal dose and the first three doses for children at 2, 3 and 4 months of age. At 9 months of age these children received 100,000 IU vitamin A supplementation with MV vaccine. Therefore, the 352 infants recruited from the EPI-linked study all received vitamin A at the time of MV vaccination, either in "low" (25,000 IU) or "high" (100,000 IU) dosage. Further details on the study population and design of the EPI-linked study have been published elsewhere (WHO/CHD 1998).

The second group of infants recruited for the current study was not enrolled in the EPI-linked study. A total of 141 infants aged 9-12 months and living in the same communities as those in the EPI-linked study were recruited independently to receive MV vaccination with no concurrent treatment. Although this part of the study was not

blinded, these children were included to serve as reasonable "controls" with whom the effects from high or low dose vitamin A supplements could be compared.

Vitamin A and Vaccine:

All infants received Schwarz standard dose MV vaccine at 9.5 ± 0.1 months of age ($10^{3.6-3.8}$ pfu/dose, Pasteur Merieux vaccine, Quimica Suiza, Lima). Vitamin A (retinol palmitate with trace amounts of vitamin E in brown gelatin capsules; Tishcom Corporation) was administered orally and was indistinguishable from the vitamin E capsules used as placebo (Tishcom Corporation). Randomization and individually packaged blister packs containing capsules with identical appearance ensured that the comparison between the vitamin A supplementation groups was blinded. Mothers and children in the control group received neither vitamin A nor placebo capsules.

Blood Samples and Handling:

Venous blood (5-6mL: 1ml with serum separator and 4-5ml with lithium heparin) was obtained from all infants prior to vaccination and 3 months post-vaccination. Serum was separated by centrifugation at ~600 x g for 10 min within 1 hour of collection and stored at –20°C on site until transported to the Universidad Peruana Cayetano Heredia (UPCH) where they were stored at –70°C until used. Heparinized whole blood was stored at room temperature and processed within eight hours of collection. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque and cryopreserved in nitrogen as previously described (León M.E. *et al* 1993) until transported to McGill University in nitrogen vapor "dry" shippers where the samples were returned to liquid nitrogen until used in assays.

Plaque Reduction Neutralization (PRN) Assay:

MV-specific neutralizing antibodies were measured at the UPCH by PRN as previously described (Berry S. *et al* 1992). Results were standardized against WHO standard anti-serum (5 IU No. 66/202, WHO International Laboratory for Biological Standards, UK) and are reported as the dilution that neutralized 50% of input plaques. Pre-vaccine titers ≥ 1:8 in this assay suggest the presence of maternal antibodies. A 4-

fold or greater increase in PRN titer after vaccination indicates a positive serologic response. Post-vaccination titers ≥ 1:120 are thought to confer protection from natural disease (Chen R. *et al* 1986, Markowitz LE *et al* 1990).

Lymphoproliferation Assay (LPA):

Cell-mediated immunity (CMI) was evaluated by antigen-specific lympho-proliferation assays (LPA) as previously described (Ward B.J. *et al* 1995), although a new harvester (Tomtec harvester 96, Tomtec Inc., Hamden, CT) and beta-counter (Microbeta 1450, Wallec, Turku, Finland) were used. A subset of children from each treatment group (high, low, and control) was randomly chosen for LPA testing. Paired PBMC samples (pre- and post-vaccine) were tested for proliferative responses to Chicago-1 MV antigen (Chi-1, gift from W. Bellini, CDC, Atlanta, GA) as well as non-MV antigens: tetanus toxoid (TT, gift from B. Lantham, Boston, MA), whole pertussis antigen (PT, gift from R. Wittes, Connaught Laboratories Ltd., Willowdale, ON), and whole inactivated Bacille Calmette Guerin antigen (BCG, gift from D. Radziok, McGill University, Montreal, QC). A Vero cell monolayer lysate was used as the control antigen as previously described (Ward B.J. *et al* 1995). Results are reported as both counts per minute (cpm) and as stimulation indices (SI = mean cpm for antigen-stimulated well/cpm of the control antigen wells). Stimulation indices ≥ 2 were considered to indicate a significant proliferative response.

Statistical Analysis:

Statistical analyses were performed using Statview 5 software (SAS, Cary, NJ). Data for each parameter were first analyzed to define their distribution. Background data and LPA were compared using the paired Student t test (two-tailed). PRN results were analyzed using the nonparametric Mann-Whitney U test. Correlations were assessed using Spearman rank correlation coefficients. Results are expressed as mean ± S.E.M. and differences achieving $p \leq 0.05$ were considered to be significant.

8.4: Results

Baseline Information:

There were no significant differences between the treatment groups in age, nutritional status or any socio-economic parameter at the time of immunization (Table 1). In the control group, the male: female ratio was slightly higher than in the other two groups (Table 1). Therefore, all statistical analyses were repeated for male and female children separately. No significant deviations from the results of the overall analysis were observed.

Maternal Antibody Titers:

48% of the children in the study had detectable maternal antibodies at the time of vaccination (pre-vaccination titers ≥ 8). Female children had slightly higher maternal antibody titers at the time of vaccination in all treatment groups, but this difference did not reach statistical significance (10 ± 0.5 *vs.* 9 ± 0.6 in males, $p \leq 0.06$) (Table 2). In the low vitamin A group, the pre-vaccine antibody titers were slightly higher than in the other two groups, although these differences did not reach significance (11 ± 0.9 *vs.* 8 ± 0.5 for controls and 9 ± 0.4 in high vitamin A group). As suggested by the low mean values, the maternal antibody titers found in our study children were very low. No child had a titer > PRN 1:96. In light of the reported effect of maternal antibody status on vitamin A impact (Semba R.D. *et al* 1995), we repeated all analyses stratified by the presence or absence of maternal antibodies. Overall, maternal antibody status had very little impact on the outcome of vaccination in any group.

Humoral Immune Response to Measles Vaccine:

Of the 392 children who were followed to three months post-vaccination, 95% seroconverted to MV and 94% developed protective antibody titers. Overall mean antibody titers rose from PRN 9 ± 0.4 pre-vaccination to 1007 ± 42 post- vaccination. The seroconversion rates were almost identical in all treatment groups (Table 2). There were no significant differences in the post-vaccination titers between the groups. The seroconversion rates for children with maternal antibodies ≥ 8 were ~92% in all treatment groups (Table 2). With the exception of children in the control group, this was

similar to the seroconversion rate for children without maternal antibody. In the control group, the seroconversion rate in children without maternal antibodies was slightly higher (98% *vs.* 92%). Overall, seroconversion rates were slightly lower in female children, compared to males, regardless of treatment received (Table 2). This difference is almost certainly the result of the slightly higher maternal antibody titers in the female children and the differences disappear when only maternal antibody-negative children were compared.

Lymphoproliferative Responses to Measles Antigens:

Lymphoproliferation testing was only performed on a subset of the samples obtained from the children recruited for this study (111/493; ~20%). The children for whom lymphoproliferative response (LPR) data are available were representative of the larger groups for all demographic parameters (data not shown). Maternal antibody status and humoral responses to vaccination in this subset were also very similar to those observed in the study groups as a whole (Table 3). Overall, the proliferative responses to MV antigens were quite low both pre- and post-vaccination (SI 0.9 ± 0.07 and 1.5 ± 0.2, respectively). However, this difference was highly significant ($p \leq 0.008$). A small number of children (7%) had borderline positive LPR in the pre-vaccination samples (SI 2.9 ± 0.3) but none of these children had serologic evidence of having suffered natural measles infection. The proportion of children with positive LPR responses ($SI \geq 2$) rose to 28% after vaccination and the responses in these children were generally much more obvious (SI 3.9 ± 0.3). There were no differences in either the mean LPR values or proportion of LPR responders between the three study groups (Table 3). Overall, there was no correlation between serological and lymphoproliferative responses to MV antigens ($r = -0.12$; $p = 0.2$).

Lymphoproliferative Responses to Other Relevant EPI Antigens:

BCG is routinely given to newborns in Peru as part of the EPI. One of the groups in the current study received vitamin A simultaneously with the three infant DPT vaccines at 2, 3, and 4 months of age. It was therefore relevant to examine the potential impact of vitamin A supplementation on the proliferative responses to these antigens as

well. Overall, the children who had received vitamin A supplements (either high or low dose) had slightly higher LPR responses to TT (SI 3.5 ± 1.1 *vs.* 2.7 ± 1.3), BCG (SI 3.3 ± 0.8 *vs.* 1.7 ± 0.3) and PT antigens (SI 5.4 ± 1.2 *vs.* 3.5 ± 1.2) compared to children not receiving the vitamin. Although a number of comparisons between the high and low vitamin A groups approached or achieved borderline statistical significance, there was no consistent pattern of differences in LPR between these groups (Table 3). Since both wild-type and vaccine strain MV have the capacity to suppress cellular responses to recall antigens, it was also of interest to examine the potential impact of measles vaccine (with or without vitamin A supplementation) on LPR to other relevant vaccine antigens at 3 months after MV vaccination. Overall, LPR to BCG was unaffected but the S.I. to both TT (2.4 ± 0.6 *vs.* 3.8 ± 1.0; $p \leq 0.02$) and PT antigen (2.4 ± 0.4 *vs.* 4.6 ± 1.3) were lower at 3 months after MV vaccination compared to pre-vaccine responses.

Failure to Seroconvert:

Among the 18 children (4 male, 14 female) who did not seroconvert, 4 were in the control group, 7 received "low" vitamin A, and 7 received "high" vitamin A (Table 2). Almost all (14/18) of these children had detectable maternal antibody titers at the time of vaccination (27.5 ± 6.2). After vaccination, none of these children had positive proliferative responses to MV antigens. One child in the low vitamin A group was found to have a protective antibody titer despite "failing" to seroconvert as a result of a relatively high pre-vaccine titer (PRN 1:96) and relatively low post-vaccine titer (PRN 1:301).

Discussion:

The possibility that the administration of vitamin A concurrent with measles (or any other) EPI vaccination could interfere with vaccine efficacy is of considerable concern. Before the introduction of the live, attenuated MV vaccine in 1963, almost all children were infected with MV before 15 years of age, with devastating consequences. Although the incidence of measles has dropped dramatically since the pre-vaccine era, the global burden remains high with an estimated 36 million cases and 880,000 deaths each year (WHO 2000). Any interference with vaccine efficacy could lead to a significant increase in both MV-related morbidity and mortality.

This study was designed to determine whether or not vitamin A in high or low dose supplements administered at the time of MV vaccination would have any impact on the induction of humoral and cellular immune responses to vaccination. The results of the antibody testing are reassuring and confirm the finding of several other groups (de Francisco A. *et al* 1996, Benn C.S. *et al* 1997, Bhaskaram P. *et al* 1997, Semba R. *et al* 1997, Bahl R. *et al* 1999, Arya S. *et al* 2000). Neither the high nor the low dose vitamin A recipients were different from control children with respect to any serologic outcome of vaccination (e.g.: absolute titer, seroconversion, and proportion achieving protective titers). In contrast to the initial observation of Semba *et al*, and like other, more recent studies, we found no effect of maternal antibody status on the capacity of vitamin A supplementation to influence the outcome of MV vaccination.

Since there is a growing consensus that both humoral and cellular responses need to be considered in estimating MV vaccine-induced protection (Ward B.J. *et al* 1995, Bautista-López N. *et al* 2000), it is also reassuring that we found no evidence of a negative impact of either high or low dose vitamin A supplementation on the induction of MV-antigen specific proliferative responses. Indeed, our data suggest that LPR responses to MV and other EPI-relevant antigens were similar (MV) or slightly higher (BCG, TT, PT) in the vitamin A supplemented children compared with controls.

Although none of the children who failed to seroconvert following vaccination had evidence of a cellular response to MV antigens, recent evidence suggests that at least some of these children will have been "primed" to respond to MV antigens (Ward B.J. *et*

al 1995). Whether or not such priming can protect against or modify natural disease is currently unknown.

The findings of the current study are in agreement with the accumulating literature supporting the safety of administering either high or low dose vitamin A supplements at the same time as EPI vaccines. Specifically, we found no evidence that high (100,000 IU) or low (25,000 IU) dose vitamin A supplements had any impact on either the humoral or cellular responses to MV vaccine administered to 9-month old Peruvian children.

Table 1: Baseline characteristics of infants in measles-vitamin A study

Characteristic	Low vit A (n=165)	High vit A (n=187)	Control (n=141)	Total (n=493)
	352 children from EPI-linked study			
Vitamin A				
Maternal dose	200,000 IU	placebo	na[1]	na
Dose at 2, 3, 4 mo (DPT/OPV)	25 000 IU	placebo	na	na
Dose at 9 mo (MV vaccine)	25 000 IU	100 000 IU	none[2]	na
Sex				
Male	82	93	87	262
Female	83	94	54	231
Type of floor at home				
earth	92	112	82	286
cement (unfinished)	42	45	31	118
cement (finished)	30	30	25	85
other	1	0	3	4
Type of walls				
mats/cardboard	58	76	43	177
triplay/wood	25	19	27	71
bricks (unfinished)	54	75	41	170
bricks (finished)	26	15	28	69
other	2	2	2	6
Primary water source				
piped to dwelling	98	96	94	288
public well	15	30	11	56
truck cistern	52	61	36	149
Primary water storage				
cylinder	34	30	25	89
tank without pipe	44	56	44	144
tank with pipe	47	45	34	126
other	40	55	37	132
none	0	1	1	2
At time of MV vaccine				
Age (months)	9.7 ± 0.1[3]	9.7 ± 0.1	9.2 ± 0.05	9.5 ± 0.1
Weight (Kg)	8.6 ± 0.08	8.7 ± 0.07	8.6 ± 0.08	8.6 ± 0.05
Height (cm)	69.4 ± 0.2	69.5 ± 0.2	69.5 ± 0.2	69.5 ± 0.1
Hematocrit	32.3 ± 0.2	32.0 ± 0.2	31.9 ± 0.2	32.0 ± 0.1
% breastfeeding	89.6%	89.1%	82.3%	87.3%
Illness (≥ 1 in prior 4 wks)				
Cough	104	122	113	339
Diarrhea	57	57	72	186
ALRI or Pneumonia	11	10	6	27
Other fever	5	12	13	30

[1] na = not applicable.

[2] children in control group received nothing with MV vaccine (no placebo)

[3] mean ± standard error of mean (S.E.M.)

Table 2: Seroconversion and protection against measles after immunization[a]

	Low vitamin A	High vitamin A	Control
Seroconversion	125 of 132 (95%)	148 of 155 (95%)	101 of 105 (96%)
male	62 of 65 (95%)	76 of 76 (100%)	65 of 66 (98%)
female	63 of 67 (94%)	72 of 79 (91%)	36 of 39 (92%)
Protection	124 of 132 (94%)	143 of 155 (92%)	99 of 105 (94%)
male	62 of 65 (95%)	73 of 76 (94%)	64 of 66 (97%)
female	62 of 67 (93%)	70 of 79 (89%)	35 of 39 (90%)
Prevaccination titer ≥ 1:8[b]	69 of 135 (51%)	74 of 161 (46%)	51 of 106 (48%)
male	31 of 65 (48%)	32 of 78 (41%)	27 of 67 (40%)
female	38 of 70 (54%)	42 of 83 (51%)	24 of 39 (62%)
seroconversion	64 of 69 (93%)	68 of 74 (92%)	47 of 51 (92%)
protection	63 of 69 (91%)	64 of 74 (86%)	46 of 51 (90%)
Prevaccination titer < 1:8	66 of 135 (49%)	87 of 161 (54%)	55 of 106 (52%)
seroconversion	61 of 66 (92%)	80 of 87 (92%)	54 of 55 (98%)
protection	61 of 66 (92%)	80 of 87 (92%)	53 of 55 (96%)
No seroconversion	7 of 132 (5%)	7 of 155 (5%)	4 of 105 (4%)
prevaccination titer ≥ 1:8	5 of 7 (71%)	5 of 7 (71%)	4 of 4 (100%)

[a] These results are based on all children enrolled in the study. Seroconversion defined as ≥ four-fold increase in titer at three months post-immunization. Protection defined as post-immunization titer ≥ 1:120.

