A Study of Measles Immunopathogenesis: Investigations of High Titer Vaccination and Mechanisms of Measles-Induced Immunopathology

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In memory of my beautiful sister,

Deborah Horatio Bertley

ABSTRACT

Measles virus has been among the most important infectious agents in the history of mankind. Prior to the isolation of the virus and development of the first vaccines in the early 1960's, measles killed between 0.1 - 10% of each birth cohort in the world. Although live-attenuated measles vaccines have significantly reduced mortality by more than 99% in developed countries, ~ 1 million deaths still occur annually in the developing world, and many questions remain regarding measles immunopathogenesis, the correlates of immunity and the vaccines themselves. The excess mortality associated with the promising high titer (HT) measles vaccines, developed and introduced in the late 1980's, refocused attention on these questions and forms the framework for this thesis.

Work for this thesis began in Haiti and the Sudan, where we recruited a large number of measles HT vaccine recipients and carried out an extensive immunologic follow-up of these children, 4 - 5 years after vaccine exposure. Despite the scope of these studies (n = 643), we were unable to detect any persistent immunologic differences or abnormalities in HT recipients. Furthermore, we demonstrated, in a prospective follow-up study of Sudanese HT recipients involving almost 40,000 visits over a 4 year period, the absence of any impact of HT vaccination on measures of infant morbidity. Although these results were reassuring to those involved in the HT trials, they were also frustrating, and forced us to explore novel techniques for the detection of subtle, long-lived differences between HT and LT vaccine recipients. We established assays for a limited number of autoantibodies (myelin basic protein, Hela cell lysate and rheumatoid factor) and found significant differences in Haitian higher titer vaccine recipients.

In a parallel laboratory-based study initiated in response to the questions raised by the HT studies, we established an *in vitro* model of measles-virus infection in human peripheral blood mononuclear cells (PBMC) and demonstrated the capacity of measles virus to induce apoptosis in all PBMC subsets. We were the first to show the relative resistance of monocytes to this process and to demonstrate that measles-induced apoptosis was influenced by viral strain, vitamin A and possibly other host factors.

Finally, the clinical samples collected for this study permitted (i) the first large scale evaluation of the syncytium inhibition assay (SIA) for the measurement of measles neutralizing antibodies, and (ii) the first demonstration that early measles vaccination primes the human immune system for a more balanced humoral and cellular response upon re-exposure to measles antigens. This last study raises the possibility of using a "prime-boost" strategy to protect children below the age of routine vaccination in the developing world.

ABRÉGÉ

Le virus de la rougeole a été parmi les agents infectieux le plus important dans l'histoire de l'humanité. Avant l'isolation du virus et l'introduction d'un vaccin attenué au début des années 1960, la rougeole a tué entre 0,1 à 10% de chaque groupe de naissance à travers le monde. Bien que les vaccins contre la rougeole ont réduit la mortalité de plus de 99% dans les pays développés, plusieurs questions telles que l'immunopathogénèse de ce microbe, la corrélation entre l'immunité et les vaccins restent à élucider. L'excès inattendu de mortalité associée aux vaccins contre la rougeole à titres élevés (TE), développés et introduits à la fin des années 1980, ont de nouveau attiré l'attention à ces questions et forment les objectifs de cette thèse.

Travaillant sur le terrain en Haïti et au Soudan, nous avons recruté un grand nombre d'enfants agés de 4 à 5 ans et ayant participé à l'étude de vaccins. Utilisant une panoplie de tests immunologiques standards, nous n'avons pas pu démontrer d'anomalies immunologiques persistantes chez les individus à TE ou des paramètres immunologiques différents chez les individus à TE comparés aux enfants vaccinés contre la souche à titre bas (TB). De plus, nous avons démontré sur une période de 4 ans au Soudan, lors d'un suivi d'un groupe, l'absence d'impact des vaccins à TE sur la morbidité des enfants. Les résultats de ces études ont apporté un soulagement à tous ceux impliqués dans les essais de TE. Ces découvertes, rassurantes d'une part et frustrantes d'autre part, nous ont poussé à explorer de nouveaux moyens pouvant détecter les différences subtiles mais persistantes entre les individus à TE et TB. Nous avons établi des essais pour un certain nombre d'autoanticorps (protéine myéline de base, lysat d'antigène Hela et facteur rhumatoïde) et démontré des différences marquées entre les groupes de vaccin chez les enfants haïtiens après vaccination, qui peuvent être dues à une faille immunologique au moment de la vaccination.

Dans une étude parallèle réalisée au laboratoire en réponse aux questions soulevées par les essais de TE, nous avons établi un modèle *in vitro* d'infection du virus de la rougeole avec les cellules mononucléaires du sang périphérique humain ("PBMC"). Utilisant ce modèle, nous avons démontré que le virus de la rougeole a la capacité d'induire la mort cellulaire programmée ("apoptosis") au niveau de toutes les PBMC et que ce processus est potentiellement influencé par la souche virale, la vitamine A et probablement d'autres facteurs de l'hôte.