[b] Pre-vaccination titer ≥ 1:8 indicates presence of maternal antibodies. None of the children had pre-vaccination titers > 1:120 (indication of prior natural infection)

Table 3: Immune responses in a subset of children tested for lymphoproliferative responses (LPR) both pre-vaccination and 3-months post-vaccination. EPI-relevent antigens tested include Chi (Chicago-1 MV) (n = 111), TT (tetanus toxoid) (n = 111), BCG (bacillus Calmette-Guerin) (n = 36), and PT (pertussis toxin) (n = 77). Overall stimulation indices increased significantly for MV-antigen (* $p \leq 0.001$) and decreased significantly for TT ($^{\dagger}p \leq 0.02$) after MV vaccination.

	Low vit A	High vit A	Control	Total
PRN[1] $_{pre}$	9.2 ± 0.7 (32)	11.2 ± 2.5 (38)	8.0 ± 0.6 (41)	9.5 ± 0.9 (111)
PRN $_{post}$	1125 ± 137	1314 ± 176	834 ± 103	1092 ± 84
Mat. Ab[2]	52%	46%	46%	48%
Seroconv.[3]	97%	95%	95%	95%
Vero[4] $_{pre}$	940 ± 205 (32)	1594 ± 352 (38)	697 ± 126 (41)	1079 ± 147 (111)
Vero $_{post}$	1566 ± 284	1692 ± 318	1045 ± 173	1419 ± 152
Chi$_{pre}$	682 ± 205 (32)	1173 ± 331 (38)	540 ± 70 (41)	798 ± 132 (111)
Chi$_{post}$	1344 ± 311	2386 ± 760	1672 ± 517	1872 ± 337
SI[5] $_{pre}$	0.7 ± 0.08	0.8 ± 0.1	1.2 ± 0.2	0.9 ± 0.07*
SI $_{post}$	1.0 ± 0.2‡	1.5 ± 0.3	1.9 ± 0.4‡	1.5 ± 0.2*
SI[6] ≥ 2$_{pre}$	0%	7%	15%	7%
SI ≥ 2$_{post}$	13%	31%	38%	28%
TT$_{pre}$	1051 ± 239 (32)	3541 ± 968 (38)	1463 ± 351 (41)	2080 ± 382 (111)
TT$_{post}$	2128 ± 489	2816 ± 647	1273 ± 282	2062 ± 290
SI$_{pre}$	3.0 ± 1.0	5.3 ± 2.5	2.9 ± 0.8	3.8 ± 1.0†
SI$_{post}$	1.9 ± 0.4	3.6 ± 1.8	1.6 ± 0.2	2.4 ± 0.6†
SI ≥ 2$_{pre}$	37%	29%	33%	33%
SI ≥ 2$_{post}$	26%	24%	27%	26%
BCG$_{pre}$	1687 ± 768 (13)	869 ± 230 (12)	1651 ± 724 (11)	1403 ± 358 (36)
BCG$_{post}$	1206 ± 267	1900 ± 457	2561 ± 1493	1789 ± 429
SI$_{pre}$	4.4 ± 2.4	0.9 ± 0.2	2.5 ± 0.7	2.7 ± 0.9
SI$_{post}$	2.1 ± 0.7	5.0 ± 2.7	1.5 ± 0.6	2.8 ± 0.9
SI ≥ 2$_{pre}$	38%	8%	45%	31%
SI ≥ 2$_{post}$	29%	27%	22%	26%
PT$_{pre}$	3456±1355 (20)	1803 ± 637 (25)	1865 ± 577 (32)	2258 ± 473 (77)
PT$_{post}$	5101 ± 1545	3313 ± 1098	1723 ± 688	3313 ± 669
SI$_{pre}$	9.6 ± 4.4	1.4 ± 0.3	4.0 ± 1.4	4.6 ± 1.3
SI$_{post}$	4.1 ± 1.1	1.7 ± 0.3	1.5 ± 0.5	2.4 ± 0.4
SI ≥ 2$_{pre}$	38%	8%	45%	31%
SI ≥ 2$_{post}$	38%	36%	22%	31%

1. PRN reported as mean pfu/mL ± S.E.M.
2. Pre-vaccine PRN ≥ 1:8 taken to indicate presence of maternal antibodies.
3. Seroconversion defined as ≥ four-fold increase in PRN post-vaccine.
4. LPR reported as cpm ± S.E.M.
5. Mean stimulation index ($SI = cpm_{antigen}/ cpm_{vero\ control}$) ± S.E.M.
6. SI ≥ 2 indicate significant proliferative response.

Connecting Statement III:

The following chapter continues to discuss the association between vitamin A and measles virus. The specific focus is on the uncharacterized mechanisms than underlie the acute vitamin A deficiency (VAD) associated with MV infection and the beneficial effects of supplementation. These additional studies describe preliminary results from our *in vitro* analysis of the role of retinoids in MV infection. In this study, we investigated the hypothesis that retinoids act to limit viral replication and alter viral transcription through changes in the retinoid-signaling cascade. We have evaluated retinoid-induced changes in viral output and in expression of nuclear retinoid receptors in U937 cells.

Chapter 9 – Additional Studies

In vitro effects of retinoids on measles viral replication & synergistic effect of retinoids and measles virus on retinoid signaling cascade.

9.1: Materials and methods

Measles virus. Wild-type Chicago-1 virus (gift from W Bellini, CDC, Atlanta, GA) was seeded on African green monkey Vero cell monolayers in MEM (Wisent, St. Bruno, QC) + 5% heat inactivated fetal bovine serum (FBS, Wisent) at a m.o.i. of 0.001 for 48-96 hours. Supernatants were replaced with a minimal volume of fresh media at 50% cytopathic effect (CPE) and monolayers were freeze-thawed at 90-95% CPE. Harvests were pooled, centrifuged at 500 x g for 10 minutes and supernatants were filtered (0.22μm; Corning-Costar, Cambridge, MA). Viral titers were determined by plaque assay and ranged from 0.5 – 2 x 10^6 plaque forming units (pfu/mL). Viral stocks were aliquoted and frozen at –76°C until used in assays.

Retinoid stock solutions. Lyophilized retinoids (all-trans retinol (ROH), all-trans retinoic acid (atRA) and 9-cis retinoic acid (9cRA), all Sigma, St. Louis, MO) were solubilized to 10^{-2}M in DMSO and then further diluted to 10^{-3}M in absolute ethanol. The final ethanol concentrations in experiments was ≤ 0.1% and had no effect on PBMC viability or MV replication (data not shown). Stock solutions were stored at –76°C in the dark. Working aliquots were stored at –20°C in the dark.

Infection and treatment of cells. Human promonocytic U937 cells (American Type Culture Collection CRL# 1593, Rockville, MD) were maintained in RPMI-1640 (Wisent) with 10mM HEPES, 50μg/mL gentamicin (both Wisent) and 10% heat-inactivated FBS (10% RPMI). Viable U937 cells were stained with trypan blue and counted by hemocytometer. For infection, 1 x 10^6 U937 cells were pelleted (300 x g for 10 minutes) and resuspended with wild type Chi-1 MV at an m.o.i. of 1.0 in minimum volume. Virus particles were permitted to attach for 1.5 hours at 37°C with 5% CO_2. Following infection, the cells were washed with HBSS (Wisent) (300 x g for 10 minutes) and resuspended in 1mL 10% RPMI with or without 1 μM ROH, atRA, or 9cRA. Cells were maintained at a final concentration of 10^6 cells/mL in 15 mL polypropylene tubes (BD Bioscience (Falcon), Mississauga, ON) and incubated at 37°C and 5% CO_2 for 24-72 hours. RNA was extracted from U937 cells and supernatants were collected from

replicate cultures to quantify viral titers at various times after infection. Mock infected U937 cells and untreated cells served as negative controls.

Isolation of RNA and reverse transcription. Pelleted cells were lysed directly in the culture tubes by trituration with 500 mL of TRIzol Reagent (Gibco, Grand Island, NY) per sample. Total RNA was obtained using phenol/chloroform extraction (Sigma) and isopropanol precipitation following manufacturer's guidelines. RNA pellets were air dried, washed in ethanol, and resuspended in RNase-free water (Pharmacy, Montreal general hospital, Montreal, QC). Quality was assessed by running 1 μg on a 1.5% agarose (Gibco) gel in 1x Tris-Acetate-EDTA (TAE) buffer and quantity was measured by spectrophotometry (Beckman DU640, Beckman Coulter, Fullerton, CA). Excess DNA was removed by DNase treatment of RNA (Per 1-10μg RNA: 1U DNAse I Amplification grade in 1x DNAse reaction buffer (Gibco) for 15 minutes at room temperature followed by inactivation with 1ul 25mM EDTA (Gibco) at 65°C for 10 minutes). Prior to storage of RNA samples at –76°C, 1μg of total RNA was reverse transcribed in a 20 μL reaction mixture: 50μg/mL random hexamers ($pd(N)_6$, Amersham Pharmacia Biotech, Piscataway, NJ), 20 nM dNTP (Gibco), 1U RNAse inhibitor, 0.2 μM DTT, 1U moloney murine leukemia virus reverse transcriptase and 1X first strand buffer (all Gibco) for 1 hour at 37°C, followed by 10 minutes at 95°C in a programmable thermal cycler (PTC-100, MJ Research, Waterton, MA). cDNA was stored at –20°C until amplification by PCR.

Semi-Quantitative PCR. Messenger RNA levels for RAR α and RXR α were measured by RT-PCR using primer pairs (Medicorp, Montreal, QC) specific for each receptor gene. ***RAR-α Primers*** (Ferrari N. *et al* 1994): *Sense* (nt 460-484) 5'ACCCCCTCTACC CCGCATCTACAAG; *Antisense* (nt 685-661) 5'CATGCCCACTTCAAAGCACTTCT GC). ***RXR-α Primers*** (designed in our lab by Avi Chatterjee using MacVector, Apple, Apple): *Sense* (nt 815-838) 5' AGACCTACGTGGAGGCAAACATGG; RXR α *Antisense* (nt 1150-1175) 5'AGCTCCGTCTTGTCCATCTGCATGT, These primer pairs were designed to amplify all known isoforms of the targeted nuclear receptors. Briefly, 5μl of the cDNA product was used for each PCR amplification reaction. The

amplification was carried out in a final volume of 50 µl: 25pmol of each primer, 125 µM dNTP (Gibco), 1.5mM $MgCl_2$ and 1 U of Taq polymerase (Promega, Madisson, WI). Reactions were performed in a programmable thermal cycler (PTC-100) for 34 cycles (denaturation 30 seconds at 94°C, annealing 30 seconds at 65°C, extension 30 seconds at 72°C). 10 µl of each reaction was loaded on 2% agarose gels in TAE buffer and the PCR products were visualized by ethidium bromide staining (0.0001% v/v). The density of the bands were measured using the Syngene gel documentation system and analysed using GeneTools software (Fisher, division of synoptics, Cambridge, England). Semi-quantitative results (expressed as integrated density values) were based on comparison with the cellular housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH), as well as negative controls used in each assay (no Taq). Standardization between assays was achieved by defining the mock-infected IDV to be equal to 1 and expressing RAR-α and RXR-α IDV's as indices where IDV index = $[IDV_{\text{nuclear receptor}} - IDV_{\text{negative control}}] / [IDV_{GAPDH} - IDV_{\text{negative control}}]$. Indices are reported as mean ± standard error of mean (S.E.M.).

Plaque Assay. Viral titers in U937 cultures and stock virus preparations were measured by plaque assay. Briefly, 85% confluent Vero cell monolayers in 24-well plates (Corning-Costar) were washed with HBSS and infected with 100 µL of serial 10-fold dilutions of culture supernatant for 90 minutes at 37°C in 5% CO_2 (duplicate wells, 100µL/well diluted in HBSS). A 16% methylcellulose (ICN, Aurora, Ohio) overlay was applied to each well (1 mL) and plates were incubated at 37°C in 5% CO_2 for a further 4-5 days. The Vero monolayers were stained for 24 hours with 4% neutral red (ICN) and then fixed with 3.7% formalin (American chemicals, Montreal, QC). Visible plaques in duplicate wells were counted and results are expressed as plaque forming units per mL (pfu/mL) ± S.E.M.

Plasmid modification. A 56 base pair (bp) oligonucleotide fragment was inserted into the pCMX-hRXR-α plasmid at a unique Afe I site within the hRXR-α sequence to generate a competitor for quantitative PCR. ***Plasmid:*** pCMX-hRXR-α (gift from

V.Giguere, Royal Victoria Hospital, Montreal, QC) was amplified in competent DH-5α *Escherichia coli* (gift from B. Ficksman, Meakins Christie, Montreal, QC) and purified using QIAGEN kit protocols (QIAGEN, Mississauga, ON). Following digestion of 1 μg plasmid with 2U Afe I (1 hour at 37°C) (NEB, Mississauga, ON), free ends were dephosphorylated with 1U calf intestinal alkaline phosphatase (CIAP, Gibco) for 5 minutes at 37°C and then both reactions were stopped with 1mM EDTA (Gibco). ***Insert:*** 0.5ug/ul of each complementary 56-bp single stranded oligonucleotide (A: 5'-GAT-CTT-GGT-ACC-GAC-CTC-GGA-TCC-ACT-AGT-AAC-GGA-AGC-TTC-CGC-CAG-TGA-ATT-CA-3' and B: 5'-TGA-ATT-CAC-TGG-CGG-AAG-CTT-CCG-TTA-CTA-GTG-GAT-CCG-AGG-TCG-GTA-CCA-AGA-TC-3') were first ligated into a double stranded oligonucleotide in a programmable thermocycler (PTC-100) as follows: 95°C for 5 minutes, drop 1°C every 30 seconds to 35°C. The ends of the oligonucleotide were then phosphorylated using T4 polynucleotide kinase (NEB) for 10 minutes at 37°C the following reaction mixture: 28 pmol oligo, 1mM dATP, 10U T4 polynucleotide kinase in 1X forward reaction buffer (all Gibco). The enzyme was then inactivated at 65°C for 20 minutes. The oligos were then purified in phenol (Sigma) and precipitated using 1M sodium acetate (American Chemicals) and 2 volumes absolute ethanol. After washing, the oligos were solubilized in 0.5 mL Tris-EDTA (pH 8.0). ***Blunt-ended ligation*** of the prepared oligonucleotide into the dephosphorylated plasmid was carried out using T4 DNA ligase in a volume of 10μl (90 fmol plasmid DNA, 275 fmol oligonucleotide DNA, 10U T4 DNA ligase, 1X ligase reaction buffer (both Gibco)) for 14 hours at 14°C. The new plasmids were then amplified in DH-5α *E. coli* and tested by RT-PCR and restriction digestion (Afe I, Eco RI (Gibco)) for success of insertion.

9.2: Results

Viral output is maximal after 72 hour infection

Figure 1 shows that measles viral (MV) output peaked after a 72 hour infection. By 96 hours the viral counts had decreased, probably due to a lack of viable cells (65-80% death as measured by trypan blue exclusion). A similar pattern of viral output was observed when infections were carried out in the presence of retinoids (10^{-6}M) (Figure 1B), although total viral output was significantly decreased (see below). We chose to begin with a 10^{-6}M concentration of retinol (ROH) as this is within the physiologic range of ROH in plasma.

Retinoids decrease viral output

MV output was consistently lower in retinoid-treated cell cultures as compared to untreated cells. As shown in figure 2, 10^{-6}M ROH decreased the viral output in U937 cells (m.o.i. =1) by ~50% (± 30% S.E.M.) at 48 hours and ~35% (± 25% S.E.M.) at 72 hours. The high variability may be due to variations in cell viability and differences between viral preparations. Similar decreases in viral output (40% ± 20%) were obtained by a former student who treated cells with equimolar ROH (10^{-6}M) and retinol binding protein (RBP) (Chatterjee A., thesis manuscript). All further experiments reported herein were carried out with ROH alone due to the impurity of commercial RBP.

Serial dilutions of retinoids

We decided to do serial dilutions of some retinoids to determine the optimal concentration to lower measles viral output. To our surprise, concentrations much lower than the physiologic concentration of retinol (1-3 μM) had greater effects. The optimal effect on viral output was observed with a final concentration of 10^{-12}M ROH, and the effects could be observed at concentrations as low as 10^{-16}M (figure 3A). When we repeated these experiments using all-trans retinoic acid (atRA) (figure 3B), the optimal concentration of 10^{-8}M was close to the reported physiologic intracellular concentrations of this retinoid (10^{-9}M).

Retinoids affect the expression of the nuclear retinoid receptor transcripts

In figure 4, the data from 10-30 experiments are shown. In general, retinoids significantly decreased the levels of transcripts for both RAR-α and RXR-α ($p < 0.0001$) in uninfected U937 cells. Conversely, retinoid treatment slightly increased the levels of both RAR-α and RXR-α transcripts in MV-infected U937 cells but these differences did not reach significance. When the different retinoids were examined individually, each form (ROH, RBP, ROH and RBP, atRA, and 9-cis retinoic acid (9cRA)) significantly decreased the levels of both RAR-α (figure 5) and RXR-α (figure 6) mRNA levels in uninfected U937 cells ($p \leq 0.001$). MV infection alone did not alter transcript levels (figures 5 & 6). In MV infected cells, 9cRA and atRA appeared to increase transcription of both RAR-α and RXR-α, while ROH alone increased only the expression of RXR-α transcripts (figures 5 & 6). The other treatment groups (ROH + RBP, RBP alone) did not affect the levels of transcripts of either retinoid receptor compared with untreated, infected cells. Previous studies in our lab have demonstrated that the other 4 retinoid receptors (RAR-β, γ and RXR-β, γ) are not detectable in this cell line (Chatterjee A., thesis manuscript).

Construction of plasmid for competitive PCR

A more accurate quantification of the retinoid-induced changes in RAR-α and RXR-α transcripts can be obtained by using competitive RT-PCR. Therefore, a plasmid containing the human RXR-α gene (pCMX-hRXRα) was modified with a 56-bp insert so that the amplification product from PCR (416bp) could be distinguished from the sample amplicon (360bp). A similar plasmid for hRAR-α was already available in our lab. Figure 7 shows the plasmid map for our RXR-α construct, with the human RXR-α gene in the EcoRI site of pCMX and our 56-bp oligonucleotide inserted at the unique Afe I site within the RXR-α sequence. Figure 8 is a representative gel that clearly shows the successful insertion of the oligonucleotide into the plasmid in 10 out of 11 instances. The plasmid was then purified and stored for future use in competitive PCR.

9.4: Discussion

A clear survival benefit of vitamin A supplementation in acute MV infection has been documented in a number of studies (Fawzi W.W. *et al* 1993, Ellison J.B. 1932, Barclay A.J. *et al* 1987). However, the mechanism by which vitamin A mediates its beneficial effects remains largely unknown. A factor that may be critical in determining the clinical outcome of MV infection is the rate of replication of the virus. This is particularly true in immune cells, which are believed to help disseminate the virus throughout the body and, which play a critical role in the immune response to this (and other) pathogen. Previous studies in our lab demonstrated that physiologic concentrations of vitamin A and its derivatives (10^{-6}M ROH, atRA, or 9cRA) had no effect on the proliferation or viability of PBMC or U937 cells. Therefore, any changes in viral output likely reflect either an intrinsic change within the cells themselves or a direct effect of retinoids on MV.

In this study, we present data that shows a clear 30-50% decrease in MV output when the infection of U937 cells is carried out in the presence of ROH or atRA. Although decreases in viral output of this magnitude may not seem significant *in vivo*, even a small limitation such as this could shift the balance in favor of the immune response fighting the virus, giving the patient a better chance of survival.

We also show that concentrations far below the physiologic levels of either retinoid result in optimal decreases in viral output. Although high concentrations of retinoids can be toxic, we have not observed any cell death associated with concentrations $\leq 10^{-6}$M for any of the retinoids tested. Therefore, the greater effects on viral output of lower concentration of retinoid may be an artifact of the *in vitro* system we are working with or could indicate a saturation point, at which the cells can no longer absorb or process the "excess" retinoid. It could also be that the retinoid receptors have a "shut off" mechanism when flooded with ligand (e.g.: ROH or atRA). Finally, there may be a balance between benefit for the virus *vs.* benefit for the cells, with the optimal decreases in viral output occuring when the retinoid concentration is most beneficial to the cells and least beneficial to MV.

Since the decrease in viral output is not due to decreased proliferation or viability, we chose to look for cellular changes at the level of mRNA expression of the

nuclear retinoid receptors. These receptors are likely candidates for regulation of the vitamin A signaling cascade for several reasons. First, they act at the final stage of the signaling cascade and are highly pleiomorphic. Second, several of these receptors contain the retinoid DNA response elements within their promoter regions, suggesting that regulation may occur via feedback loops (Giguere V. 1994).

Our results from retinoid-treated U937 cells support the feedback loop theory, since the incubation of cells in the presence of retinoids approximately halved the expression of RAR-α and RXR-α transcripts in uninfected cells (40% ± 15% and 50% ± 20% respectively, $p \leq 0.0001$). All of the retinoids tested (ROH, atRA, 9cRA, ROH + RBP) decreased the expression of the transcripts of both nuclear receptors (30-60%, $p \leq 0.001$). Real time PCR or competitive PCR using the plasmid we constructed will allow for more accurate quantification of the changes in transcript expression. The decrease induced by RBP is possibly due to ROH that remains bound even after purification. It is also possible that this as yet poorly characterized binding protein elicits a retinoid-response in the absence of its ligand if it is present in high concentrations. However, further studies would be needed to confirm this.

MV infection of U937 cells did not induce changes in the expression of the nuclear retinoid receptors at the mRNA level. However, the presence of MV did have an effect on the retinoid-induced changes in transcript levels. Cells simultaneously infected with MV and treated with retinoids had no decrease in RAR-α or RXR-α mRNA levels. In fact, for atRA and 9cRA, the levels of transcripts for both RAR-α and RXR-α actually increased slightly in MV infected cells. It appears, therefore, that MV can significantly influence retinoid-induced changes in the signaling cascade. Whether maintaining the levels of nuclear retinoid receptors at the levels observed in untreated cells is induced by or adaptive to MV remains unclear. Future experiments that directly test this hypothesis include: (1) Confirming that changes at the transcriptional level translate to changes in expression of nuclear retinoid receptor proteins using Western blotting; (2) Characterizing the particular retinoid signaling pathway involved in MV-infected cells. This includes repeating viral output experiments in cell lines overexpressing or lacking RAR-α or RXR-α and testing a panel of nuclear retinoid receptor agonists and antagonists; and (3) Looking at the effect of both natural retinoids

and their analogs on MV-induced apoptosis of PBMC, particularly the monocytic cells that appear to be resistant to MV-induced apoptosis (see manuscript I).

In conclusion, we have observed that retinoids decrease viral ouptut from MV infected U937 cells by 30-50%. Although MV alone had no impact on RAR-α or RXR-α transcript levels, MV-infected U937 cells did not respond to retinoid supplementation with reduced nuclear receptor transcription.

Figure 1: (A) U937 cells were infected with Chicago-1 measles virus (MV) at a multiplicity of infection (m.o.i.) of 1 for 24, 48, 72, or 96 hours and the viral output in the supernatant was measured by plaque assay. Results (n=3) are expressed as plaque forming units (pfu/mL) +/- standard error of mean (S.E.M). **(B)** Infected U937 cells were treated with 10^{-6}M retinoids (RET: all trans retinoic acid, 9-cis retinoic acid, or retinol) for the duration of infection (n=3).

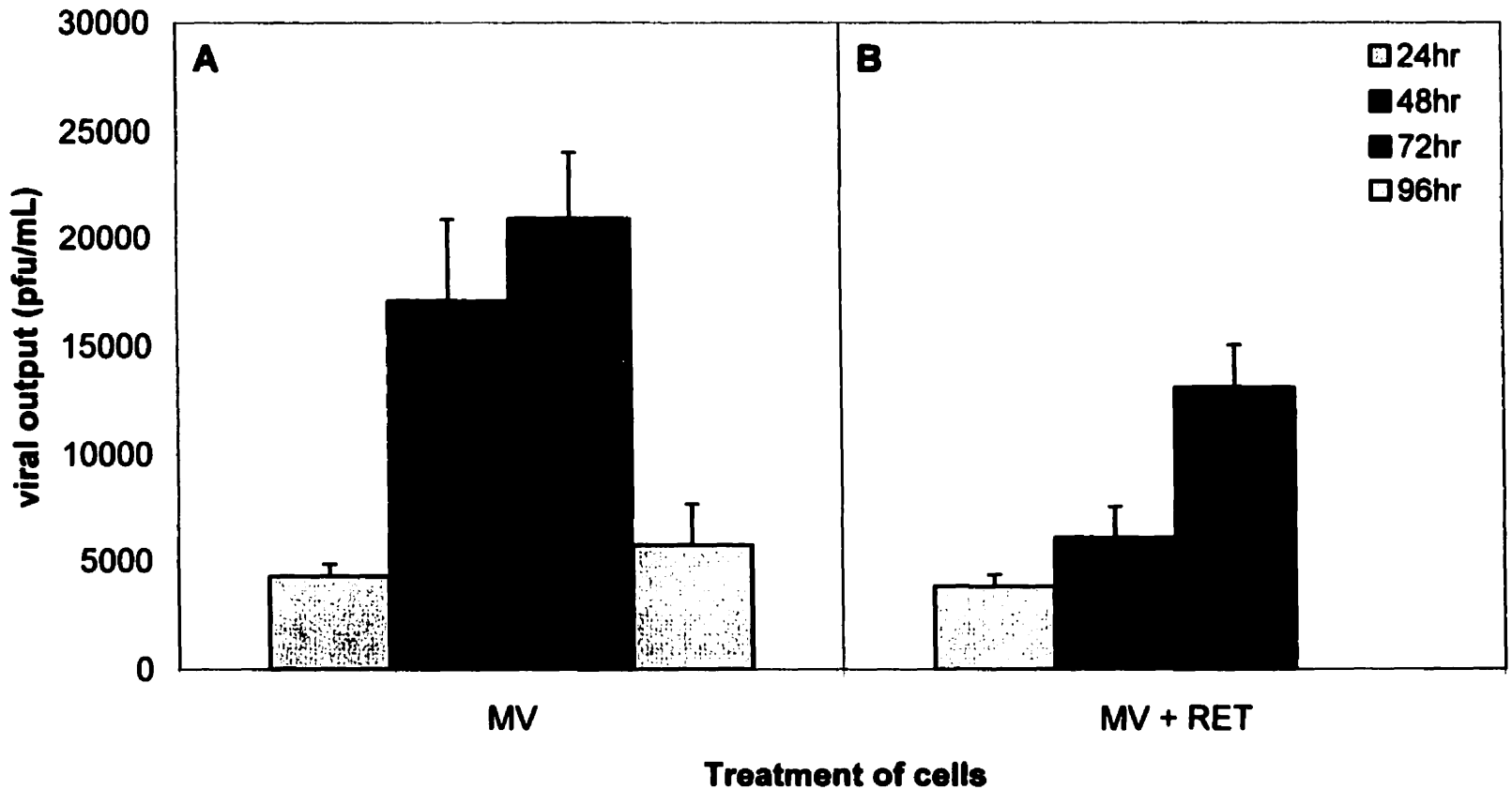


Figure 2: Measles viral (MV) output (pfu/mL) in untreated and retinol-treated (10^{-6}M ROH) U937 cells. Viral outputs in supernatants collected after 48 and 72 hour infections (m.o.i. = 1) were measured by plaque assay (n=3). Retinol treatment decreased viral output by ~50% at 48 hours (* $p < 0.07$) and ~35% at 72 hours after infection (** $p < 0.08$).

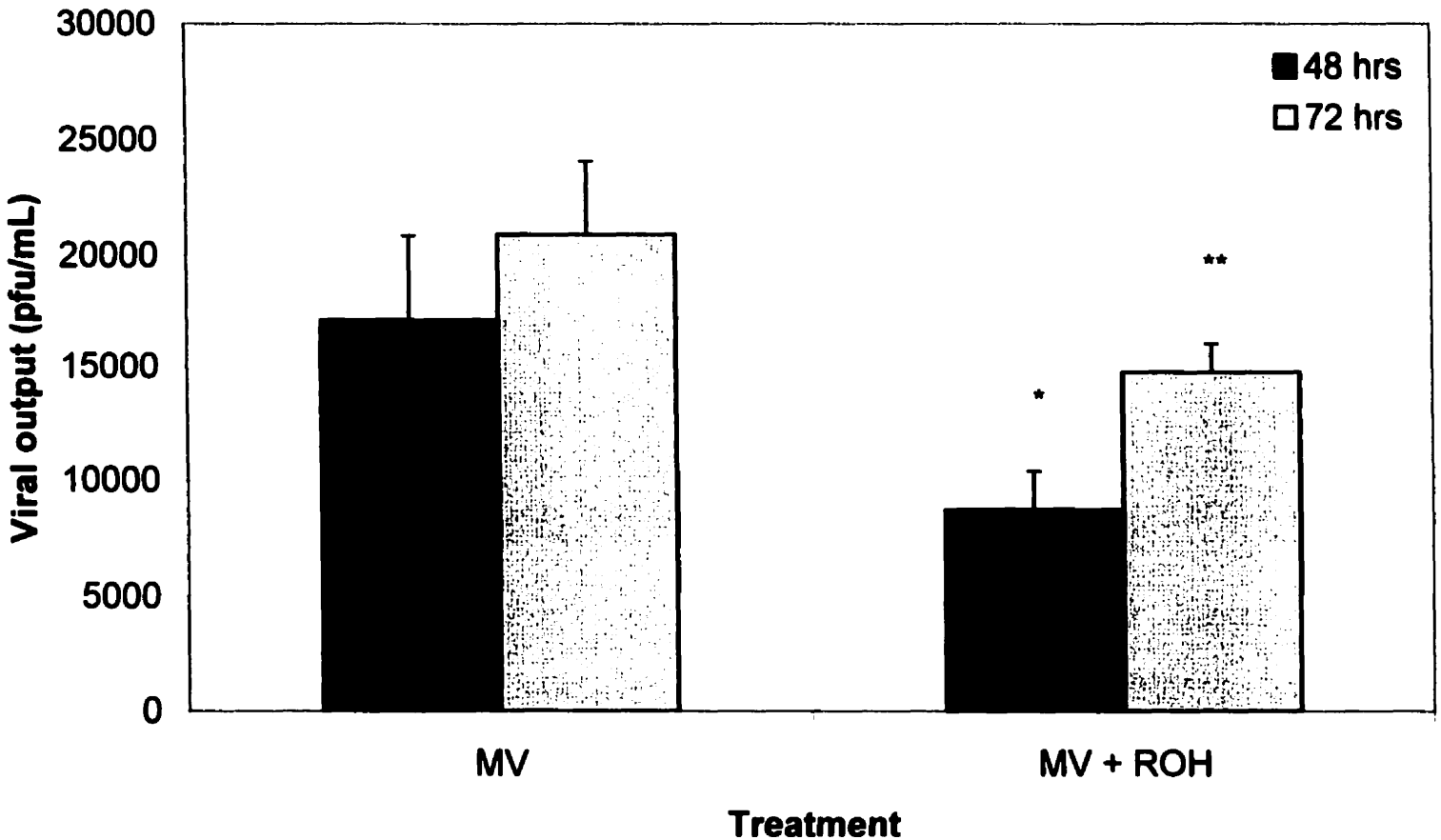


Figure 3: Serial dilutions of (A) retinol and (B) all-trans retinoic acid were tested for their effect on viral output during a 72 hour infection (m.o.i.=1) of U937 cells with Chicago-1 virus (MV) (n=2). Viral output from MV alone was set at 1 to allow comparison between experiments. Results expressed as viral output index +/- S.E.M. (= pfu/ml of sample/ pfu/mL of MV alone). Retinoids at 10^{-4}M were toxic to the cells.

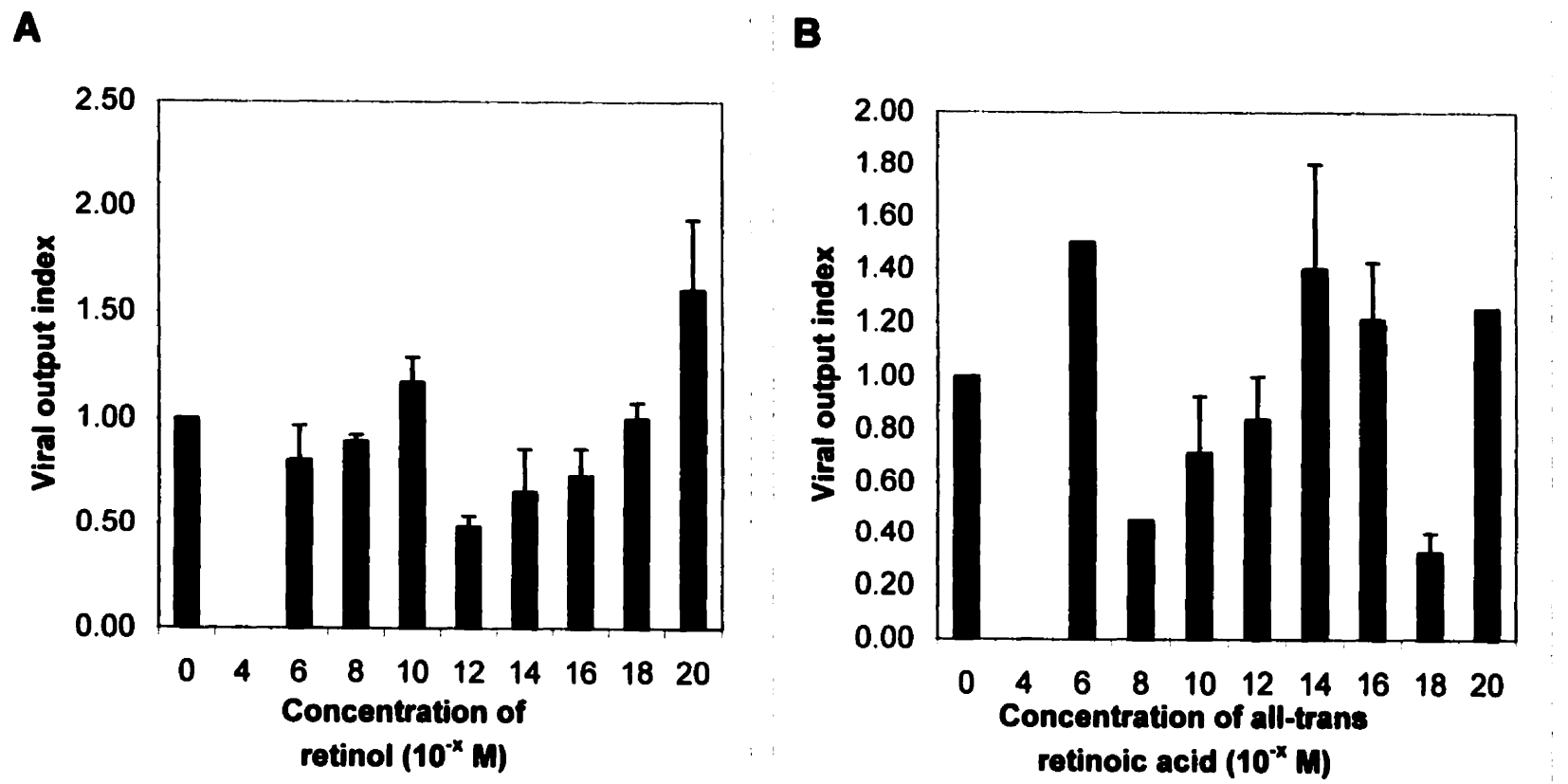


Figure 4: RT-PCR was performed on RNA extracted from U937 cells after a 24 hour measles-infection (m.o.i.=1) with or without retinoid treatment (10^{-6}M) to detect levels of RAR-alpha and RXR-alpha mRNA. Mock infected cells incubated with or without retinoids served as controls. Retinoids decreased transcription of both receptors compared with untreated cells (* $p < 0.0001$). In measles infected cells, receptor levels increased upon retinoid treatment compared with uninfected, treated cells (** $p < 0.006$, *** $p < 0.0002$).

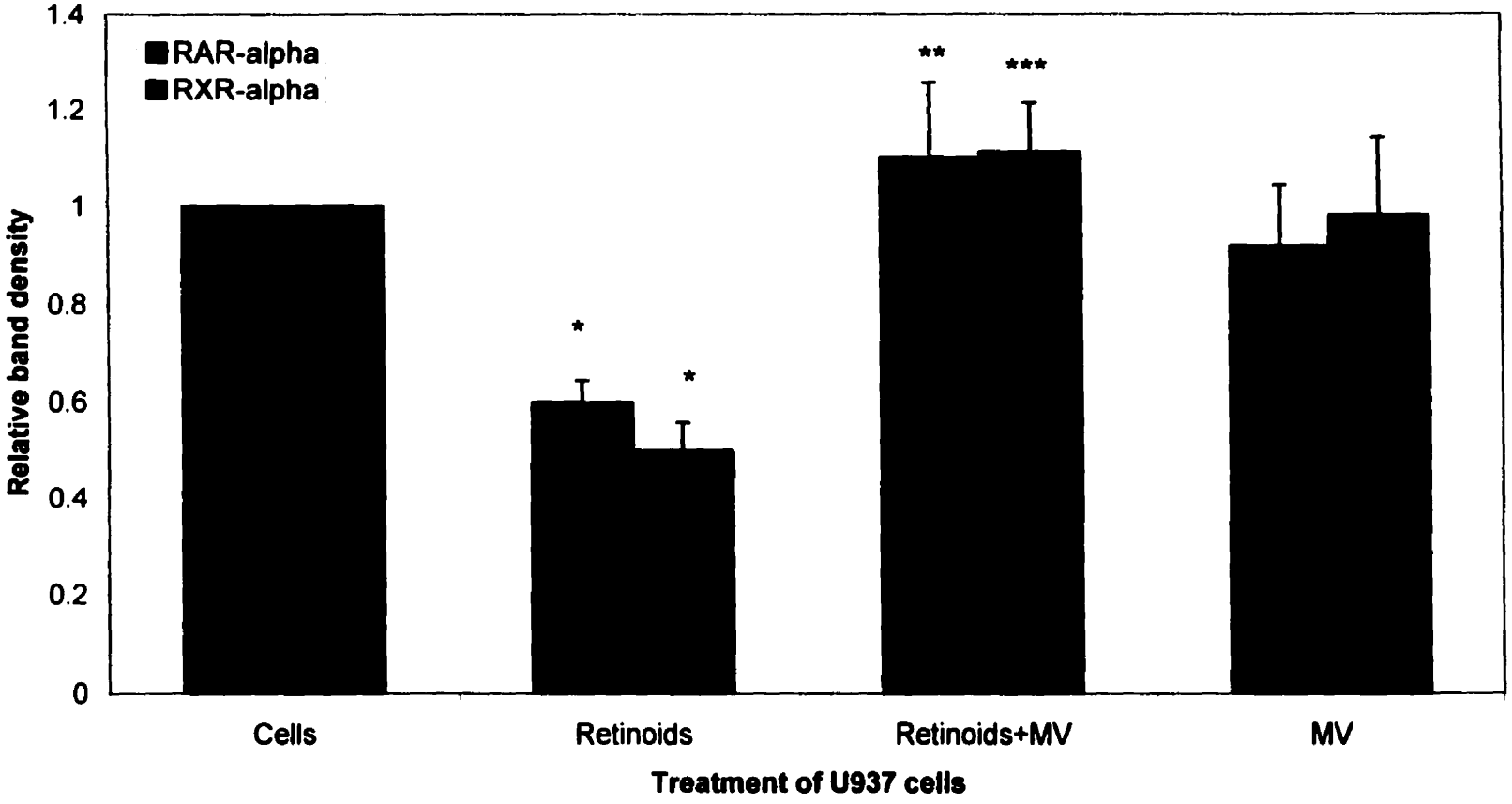


FIGURE 5: Expression of RAR-alpha mRNA. MV-infected and mock-infected U937 cells (moi=1) treated with retinoids (10^{-6}M) for 24 hours. RNA extracted from the cells was tested by RT-PCR for RAR-alpha mRNA expression. Overall, retinoids decreased transcript levels in uninfected cells and had no effect or caused a slight increase in MV-infected cells.

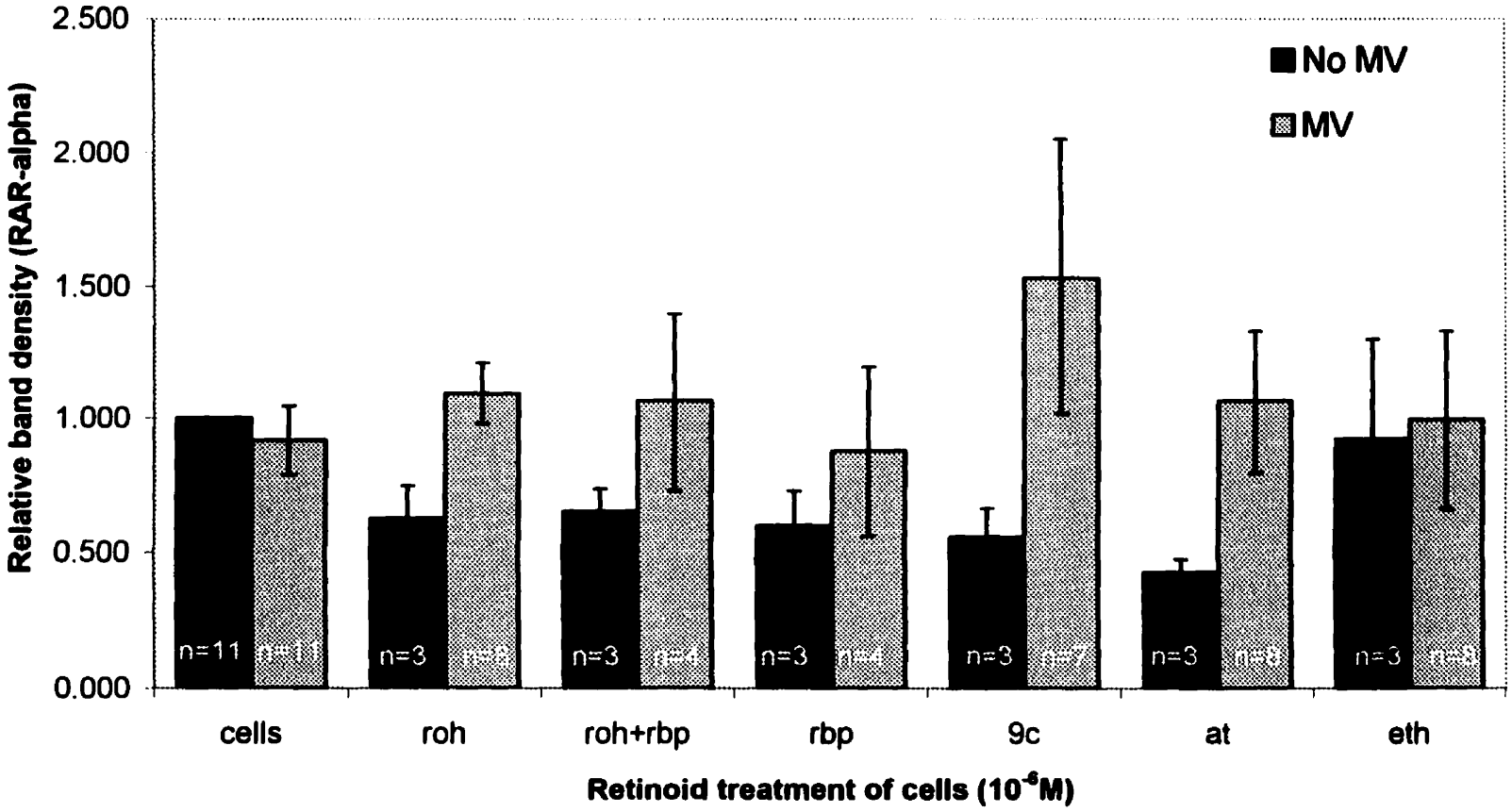


FIGURE 6: Expression of RXR-alpha mRNA. MV-infected and mock-infected U937 cells (moi=1) treated with retinoids (10^{-6}M) for 24 hours. RNA extracted from the cells was tested by RT-PCR for RXR-alpha mRNA expression. Overall, retinoids decreased transcript levels in uninfected cells and had no effect or caused a slight increase in MV-infected cells.

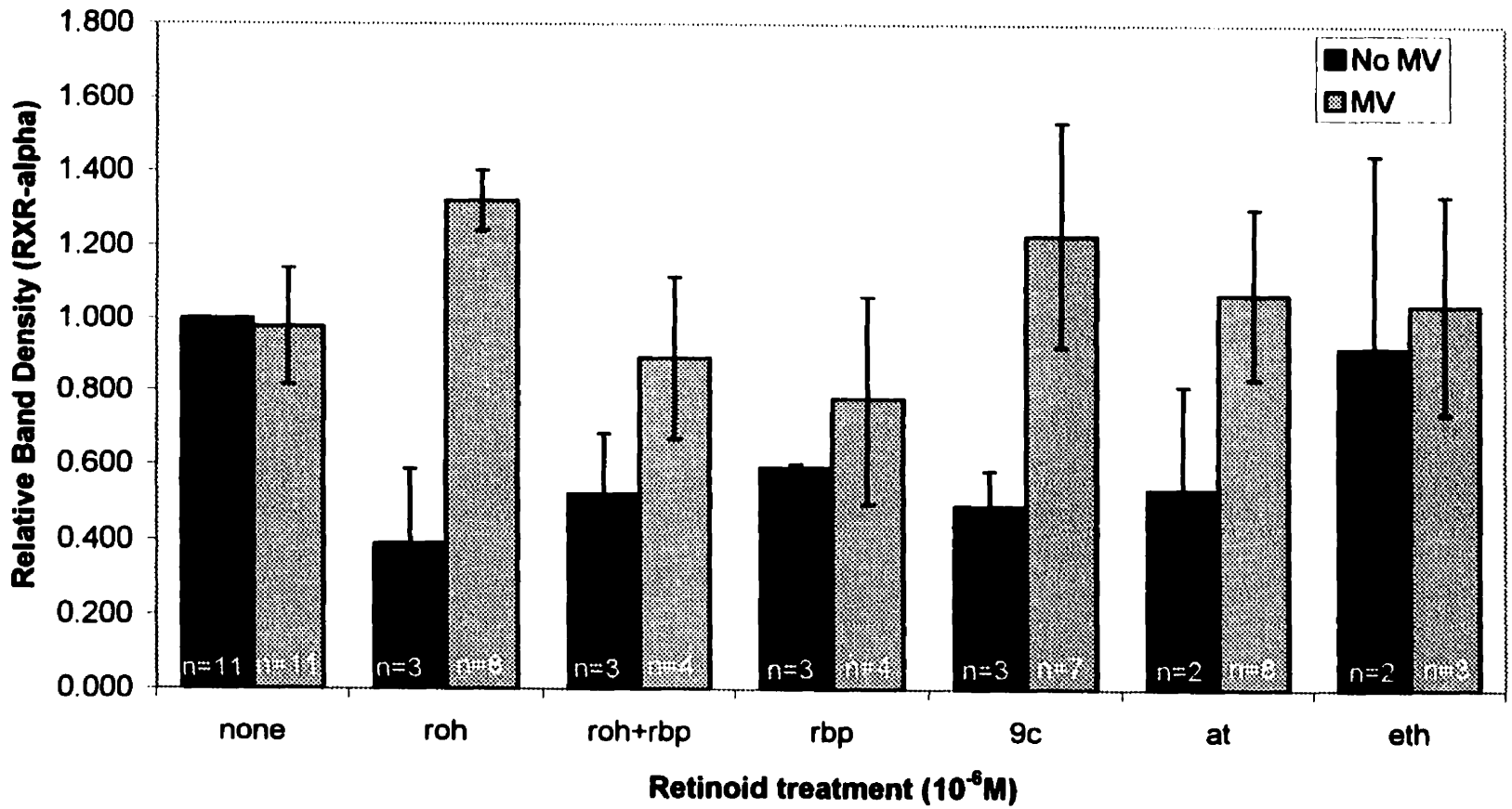


Figure 7: Cartoon of plasmid map for pCMX-RXRα*. Plasmid was constructed by inserting a 56bp segment into the unique Afe I site in the human RXRα gene located in the original pCMX-RXRα.

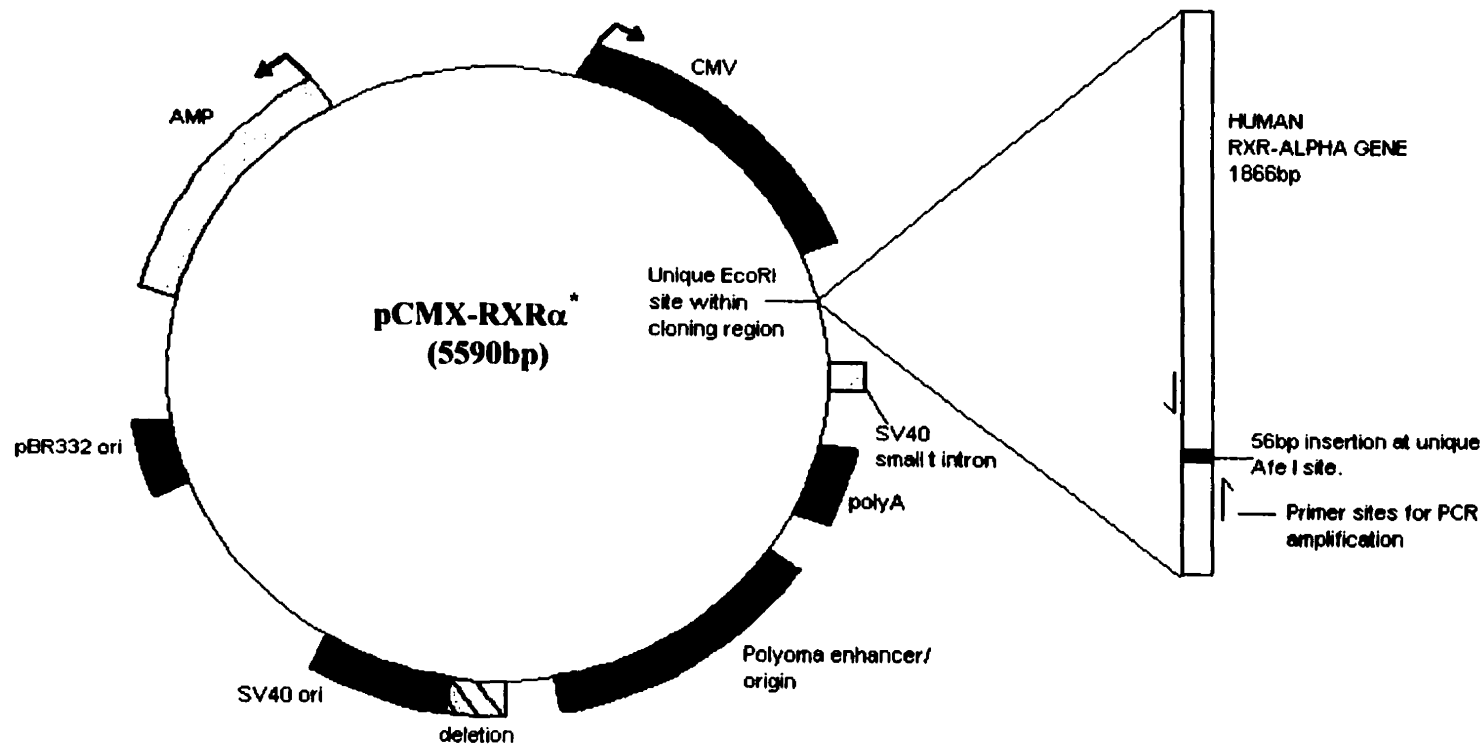
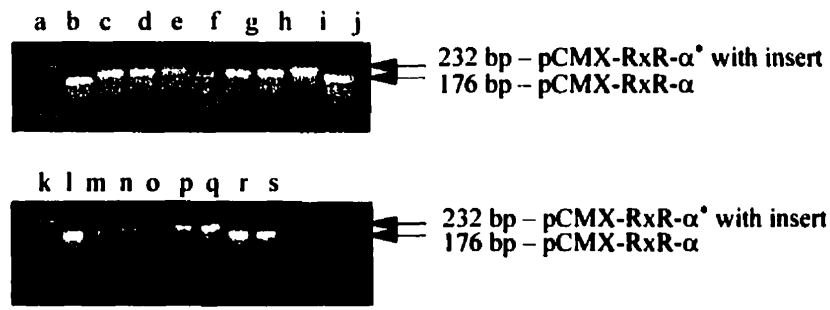


Figure 8: Successful insertion of 56bp segment into hRXR-α gene within pCMX construct. Confirmation by PCR. Lanes a,k = 100bp DNA ladder. Lanes b, f, j, l, o, r = original pCMX-RXR-α plasmid. Lanes c-e, g-i, m-n, p-q, s = newly constructed plasmid minipreps. Note that all plasmids in minipreps had successful insertion of segment, with the exception of the plasmid run in lane s.



SECTION III:

FUTURE DIRECTIONS, SUMMARY, APPENDIX I

Chapter 10: Future Directions and Discussion

This master's involved a series of studies with a common goal: to increase our understanding of the immune response to both natural measles (MV) infection and MV vaccination, with a special emphasis on the only known treatment for MV infection, vitamin A.

Measles virus and apoptosis

The apparent differences between wild-type viruses and vaccine-strain viruses in their ability to replicate in human peripheral blood mononuclear cells (PBMC) and to induce apoptosis may reflect intrinsic differences in pathogenicity. Although subtle changes in viral structure due to different passage histories might plausibly account for these differences at the cellular level, recent studies have shown variable viral growth and virus-induced cell death among even closely related MV strains (Auwaerter PG *et al* 1999, Valsamakis A *et al* 1999, 2001). Detailed phylogenetic studies will be required to investigate this possibility.

Apoptosis and viral output also varied considerably between the PBMC obtained from different individuals. These differences between individuals were consistently seen within each experiment (i.e. rank order) and over time. These observations suggest that the level of PBMC permissiveness to MV infection and/or replication may vary. This might help explain the wide range of clinical manifestations of natural MV infection. However, we do not yet know whether or not increased apoptosis is correlated with less severe clinical presentations. On the one hand, removal of human immune cells could facilitate viral growth. Conversely, programmed death of infected cells could limit viral growth and spread. Correlating viral output with susceptibility to MV-induced PBMC apoptosis in individuals with defined clinical outcomes would help to resolve these issues. Studies correlating apoptosis and viral loads using a panel of viruses in animals would also increase our understanding of these events. However, only monkeys have CD46, the MV receptor, making such studies very expensive. Transgenic mouse models (e.g.: CD46 transgenic mice, huSCID mice) are emerging but even mice that allow MV replication lack the clinical symptoms observed in humans and monkeys.

The superiority of cord blood lymphocytes in supporting MV growth (Tishon A *et al* 1996) suggests that an 'age' factor may be involved. It would be interesting to study apoptosis in cord blood mononuclear cells (CBMC) as well as PBMC from individuals over a range of ages to further characterize this 'factor'.

The mechanism of MV-induced apoptosis remains unknown, although viral protein expression and replication seem to be required (Fugier-Vivier I. *et al* 1997). Many studies have shown that different apoptotic pathways can be triggered by different stimuli (e.g. viruses *vs.* ultraviolet radiation *vs.* starvation). Experiments in our lab have shown that the level of Fas (CD95) is increased significantly on the surface of MV-infected cells. This upregulation suggests a possible mechanism by which MV triggers programmed cell death (PCD). However, other surface molecules (TNF, TNF-R, CD95L, TRAIL) and the cytoplasmic caspases will need to be examined to fully understand the cascade of events leading to MV-induced apoptosis.

It would also be interesting, in light of our focus on vitamin A and MV, to investigate whether vitamin A treatment of MV-infected PBMC would 'rescue' dying cells or push them to apoptose more rapidly. Preliminary results using the TUNEL assay suggest that vitamin A accelerates the PCD induced by MV (Chatterjee A., thesis manuscript). The effect of retinoids on the monocyte subpopulation would be of particular interest since we found these cells to be relatively resistant to MV-induced apoptosis. A panel of retinoids could be screened to gain further insight into the involvement of the highly pleiomorphic retinoid signaling cascade.

II: Measles vaccine and vitamin A

The absence of a negative effect on vaccine efficacy in the vaccine-based trial support the co-administration of vitamin A and live, attenuated MV vaccine: two individual interventions with remarkable impact on child survival in the developing world. Even children with detectable maternal antibodies to MV seroconverted at very acceptable levels following vaccination with or without high or low dose vitamin A supplements. These data confirm the results of other clinical trials that found no adverse effects in administering vitamin A supplements at the time of MV vaccination in 9-

month olds. Our trial extends the findings of the prior studies with the inclusion of cellular responses to measles and other EPI-relevant antigens.

III: Measles virus and vitamin A

Finally, our preliminary results from studies on the retinoid signaling cascade present many promising avenues of research to pursue.

First, it appears that retinoids themselves have the capacity to modulate the transcription of their own nuclear receptors. The presence of feedback loops have been suggested in literature since retinoid response elements have been identified upstream of many of the genes involved in the retinoid signaling cascade (in humans: RAR-α2, RAR-β2, RAR-γ2) (Giguere V. 1994). Results from Western blots will confirm whether these changes at the level of mRNA reflect changes at the protein level and real time or competitive PCR will provide more accurate quantification of the changes at the mRNA level.

Second, this retinoid-induced modulation does not appear to occur in MV-infected cells. Whether maintaining adequate levels of nuclear retinoid receptors is induced by or adaptive to MV remains unclear. Studies are currently underway in our lab to further characterize the relationship between the retinoid signaling cascade and MV. Many nuclear retinoid receptor agonists and antagonists have been developed in the search for cancer therapies and treatments for infections (Chambon P 1996, Napoli J.L. 1993). Testing a panel of both natural and synthetic retinoids should permit the selective triggering (or inhibition) of the particular retinoid signaling pathway involved in MV infection. Examining nuclear receptor expression in cells lacking or overexpressing either RAR-α or RXR-α may also help characterize the pathway involved. Preliminary data using cell lines lacking the RAR-α receptor suggest a central role for this nuclear receptor in the response to MV. The effects of retinoids on viral output and apoptosis may provide more direct evidence (e.g. biology) to corroborate the results from RT-PCR and Western blots.

To date, our studies have focused on the nuclear retinoid receptors. This focus was chosen because the nuclear retinoid receptors are the most pleiomorphic element/step in the retinoid signaling cascade. However, other elements in the retinoid

signaling cascade may also be involved in the response to measles infection. Therefore, expanding our studies to include the cellular retinoid binding proteins (CRBP) and the cellular retinoic acid binding proteins (CRABP) may also provide interesting data.

Most of these retinoid studies were carried out in infected pro-monocytic U937 cells. Repeating and expanding these studies in PBMC and individual PBMC subsets will provide results that are more relevant to the situation *in vivo*. Real time or competitive PCR using the plasmid designed in our lab can be performed to provide a more accurate quantitative measure of the changes in transcription.

Chapter 11: Summary and concluding remarks

Our understanding of MV infection is currently lacking in several important areas. We do not yet understand fully the mechanisms by which lifelong immunity is achieved after natural infection or the potent and prolonged immunosuppression that complicates infection (Griffin D.E. *et al* 1994). The true duration of protection after vaccination and the mechanism(s) by which MV vaccines fail also remain unknown. Little is known about the correlates of immunity after either natural disease or vaccination (Ward B.J. *et al* 1995). Further, the mechanisms that underlie the 50% reduction in MV-associated mortality with the only treatment available, vitamin A, remain uncharacterized.

Our data confirms the safety of administering vitamin A supplements concurrent with MV vaccination in 9-month old children. This supports the 1987 decision of the EPI to link vitamin A supplementation and MV vaccination in areas of endemic vitamin A deficiency. Our work shows that MV can induce high levels of apoptosis in human PBMC whereas previous studies have failed to demonstrate such an effect. Differences in individual susceptibility to MV-induced apoptosis may explain these discrepant results. The facts that the induction of apoptosis was dependent on active viral replication and was also strain-dependent are also important contributions to the understanding of the correlates of immunity and immunosuppression of MV infection. Finally, the changes in nuclear retinoid receptor transcription in both retinoid- and MV-infected cells suggests a direct role for the retinoid signaling cascade in the reduced viral output observed in retinoid treated cells. Further studies of the effect of retinoids on MV-induced apoptosis and viral output in PBMC and other cell lines, and the related changes in the retinoid signaling cascade will hopefully permit the full characterization of the mechanism underlying the clinical benefits of vitamin A treatment in measles.

Connecting Statement IV:

As a final addition to this thesis, appendix I contains a manuscript submitted for publication that examines the MV antigen-specific cytokine responses before and after MV vaccination. This returns the focus back to MV itself, and to the potent immunomodulation associated with both natural MV infection and vaccination. Some contributors to MV-associated immunosuppression have already been discussed in this thesis. In manuscript I, we demonstrated a role for MV-induced apoptosis in immunosuppression while in manuscript II and the additional studies, we touched upon the immunomodulation resulting from MV-induced vitamin A deficiency. This final manuscript focuses on the disturbances in cytokine production patterns that follow both natural infection and MV vaccination. The results demonstrate an association between Th1 and Th2 cytokine production and the induction of cellular or humoral immune responses, respectively, and raise the possibility that MV antigen-specific cytokine production profiles might be useful in predicting immune response generated by MV vaccination.

Appendix 1

Cytokine measurements as predictors of immune response after measles vaccination.

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Running head: Predictors of immune responses to MMR

Keywords: Measles, Measles vaccine, Cytokines, Neutralizing antibodies, Lymphorpoliferative response

Abstract

***Introduction*:** Measles is a potent immunomodulatory virus. The binding of measles virus to its receptor (CD46) down-regulates IL-12 production and causes a broad disturbance of normal cytokine production patterns that has been documented in both natural disease and following vaccination. In aggregate, these studies suggest that measles initiates a Th0/Th1 response that rapidly evolves to a Th2-predominant response. The roles of Th1 and Th2 cytokines in the induction of cellular and humoral responses, respectively, raises the possibility that measles antigen-specific cytokine production profiles might be useful in predicting the pattern of immune response generated by measles vaccination. ***Materials and Methods*:** We evaluated cytokine production patterns in a total of 88 children at various times after vaccination or revaccination with MMR: 60 children undergoing routine primary immunization at 12 months of age were studied before vaccination and between 5 weeks and 6 months after vaccination; 18 children were studied between 5-15 years after routine vaccination; 10 previously vaccinated children between 5 and 13 years of age with low or absent antibody titers were studied immediately prior to revaccination and at 1 and 3 months after revaccination. Cytokine production was evaluated in three ways: culture supernatant (protein) levels by ELISA following *in vitro* stimulation of PBMC with measles antigens; cytokine mRNA expression in PBMC by RT-PCR ELISA following measles antigen stimulation *in vitro*; spontaneous intracellular (ic) cytokine levels in peripheral blood mononuclear cells (PBMC) *ex vivo* by FACS analysis. ***Results*:** Little or no measles antigen-specific cytokine production could be detected in children before vaccination/revaccination, and all cytokine measures were markedly lower in children who failed to mount lymphoproliferative response to measles antigens after vaccination/revaccination. Measures of IFN-γ and IL-5 production were generally increased at most time points after vaccination (supernatant protein, mRNA and ic protein levels). Supernatant protein levels and mRNA expression for IL-10 and IL-12 also generally increased after vaccination. Children who responded to vaccination with the production of both antibodies and lymphoproliferation to measles antigens ($Ab^{hi}CMI^{hi}$) had significantly higher levels of IFN-γ in culture supernatants at 5-8 weeks ($p=.06$), and 5-13 years after vaccination ($p<.04$) compared with children who generated (or maintained) antibody responses alone ($Ab^{hi}CMI^{lo}$). Antigen-specific IFN-γ mRNA levels ($p<.03$) and *ex vivo* levels of spontaneous IFN-γ levels in $CD8^+$ T cells ($p<.02$) were also higher in the $Ab^{hi}CMI^{hi}$ children at 5-8 weeks after vaccination. Supernatant protein levels and mRNA expression for IL-10 and IL-12, as well as icIL-2

levels in $CD4^+$ T cells were also increased in the $Ab^{hi}CMI^{hi}$ children. Cytokine production profiles were not influenced by gender. ***Conclusions***: Measles vaccination/revaccination induces a Th0/Th1 response that is dominated by detectable antigen-specific IFN-γ production for many years after vaccination. The initial response to measles vaccination/revaccination includes increased IL-10 and IL-12 production and evolves over time (3-6 months) to include increased IL-5 production. These findings suggest that the induction of measles antigen-specific IFNγ production is a good surrogate marker for a balanced ($Ab^{hi}CMI^{hi}$) response to measles vaccination.

Introduction

Prior to the emergence of human immunodeficiency virus, measles was widely recognized as capable of potent immunomodulatory action [1]. Indeed, the immediate immunological impact of these two viruses is similar in many ways [2, 3]. Natural measles virus infection is characterized by a wide range of immunologic abnormaliteis including profound lymphopenia, loss of delayed-type hypersensitivity responses, loss of mitogen- and antigen-stimulated lymphoproliferation and decreased NK cell function. Immunosuppression during the acute phase of the infection can be sufficiently pronounced to cause remission in a variety of childhood autoimmune diseases [4]. This immunosuppression is thought to account for much of the morbidity and mortality associated with measles, through susceptibility to secondary bacterial and viral infections. The period of clinically relevant immunosuppression is not precisely known, but young children who survive measles appear to be at increased risk of dying for months to years after the acute infection has apparently resolved. Intensive study over the past decade has suggested a number of mechanisms that may plausibly contribute to measles-induced immune suppression. These include apoptosis of thymocytes, antigen-presenting and effector cells, production of as yet poorly characterized soluble suppressor factors, virus-induced cell cycle arrest in immune cells and disruption of normal cytokine production patterns.

The last possiblitity is particularly intriguing since the binding of the measles virus to its cellular receptor (CD46, a complement regulatory protein) has recently been reported to down-regulate monocyte production of the crucial Th1 cytokine IL-12 [5]. This finding suggests a molecular mechanism for the earlier and seemingly paradoxical observations of diffuse immune cell activity druing measles [6, 7, 8, 9] with polyclonal B cell activation [10] but general suppression of many aspects of cell-mediated immunity [11, 12, 13, 14, 6, 15, 16]. Studies in natural infection demonstrate that early evidence of $CD8^+$ T cell activity with massive IFN-γ production followed by $CD4^+$ T cell activity and massive IL-4 production [8, 17, 9, 18, 19] have lent further support to the concept that measles, like HIV [2, 3], acts to deviate immune responses to a predominant Th2 pattern [20]. An early study in adults revaccinated due to low antibody titers tended to support this concept and suggested that an exaggerated or early Th1‡Th2 switch might occur following vaccination [18]. This hypothesis was also thought to account for the relative difficulty that early investigators had encountered when attempting to ellicit measles antigen-specific lymphoproliferative or DTH reponses [21, 22, 23, 24, 25, 26, 27]. More recently, several laboratories have overcome this obstacle and have reported a capacity to measure measles-specific lymphoproliferation after vaccination [28, 29, 30,

31, 32, 33], revaccination [34] and natural disease (unpublished). Several of these studies included measures of cytokine production and demonstrated an overall increase in antigen-specific IFN-γ production following vaccination. In only a single study of children after bone marrow transplantation correlations were sought between cytokine production and the pattern of immune response (e.g.: humoral or cellular). These investigators observed that pre-existing lymphoproliferative responses to measles antigens (with IFN-γ production) significantly inhibited the sero-response to booster vaccination.

In the current study, we sought to determine the value of early, post-vaccination cytokine measurements in determining the balance between cellular and humoral responses following MMR as well as the stability of vaccine-induced, antigen-specific cytokine profiles over time.

Materials and methods

Study population

In the context of a detailed study of immune responses generated by measles vaccine, we recruited in community clinics in Canada healthy, 12-month-old children (EARLY VAX group) undergoing routine vaccination with MMR (Merck, Sharp & Dohme, West Point, PA) , as well as 6- to 15-year-old children who had received primary measles vaccine when they were 12 months of age (LATE VAX and REVAX groups). After informed consent was obtained from the parents of the children, blood samples were collected from 60 children of the EARLY VAX group on the day of vaccination, as well as 1-8 weeks and 6 months after vaccination. In the LATE VAX group, samples were taken from 18 children 5-13 years after primary measles vaccination, and in the REVAX group samples were taken from 10 children 1-3 months after measles revaccination. Characteristics of these samples have been described elsewhere [33, 34, 35].

Sample handling

After removal of the plasma from the samples, blood cells were washed twice in 10 ml of Hanks buffered salt solution (HBSS, Life Technologies, Grand Island, NY). Peripheral blood monomuclear cells (PBMC) were isolated using Ficoll-Paque (Pharmacia, Piscataway, NJ), as previously described [34]. Isolated PBMC were aliquoted and cryopreserved at -196°C (liquid N_2).

Measurement of the humoral and lymphoproliferative responses

The plaque reduction neutralization (PRN) test and the lymphoproliferative assay were carried out as described elsewhere [33].

Cytokine measurements

Because there is some controversy regarding the best way to measure cytokine levels [36], cytokines were measured with three different methodologies in order to acquire as much information as possible with limited PBMC culture material. Culture supernatant (protein) levels were measured by ELISA following *in vitro* stimulation of PBMC with measles antigens; Cytokine mRNA expression in PBMC was measured with RT-PCR ELISA following measles antigen stimulation *in vitro;* and spontaneous intracellular (ic) cytokine levels in PBMC were measured *ex vivo* by FACS analysis. However, not all cytokine assays were performed for every sample. In the EARLY VAX group, protein production was measured in 31-60 young children, mRNA cytokine

expression in 7-20 children, and spontaneous intracellular cytokine levels in 31 children. In the LATE VAX group, protein and mRNA cytokine levels were measured in 18 children, and in the REVAX group these two measurements were performed in 10 children.

Antigen preparation

Measles antigen, obtained from wild-type (CHI-1; gift from W Bellini, CDC, Atlanta) was prepared as previously described [34]. Briefly, Vero cell monolayers were infected at a low multiplicity of infection (MOI = 0.01) and were harvested at peak cytopathic effect after one freeze-thaw cycle. Culture lysates were clarified (600 x g for 10 minutes), filtered (0.22 μm) and pooled before centrifugation at 29,000 x g for 2 hours at 4°C. The viral pellet was resuspended in ice-cold PBS, lightly sonicated and stored at -70°C until used in assays. A clarified and filtered Vero cell lysate served as the control antigen. Antigen protein concentrations were estimated using a modified Bradford assay (BioRad Laboratories, Hercules, CA).

Preparation of PBMC

Control and measles antigens were coated on the same 24-well flat-bottomed polystyrene plates (NUNC, Roskilde, Denmark). Cryopreserved PBMC were quick-thawed and washed once in HBSS and resuspended at 2.5 x 10^6 cells/mL. PBMC were distributed in control and measles antigen wells (1 mL/well). Cell cultures were incubated for 96 hr at 37°C in a humidified atmosphere containing 5% CO_2.

Cytokine ELISAs

Supernatants for cytokine measurements were collected 96 hr after the measles and control antigen stimulation. The volume of supernatant that could be collected varied from 0.35 to 0.55 mL, which allowed 3-5 determinations to be made for most of the samples. Supernatant cytokine levels were determined by capture ELISA using commercial (IL-4, IL-5, IL-10, IL-12, IFN-γ and TNF-α) antibody pairs, according to the manufacturer's instructions (Pharmingen, San Diego, CA). Briefly, flat-bottomed plates (Immulon4, FB; VWR Canlab, Mississauga, ON) were coated overnight at 4°C with appropriate antibodies at 2-10 μg/mL in $NaHCO_3$, pH 8.2. (Table C). The plates were blocked with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Grand Island, NY) in PBS (100 μL/well) at room temperature for 30 min and washed three times in 0.05% Tween-20 (American Chemicals Ltd., Montreal, QC) in PBS (PBS/Tween). The test samples were used neat and serial dilutions of the standard curves

in blocking buffer were added to the plates (50 µL/well) and incubated overnight at 4°C. The standard curves were obtained by using recombinant cytokines for the cytokine of interest (PharMingen) (Table D). Biotinylated second-step antibodies (PharMingen) (Table E) were diluted in PBS/Tween, and incubated at room temperature for 1 hr (100 µL/well). The plates were washed six times and streptavidin-HRP (1/1500: 100 µL/well; Cedarlane, Hornby, ON) was addedand incubated for 1 hr at room temperature. After eight washes, the cytokine plates were developed with the ABTS (Boehringer Mannheim Canada, Laval, QC) and 30% H_2O_2 for ~20 min and the optical density (OD) of the reaction was read at 405 nm. The results are reported in picograms, based on extrapolation from the appropriate standard curve.

Measurement of measles-specific cytokine mRNA

RNA extraction

After incubation, culture plates were centrifuged briefly to precipitate cultivated cells in the bottom of the wells, and to facilitate collection of the supernatant. Cells were lysed directly in the culture plates by trituration with 500 ml of Trizol/well (Life Technologies, Grand Island, NY). Total cellular RNA was obtained using phenol/chloroform extraction and isopropanol precipitation, following the manufacturer's (Life Technologies) instructions. RNA pellets were dried briefly and kept at -70°C until reverse-transcription was performed.

Reverse-transcription

Total cellular RNA was reverse-transcribed using Monoley murine leukemia virus reverse transcriptase (2.5 µL M-MLV, Transcriptase-Superscript II: Life Technologies), 1mM each of the dNTPs (dATP, dCTP, dGTP, and dTTp), RNase inhibitor (1 U/mL Rnasin, Promega, Madison, WI), 5mM random hexomer primers, 0.01 M DTT, and 5 µL of 5X first strand buffer (250 mM Tris-HCL, 375 mM KCL, 15 mM $MgCl_2$, pH 8.3) in a final volume of 20 µL. After 60 min at 42°C, reverse transcriptase activity was terminated by raising the reaction temperature to 95°C for 5 min, after which the tubes were quickly chilled on ice. The final cDNA product was stored at -20°C for subsequent amplification by PCR.

PCR amplification

Cytokine transcript levels were measured using a modification of a semiquantitative reverse transcriptase PCR technique described previously [37]. Briefly, 5 µL of the cDNA product (diluted 1:4) was added to a PCR reaction mixture containing

3 µl 10X buffer (500 mM KCl, 100 mM tris-HCl, pH 8.3), 1 µl dNTPs each at 10 mM, 1 unit Taq DNA polymerase (Promega, Madison, WI), and 1 µl each of 20 µM biotin-labeled 5' and unlabeled 3' primers in a total volume of 50 µl. PCR was performed by first incubating the reaction mixture at 95°C for 4 min, followed by temperature cycling at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Optimal PCR reaction conditions were defined for each cytokine primer pair [38, 39, 40] such that the relationship between the input cDNA and the final PCR product was appropriately linear. Controls were included in each assay to confirm that no contamination of the cDNA had occurred. The number of PCR cycles selected and the biotin-labeled 5'-primers and unlabeled 3'-primers used for each cytokine are described in Table A. The amplified DNA was analyzed by hybridization with internal cytokine-specific probes (see below). Amplification of the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPD-H) served as an internal control for the the amount of cDNA from each sample.

ELISA for detection and quantitation of PCR products

For the detection of PCR products, 96-well microtiter plates (Immulon2 RB; VWR Canlab, Mississauga, ON) were coated with 100 µL of avidin diluted in carbonate-bicarbonate buffer (1 µg/mL; Sigma, Saint Louis, MO) overnight at 4°C. After 3 washes with PBS/Tween, 5 µL of biotin-labeled PCR-amplicons were diluted in 20 µL of hybridization buffer (HB) and allowed to bind to previously avidin-coated well at room temperature for 30 min. The DNA was denatured by a brief 1N NaOH wash at room temperature. After six washes with Tris-buffered saline in 0.05% Tween-20 (American Chemicals Ltd., Montreal, QC) (TBS/T), the avidin-bound DNA strands were hybridized at 42°C for 15 min with cytokine-specific internal probes, end-labeled with fluorescein (Table B). After hybridization, the plates were washed and incubated with 0.75 x 10^{-4} U/mL alkaline phosphatase-conjugated anti-fluorescein (anti-FL FAB; Boehringer Mannheim, Laval, QC) for 15 min at 37°C; bound antibodies were detected with an alkaline phosphatase substrate and amplifier (ELISA amplification kit; Life Technologies). The ELISA plates were then read at 490-495 nm on a microplate reader. The resulting OD values for each cytokine were normalized to the GAP-DH value for the same sample (e.i., cytokine OD/GAP-DH OD).

Spontaneous intracellular cytokine production

Cell stimulation and flow cytometry

Cryopreserved cells were thawed at 37°C and washed twice in HBSS (Life Technologies). Approximately 2 x 10^6 cells/ml were plated in a 24-well flat-bottoned plate (Falcon). PBMC were stimulated with Phorbol 12-Myristate 13-Acetate (10 ng/ml PMA; Sigma, Saint Louis, MO) and Ionomycin (1 mg/ml, Sigma) in the presence of Brefeldin A (10 mg/ml, Sigma) for 6 hr at 37°C in 5% CO_2. Non-stimulated cells (negative control) were cultured in the presence of Brefeldin A only. The cells were harvested, washed and transfered to 5 mL polysterine tubes (Falcon 2052) for immediate staining. PBMC were stained with FITC-anti-CD4, FITC-anti-CD14, and PerCP-anti-CD19 or PerCP-anti-CD8 (Becton Dickinson), then fixed with 4% (w/v) paraformaldehyde (Sigma) pH 7.4-7.6, permeabilized with 0.1% (w/v) saponin (Sigma) in PBS pH 7.4-7.6, and subsequently stained with 0.25 mg of PE-anti-human IFN-γ, IL-2, IL-4, IL-5 or IL-12 antibody (PharMingen, CA). The incubation time for fixation, permeabilization and staining was 15 min each at 4°C. After washing with saponin, the cells were analyzed using a FACScan (Becton Dickinson) with CELLQuest software (Becton Dickinson). Flow cytometric analysis was performed as previously described [41]. Briefly, for each sample, data for 15,000 events were acquired by using log-amplified fluorescence and linearly amplified side- and forward-scatter signals. All samples were analyzed by setting appropriate forward- and side-scatter gates around the lymphocyte and monocyte populations. For all analyses, cell populations stained with negative control MAbs (Becton Dickinson) were entirely contained within the region corresponding to the first 1.0-1.1 logs of fluorescence intensity.

Statistical Analysis

Statistical analyses were performed using Statview 512 software (SAS, Cary, NJ). Data for each parameter were first analyzed to define their distribution. Cytokine responses in individual patients before and after vaccination were compared using the paired Student t test; the unpaired t test and the nonparametric Mann-Witney U test were used for comparisons between groups. Correlations were assessed using Spearman rank correlation coefficients. Results were considered significant at the level of $p \leq 0.05$.

Table A: PCR primers for cytokine amplification

Cytokine	Cycles	biotin-labeled 5'-primers	unlabeled 3'-primers
GAP-DH	23	5'-CCC TTC ATT GAC CTC AAC TAC ATG GTT-3'	3'-AGT CTT CTG GGT GGC AGT GAT GGC-5'
IFN-γ	25	5'-GCA GGT CAT TCA GAT GTA GCG-3'	3'-CAG CCA TCA CTT GGA TGA GTT-5'
IL-2	30	5'-CTG GAG CAT TTA CTG CTG GAT-3'	3'-ATG GTT GCT GTC TCA TCA GCA-5'
IL-4	30	5'-GAC ACA AGT GCG ATA TCA CCT-3'	3'-AAG TTT TCC AAC GTA CTC TGG-5'
IL-5	30	5'-GCT TCT GCA TTT GAG TTT GCT AGC-3'	3'-TGG CCG TCA ATG TAT TTC TTA AAG-5'
IL-10	30	5'-CTG AGA ACC AAG ACC CAG ACA TCA A-3'	3'-CAA TAA GGT TTC TCA AGG GGC TGG-5'
IL-12	30	5'-CAT TCG CTC CTG CTG CTT CAC-3'	3'-TAC TCC TTG TTG TCC CCT CTG-5'

Table B: Sequence of the probes used for the detection and quantification of PCR products for cytokines.

Cytokine	Probe sequence
GAP-DH	5'-TCT GCT GAT GCC CCC ATG TTC GTC ATG GGT GTG-3'
IFN-γ	5'-ACT GAC TTG AAT GTC CAA CGC AAA GCA-3'
IL-2	5'-AAG AAT CCC AAA CTC ACC AGG ATG CTC ACA-3'
IL-4	5'-CAT CTT TGC TGC CTC CAA GAA CAC-3'
IL-5	5'-CTC TGC TGA TAG CCA ATG AGA CTC TGA GGA-3'
IL-10	5'-GCC ATG AGT GAG TTT GAC ATC-3'
IL-12	5'-TAA GAC CTT TCT AAG ATG CGA GGC CAA-3'

Table C: Antibodies used for the detection of the cytokines by ELISA.

Cytokine	Description
IFN-γ	mouse anti-human MAb NIB42, 5 μg/mL
IL-4	anti-human IL-4 MAb 8D4-8, 5 μg/mL
IL-5	anti-human IL-5 MAb TRFK5, 2 μg/mL
IL-10	anti-human/viral IL-10 MAb JES3-9D7, 5 μg/mL
IL-12	anti-human IL-12 MAb G161-566 and G161-159, 10 μg/mL
TNFα	anti-human TNFα MAb1, 2 μg/mL

Table D: Recombinant human cytokines used for standard curves

Cytokine	Description
IFN-γ	2616KC, 100 ng/vial
IL-4	19641V, 5μg at 33μg/mL
IL-5	19651V, 5μg at 33μg/mL
IL-10	19701P, 5μg at 33μg/mL
IL-12 (p70)	19721V, 5μg at 33μg/mL
TNFα	19761T, 10mg

Table E: Biotinylated second-step antibodies used for cytokine ELISAs

Cytokine	Description
IFN-γ	anti-human IFN-γ 4S.B3, 2 μg/mL
IL-4	anti-IL-4 MP4-25D2-biotin, 2 μg/mL
IL-5	anti-IL-5 JES1-5A10, 1 μg/mL
IL-10	anti-IL-10 JES3-12G8, 1 μg/mL
IL-12	anti IL-12 C8.6, 1.5 μg/mL
TNFα	anti-TNFα MAb11, 1 μg/mL

Results

Study subjects and the immune response to vaccination

Cytokine measurements were performed on one or more samples obtained from each of a total of 88 children. Samples sets were available for three time points for 60 children undergoing routine MMR vaccination at 12 months of age (EARLY VAX): pre-vaccination, between 1-8 weeks after vaccination, and 6 months after vaccination. The mean age at vaccination in these children was 416 ± 2.9 days (range 395-488 days). Sample sets were also available from 10 older children found to have sub-protective antibody titers undergoing revaccination with MMR (REVAX): prevaccination and 1 and 3 months post-vaccination. The mean age of these children at revaccination was 9.4 ± 3.4 (4 boys, 6 girls). Based upon IgG avidity and IgM determinations (unpublished), 4 of these children generated primary responses to revaccination and the remaining 6 mounted secondary responses to revaccination. Finally, single blood samples were available from 18 older children between 5 and 13 years of age (mean 9.5 ± 4.5 years: 7 boys, 11 girls) who had received routine MMR vaccination at 12 months of age and had had unknown contact with natural disease since the time of vaccination (LATE VAX).

Immune response to measles antigens

Neutralizing antibody titers and lymphoproliferative responses to measles antigens for these children have been previously reported (Table 1). Briefly, the young children followed during routine immunization (EARLY VAX) had very low levels of maternal antibody at the time of vaccination (mean PRN 10 ± 1.1) although antibodies were detectable in 46%. Neutralizing antibodies rose rapidly after vaccination to reach high titers at 6 months after vaccination (PRN 1820 ± 210). Seven percent failed to generate PRN titers thought to be protective (PRN ≥ 120) [42]. Lymphoproliferative responses to measles antigens could be detected in 61% of these children between 5 weeks and 6 months after vaccination (mean SI 5.6 ± 1.2). Maternal antibody status at the time of initial vaccination was unknown for all of the older children included in this study. One third of the older children undergoing revaccination (REVAX) had low titers of pre-existing neutralizing antibodies. Titers rose rapidly upon revaccination to high titers at 1 month after revaccination (598 ± 123) but fell equally rapidly to more modest levels by 3 months after revaccination (PRN 268 ± 107). At 3 months after revaccination, 4/10 (40%) had PRN titers < 120. Lymphoproliferative response to measles antigens were detectable in 4/10 (40%: SI 4.9 ± 0.9) of these children before revaccination and strong responses could be detected in all but one child at 3 months after revaccination (91%: SI 12.7 ± 2.3). Antibody titers in the single samples obtained

4-12 years after vaccination (LATE VAX) were modest (PRN 465 ± 116) and 4/18 (22%) of the children had PRN titers < 120. Almost three quarters (13/18) of these children had detectable lymphoproliferative responses to measles antigens (74%). Note that the proportions of both groups of older children with detectable lymphoproliferative responses are higher than reported in the original studies (78% and 66% respectively) since children with little or no lymphoproliferative responses were found to produce little or no detectable cytokine responses. As a result, we included slightly more samples with detectable measles-specific lymphoproliferative in the cytokine studies. The small number of young children (n=2) with no evidence of either humoral (PRN < 8) or cellular responses (SI < 3) had no cytokine responses to measles antigens and were excluded from analyses. The remaining children all had at least some detectable antibody response (PRN ≥ 8). For the purposes of the discussion below, the children have been divided into two groups based upon the presence ($Ab^{hi}CMI^{hi}$) or absence ($Ab^{hi}CMI^{lo}$) of a detectable lymphoproliferative response.

Cytokine determinations

Relatively small numbers of PBMC were available for study from the young children undergoing routine immunization (0.5–3 x 10^6/sample). As a result, not all of the cytokine determinations are available for each sample (e.g.: supernatant protein, mRNA and intra-cellular staining in this order of priority). Similarly, not all cytokines could be measured for the smaller samples (i.e. order of priority for both supernatant protein levels and mRNA expression was IFN-γ, IL-10, IL-12, IL-5, IL-4, IL-2, TNFα). For the intracellular cytokine staining (performed only with PBMC obtained from the younger children, EARLY VAX, between 5-8 weeks after immunization), the order of priority was IFN-γ, IL-2, IL-4, IL-5, IL-12.

Cytokine levels in supernantants from measles antigen-stimulated cultures

The overall cytokine 'profile' in measles antigen-stimulated cultures was similar in the three groups of children studied and did not appear to evolve to any great extent during the 3-6 month follow-up periods after either primary vaccination in 12 month olds (EARLY VAX) or revaccination in older children (REVAX) (Table 2). Measles antigens induced little cytokine production in children prior to vaccination (EARLY VAX) or revaccination (REVAX). By far, the most prominent response was the production of antigen-specific IFN-γ at all times after vaccination in all groups. Low levels of antigen-specific IL-10 production were detectable in the EARLY VAX children

at all time points after vaccination, and antigen-specific IL-5 production was detected in the older children (both REVAX and LATE VAX groups). There were no differences in the cytokine production profiles between the older children who mounted either primary or secondary responses to revaccination. There were no significant gender differences in the cytokine production profiles in any of the groups studied. Levels of other cytokines show no particular pattern.

Cytokine mRNA expression in measles antigen-stimulated PBMC

Overall, the cytokine mRNA expression profiles in measles antigen-stimulated PBMC paralleled the findings in the culture supernatants. Little or no measles antigen-specific cytokine mRNA was expressed prior to initial vaccination (EARLY VAX) or revaccination (REVAX). However, between 1-6 months after primary vaccination, antigen stimulation resulted in the expression of large amounts of IFN-γ mRNA and significant increases in IL-10 and IL-12 mRNA expression (Table 3). Between 1-3 months after revaccination, *ex-vivo* measles antigen stimulation resulted in sharp increases in IFN-γ mRNA expression ($p = .08$) and lesser, but noticible increases in IL-5 mRNA expression. The LATE VAX group showed strong induction of IFN-γ in response to measles antigen stimulation.

Spontaneous intracellular cytokine levels in PBMC after vaccination

Intracellular (ic) cytokine determinations were only performed in selected samples from the EARLY VAX children. Compared with prevaccination levels, icIL-2 showed decreased levels in circulating $CD4^+$ and $CD8^+$ ($p < .02$) T cells. $CD8^+$ T cells showed increased levels of icIL-4 ($p < .04$) between 5-8 weeks after vaccination. Also, monocytes showed increased levels of icIL-12 ($p < .04$) after 5-8 weeks. Other changes in the icCytokine levels did not reach statistical significance (Table 4).

Cytokine profiles and patterns of immune response

Since children who had no detectable responses were excluded and PBMC numbers did not permit the determination of all cytokine level in all samples, the total number of children in each group who could be included in this analysis was slightly reduced. In this analysis, 62% of children for whom supernatant cytokine information was available for at least one time point after vaccination/revaccination had significant humoral as well as lymphproliferative responses to measles antigens ($n = 24$; $Ab^{hi}CMI^{hi}$). The remaining 38% only showed evidence of a humoral response ($n = 15$;

$Ab^{hi}CMI^{lo}$). The children for whom mRNA (70% $Ab^{hi}CMI^{hi}$ and 30% $Ab^{hi}CMI^{lo}$) and ic cytokine data (71% $Ab^{hi}CMI^{hi}$ and 29% $Ab^{hi}CMI^{lo}$) were available were similarly grouped. Comparisons between those who generated antibodies alone and those who mounted both humoral and cellular responses to measles antigens revealed striking differences in all of the groups studied (EARLY VAX, LATE VAX and REVAX). Compared with $Ab^{hi}CMI^{lo}$ children, $Ab^{hi}CMI^{hi}$ children had higher levels of IFN-γ in culture supernatants ($p = .07$) and ic staining in $TD8^+$ T cells ($p < .02$) between 5-8 weeks after vaccination as well as higher IFN-γ mRNA expression at 5-8 weeks ($p < .04$) and 4-12 years after primary vaccination and at 1-3 months after revaccination ($p < .04$) (Figures 1 and 2). Six months after primary vaccination, the $Ab^{hi}CMI^{hi}$ EARLY VAX children had lower supernatant levels of both IL-10 ($p < .03$) and IL-12 ($p < .04$) compared with the $Ab^{hi}CMI^{lo}$ children, but paradoxically higher mRNA expression for these same cytokines. However, the number of cytokine mRNA determinations that could be made in this group was very limited (n = 1-5). Five to eight weeks after primary vaccination, the ic level of IL-2 in $CD4^+$ T cells was markedly higher in the $Ab^{hi}CMI^{hi}$ EARLY VAX children, as compared with those who generated antibodies alone ($p < .02$).

Influence of maternal antibodies on measles antigen-specific cytokine production

Maternal antibodies are known to be a significant negative factor in the development of humoral responses after vaccination. Almost half (47%) of the children in the EARLY VAX group had low levels of maternal antibodies at the time of vaccination. Although children with maternal antibodies had slightly lower PRN titers at 6 months after vaccination (1447 ± 231 vs. 2168 ± 325 in those without detectable antibodies at the time of vaccination) and slightly higher lymphoproliferative responses overall (6.3 ± 2.5 vs. 4.7 ± 0.9), only differences in their antibody response reached statistical significance ($p < .05$). When in EARLY VAX children the analysis of cytokine profiles and immune response pattern was repeated with only those children who were negative for maternal antibodies, strong positive correlations were observed between the $Ab^{hi}CMI^{hi}$ pattern of response and the expression of both measles virus antigen-specific IL-10 mRNA ($r = .8$; $p < .05$) and culture supernatant IL-12 (all at 6 months after vaccination, $r = .6$; $p < .02$).

Discussion

Until very recently, the immune response to measles vaccination was evaluated on the basis of antibody production alone. Primary and secondary vaccine failure continue to be defined by the presence or absence of antibodies at different times after vaccination [43, 44, 45, 46, 47] and protection is determined by an antibody titer 'cut-off' [42]. However, it has long been known from experiments of nature (e.g.: agammaglobulimemia) [48] and inadvertant vaccination of immunocompromised subjects [49], that cellular immunity is critical for recovery from acute measles and sufficient for long-term protection from re-infection. Furthermore, evidence has steadily accumulated from case reports [50], epidemiologic observations [51] and animal studies [52] which indicates that some individuals without detectable antibody titers after vaccination are nonetheless protected from natural measles. We and others have recently demonstrated that lymphoproliferative responses to measles antigens can be readily measured following measles vaccination but are present in only 50-65% of vaccinated children between 6-12 months of age in the developed world. Interestingly, the proportion of chidlren who mount this response is much lower (20-25%) in the developing world [53, 54], despite the fact that antibody responses are excellent in both settings (e.i., 90-95% seroconversion rates with high antibody titers). We have also observed that a small proportion of children in the developed world develop lymphoproliferative responses to measles antigens with little or no antibody production ($Ab^{lo}CMI^{hi}$ pattern) [34]. The proportion of children with this pattern of response actually increases with time after routine vaccination suggesting that, in some individuals, the cellular memory for measles antigens may be more durable than the humoral response [33].

Although the full clinical significance of the pattern of immune responses after vaccination (e.g.: $Ab^{hi}CMI^{hi}$, $Ab^{hi}CMI^{lo}$, etc.) remains to be determined in longitudinal studies of protection, we wished to determine if early cytokine production patterns following vaccination were associated with a particular long-term pattern of response. We were especially interested to know if recognizable Th1 or Th2 cytokine profiles would be correlated with either CMI- or antibody-predominant responses, respectively. As noted above, several groups of investigators have evaluated vaccine-induced cytokine production and noted increased antigen-specific IFN-γ production by PBMC following MMR vaccination. However, only Pauksen et al. [29] attempted to correlate the production of cytokines with the pattern of immune response. Studying children who had undergone bone marrow transplantation, these investigators found a strong association between pre-existing lymphoproliferative responses to measles antigens and IFN-γ

production by PBMC following MMR vaccination. In the current study, we demonstrate conclusively that the capacity to produce IFN-γ in response to measles antigens is a strong predictor of the development of a balanced ($Ab^{hi}CMI^{hi}$) response to vaccination in healthy children. Our data also suggest that $CD8^+$ T cells participate actively in the production of this cytokine early after vaccination and that the antigen-specific production of IFN-γ is a durable aspect of the cellular response to vaccination, lasting for at least 10 years. Children who mounted balanced ($Ab^{hi}CMI^{hi}$) responses also had increased IL-2 levels in $CD4^+$ T cells as well as evidence of increased antigen-specific IL-10 and IL-12 production during the first months after vaccination, confirming the Th0/1 nature of the initial response. Subsequent increases in antigen specific IL-5 production at 5-13 years after vaccination lend support to the concept that measles-specific responses evolve over time towards a Th2-type pattern [9, 18, 19]. It is important to point out that we did not find a particular cytokine profile associated with the 'failure' to generate antigen-specific lymphoproliferative responses to vaccination (i.e., children with $Ab^{hi}CMI^{lo}$ did not make excessive amounts of IL-10 or other Th2 cytokines).

These findings not only help to describe the immunologic events that accompany measles vaccination but may also be of use in the development of new measles vaccines. Although measles is targeted for erradication by the WHO by the year 2010 [55], measles vaccination will need to continue for a considerable time even after documented erradication. Issues of vaccine safety will become paramount during this time and even the extraordinarily rare association of the complications associated with the current live, attenuated vaccines will become unacceptable. On the basis of this work, new vaccines that elicit antigen-specific IFN-γ responses might be predicted to induce both humoral and long-term cellular memory for measles antigens.

Table 1:
Humoral and lymphoproliferative responses to measles vaccination and revaccination: Measles-specific neutralizing antibodies lymphoproliferative response in 60 EARLY VAX. 18 LATE VAX and 10 REVAX children. Measurements were performed before (Prior) measles vaccination or revaccination and several times after vaccination or revaccination. PRN values ≥ 120 are thought to protect against symptomatic measles virus infection (Chen RT et al. 1989) and stimulation indices (SI) ≥ 3 are thought to be significant. NA indicates not available.

	EARLY VAX	LATE VAX	REVAX
PRN titers			
Prior	10 ± 1.1	NA	22.2 ± 13.09
1-2 months	1237 ± 169	NA	598 ± 123
3 months	NA	NA	268 ± 107
6 months	1820 ± 210	NA	NA
4-12 years	NA	465 ± 116	NA
LP response (SI)			
Prior	1.4 ± 0.1	NA	2.9 ± 0.7
1-2 months	4.5 ± 0.8	NA	23.6 ± 8.0
3 months	NA	NA	8.7 ± 3.6
6 months	5.6 ± 1.2	NA	NA
4-12 years	NA	8.4 ± 2.5	NA

Table 2:
Cytokine protein measurements before and after measles vaccination or revaccination: Measles-specific protein cytokine release measured in EARLY VAX, LATE VAX and REVAX children. Measles-specific cytokine protein concentration was detected in PBMC supernatant culture by ELISA. The results are reported in picograms/ml minus the control culture values. Negative values indicate measles-antigen specific cytokine concentration that were lower than concentrations in the control. Measurements were performed before (Prior) measles vaccination or revaccination and at several times after vaccination or revaccination. NA indicates not available. Measurements are reported as mean values ± the standard error of the mean (SEM).

	EARLY VAX	LATE VAX	REVAX
Prior			
IFN-γ	33.8 ± 47.1	NA	518 ± 426
IL-10	34.7 ± 33.4	NA	-266 ± 159
IL-12	62 ± 33	NA	.031 ± .19
IL-5	114 ± 28	NA	-5.5 ± 8.3
IL-4	-6.7 ± 5.4	NA	NA
IL-2	NA	NA	NA
TNF-α	112 ± 40	NA	31 ± 28
1-2 months			
IFN-γ	263 ± 97	NA	997 ± 317
IL-10	118 ± 33	NA	123 ± 75
IL-12	104 ± 46	NA	-.91 ± .24
IL-5	101 ± 27	NA	69± 17
IL-4	5.5 ± 5.3	NA	NA
IL-2	NA	NA	NA
TNF-α	101 ± 32	NA	42 ± 17
3 months			
IFN-γ	NA	NA	942 ± 232
IL-10	NA	NA	11 ± 11
IL-12	NA	NA	-.5 ± .5
IL-5	NA	NA	59 ± 18
IL-4	NA	NA	NA
IL-2	NA	NA	NA
TNF-α	NA	NA	77 ± 40
6 months			
IFN-γ	168 ± 128	NA	NA
IL-10	52 ± 21	NA	NA
IL-12	21 ± 23	NA	NA
IL-5	71 ± 18	NA	NA
IL-4	.24 ± .18	NA	NA
IL-2	NA	NA	NA
TNF-α	18 ± 25	NA	NA
4-12 years			
IFN-γ	NA	145 ± 203	NA
IL-10	NA	-26 ± 20	NA
IL-12	NA	-.2 ± .2	NA
IL-5	NA	-6.2 ± 8.6	NA
IL-4	NA	.05 ± .04	NA
IL-2	NA	.4 ± .3	NA
TNF-α	NA	15.8 ± 6.8	NA

Table 3:

Cytokine mRNA expression before and after measles vaccination or revaccination: Measles-specific mRNA cytokines measured in EARLY VAX. LATE VAX and REVAX children. Levels of mRNA were detected in PBMC by semi-quantitative RT-PCR ELISA. Relative ratios were calculated as the mean OD in measles virus antigen-stimulated cells, divided by the mean OD for GAP-DH. Measurements were performed before (Prior) measles vaccination or revaccination and at several times after vaccination or revaccination. NA indicates not available. Measurements are reported as mean values ± SEM.

	EARLY VAX	LATE VAX	REVAX
Prior			
IFN-γ	.16 ± .10	NA	.45 ± .88
IL-10	.25 ± .06	NA	-.09 ± .52
IL-12	.22 ± .13	NA	-.03 ± .19
IL-5	.03 ± .01	NA	-.35 ± .48
IL-4	.14 ± .05	NA	NA
IL-2	.14 ± .05	NA	NA
TNF-α	NA	NA	.46 ± .22
1-2 months			
IFN-γ	1.60 ± .70	NA	3.9 ± 1.9
IL-10	1.10 ± .60	NA	-1.6 ± 2.1
IL-12	.30 ± .06	NA	-.19 ± .24
IL-5	.25 ± .09	NA	.94 ± .54
IL-4	1.30 ± 1.01	NA	NA
IL-2	.40 ± .30	NA	NA
TNF-α	NA	NA	-.53 ± .35
3 months			
IFN-γ	NA	NA	5.40 ± 1.80
IL-10	NA	NA	2.30 ± 1.40
IL-12	NA	NA	-.50 ± .50
IL-5	NA	NA	1.20 ± .60
IL-4	NA	NA	NA
IL-2	NA	NA	NA
TNF-α	NA	NA	.83 ± .55
6 months			
IFN-γ	1.50 ± .60	NA	NA
IL-10	.49 ± 13	NA	NA
IL-12	.36 ± .20	NA	NA
IL-5	.27 ± .05	NA	NA
IL-4	.45 ± .30	NA	NA
IL-2	.02 ± .01	NA	NA
TNF-α	NA	NA	NA
4-12 years			
IFN-γ	NA	.89 ± .23	NA
IL-10	NA	-.22 ± .24	NA
IL-12	NA	-.20 ± .22	NA
IL-5	NA	.10 ± .30	NA
IL-4	NA	.05 ± .05	NA
IL-2	NA	.36 ± .30	NA
TNF-α	NA	.26 ± .06	NA

Table 4:

Intracellular cytokine levels in EARLY VAX children group measured before and after MMR vaccination.

	Prevaccination	5-8 weeks after vaccination
IFN-γ		
CD4	2.3 ± .9	2.9 ± 1.3
CD8	3.9 ± .6	4.8 ± .8
IL-2		
CD4	5.8 ± 1.6	3.8 ± 1.2
CD8	3.4 ± .6	2.3 ± .6
IL-4		
CD4	1.8 ± .7	3.5 ± 1.3
CD8	4.8 ± 1.5	8.0 ± 10.8
IL-5		
CD4	7.8 ± .7	1.8 ± .4
CD8	3.7 ± .8	2.8 ± .6
IL-12		
Monocytes	27.1 ± 6.5	20.2 ± 3.8
B cells	6.0 ± 4.2	8.9 ± 3.4

Figure 1: Cytokine measurements in children with $Ab^{hi}CMI^{hi}$ and $Ab^{hi}CMI^{lo}$ responses 5-8 weeks, 6 months after vaccination, and in 6- to 15-year-old children. a) Measles-specific cytokine protein concentration was detected in PBMC supernatant culture by ELISA. The results are reported in picograms/ml minus the control culture values ($p = .07$, $p < .03$, $p < .04$, $p < .04$, $p < .002$). b) Measles-specific mRNA levels were detected in PBMC by semi-quantitative RT-PCR ELISA. Relative ratios were calculated as the mean OD in measles antigen-stimulated cells, divided by the mean OD for GAP-DH ($p < .03$, $p = .09$, $p = .33$, $p < .04$, $p < .05$).

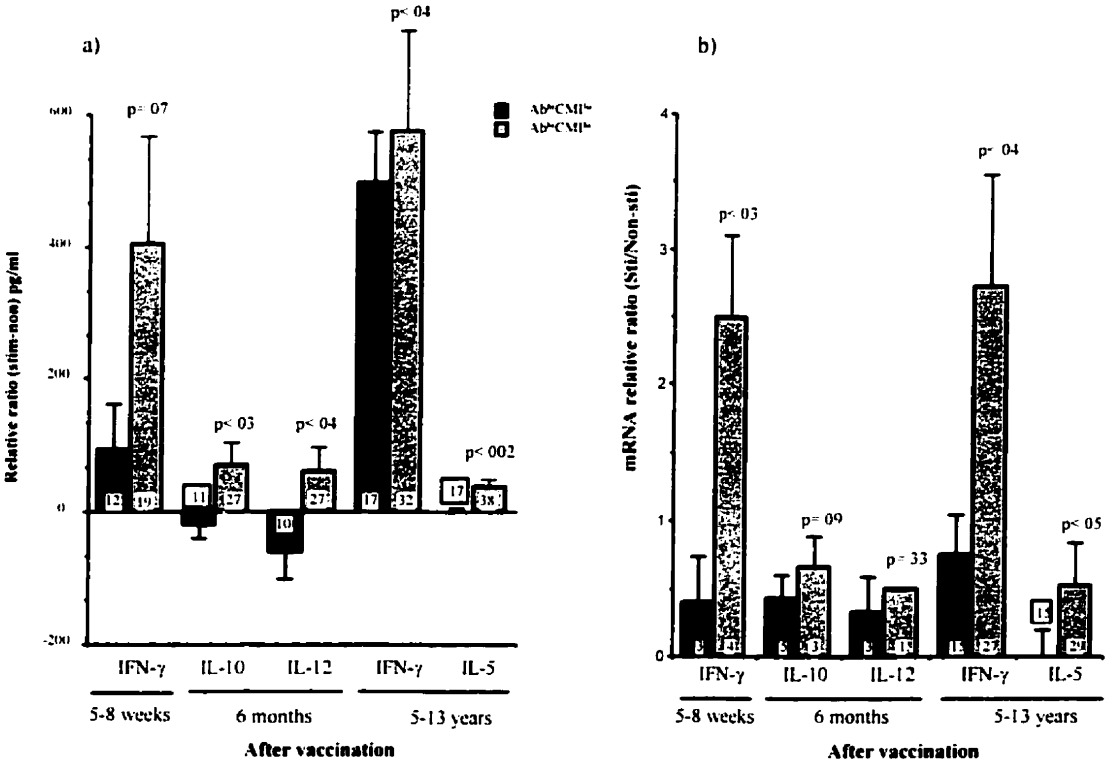
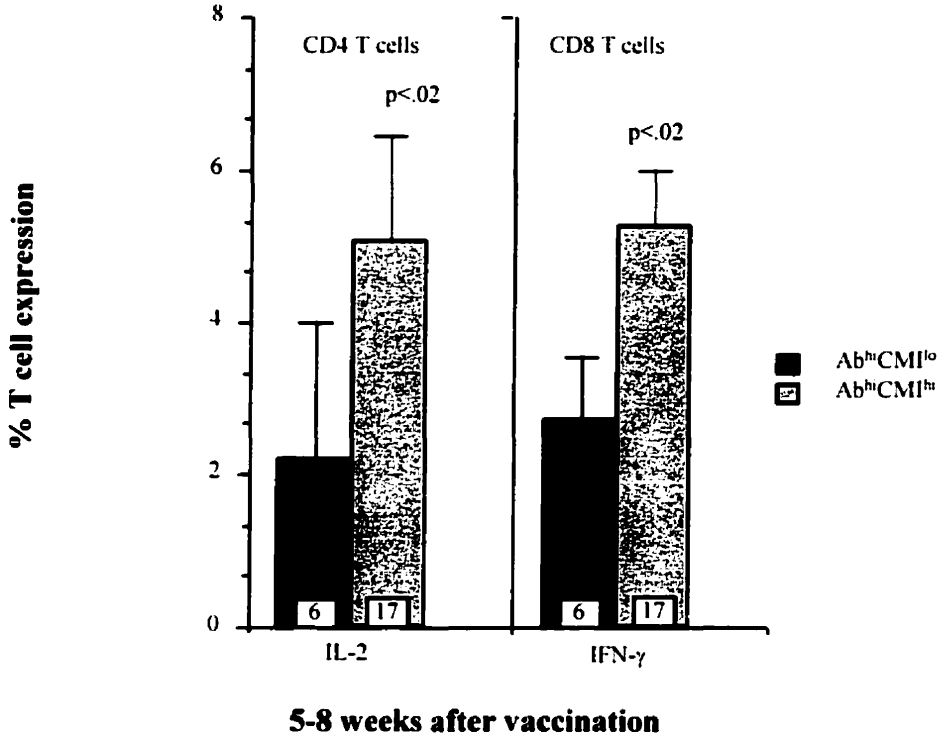


Figure 2: Intracellular cytokine production in children with $Ab^{hi}CMI^{hi}$ and $Ab^{hi}CMI^{lo}$ responses at five to eight weeks after MMR vaccination. The percentage of T cells producing cytokines was detected by *ex-vivo* intracellular staining ($p<.02$, $p<.02$).



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