Finallement, ce travail a permis une première évaluation à grande échelle de l'essai d'inhibition du syncytium pour la mesure des anticorps neutralisants de la rougeole et a permis de démontrer qu'une exposition précoce au virus de la rougeole amorce le système

immunitaire de l'homme à une réponse humorale et cellulaire plus équilibrée. Cette dernière étude soulève la possibilité d'utilisation d'un premier rappel pour immuniser les petits enfants dans les pays en voix de développement.

ACKNOWLEDGMENTS

The birth, development and completion of this project were made possible only through the dedication and constant support from my supervisor and mentor Dr. Brian J. Ward. For this, and for the multitude of experiences (including international health field-work) that I have gained through our relationship, I would like to say "thank you". I am undoubtedly better because of them.

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Finally, I would like to dedicate this thesis to my family: My parents for their unparalleled example of academic achievement and inexhaustible work ethic. I infinitely value this example and have learned immeasurably from you both - thank you. My brothers and adopted sisters, Albert, John, Martha and Karen for their undying support and confidence in me. My sister, Deborah - thoughts of your very own struggle have inspired me to labor on at this sometimes arduous task.

Frederic Marcus Nkrumah Bertley,
Montreal, QC, 1999

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THESIS OFFICE STATEMENT

Candidates have the option of including, as part of the thesis, the text of paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis. If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more that a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirement of the "Guidelines for Thesis Preparation". The thesis must included: a Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment 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. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.

STATEMENT OF ORIGINALITY

MANUSCRIPT I:

This is the first large-scale comparison of the technically difficult and time consuming plaque reduction neutralization (PRN) assay with the much simpler syncytium inhibition assay (SIA). The two tests had previously been compared using only a small number of samples (n = 25) (Forthal DN et al 1994). We tested 594 samples from a wide variety of settings and optimized the SIA for the measurement of both high and low titers of neutralizing antibodies.

MANUSCRIPT II:

This manuscript is the only immunologic follow-up of the HT measles vaccine recipients in Haiti. This report follows the publication of the original HT vaccine efficacy trial results in Haiti (Job JS et al 1991) and in the initial follow-up study that demonstrated increased mortality in the HT vaccine recipients (Holt EA et al 1993).

MANUSCRIPT III:

The Sudanese HT measles vaccine trial was unique in two respects: (i) it was the only HT vaccine trial that was placebo-controlled and (ii) it was the only HT trial that included long-term, prospective follow-up for potential benefit or adverse events. This manuscript is the only immunologic follow-up of the Sudanese HT measles vaccine recipients.

MANUSCRIPT IV:

As noted above, the Sudan study incorporated a prospective follow-up of the HT vaccine recipients in the original study design. This manuscript presents bi-monthly morbidity data collected over a four year period after vaccination. This is the only prospective study of morbidity following HT measles vaccination.

MANUSCRIPT V:

This manuscript describes the first use of autoantigen reactivity as a potential marker for long-term immunologic disruption initiated by HT measles vaccination. Very little is known about the influence of childhood infections and vaccinations on the induction of autoantibodies or reactivity to self-antigens.

MANUSCRIPT VI:

In this report we demonstrate, for the first time, that measles virus can induce massive apoptosis of peripheral blood mononuclear cells (PBMC) as well as explore the role of viral strain in this process. An appendix demonstrating the effect of vitamin A on the kinetics of measles-induced apoptosis is included.

MANUSCRIPT VII:

This report is the first demonstration of immunologic "priming" by early measles vaccination. In this study, children who would normally be classified as "vaccine failures" are shown to mount a more balanced humoral and cellular response upon re-exposure to measles virus antigens.

STATEMENT OF AUTHORSHIP

I participated in the development and completion of the Haiti and Sudan HT measles vaccine immunologic follow-up studies in several different capacities including: (i) **study design** and **field work** in Haiti and Sudan (e.g. study-site preparation, administration and logistical planning, establishment of follow-up clinics, and troubleshooting), (ii) **blood collection** and **processing** in laboratories in Haiti and Sudan (e.g. plasma separation and cryopreservation of peripheral blood mononuclear cells), (iii) **preparation** and **transportation** of samples to Canada and (iv) **all laboratory work** performed on the collected samples, with the exceptions noted below.

The work presented in Manuscripts II, III, V, VI, and VII was performed by me under the guidance of Dr. Brian J. Ward, with the following exceptions: in Manuscript II, the WBC counts, differential counts and DTH measurements were performed by Haitian technicians, BJW (McGill) and RM (Johns Hopkins University) respectively. In Manuscript VII, the PRN assay was performed in the virology laboratory of the London School of Hygiene and Tropical Medicine.

As a contributing author for Manuscript I, I performed a substantial number of the assays reported and participated in the analysis of the data and in writing the manuscript. In Manuscript IV my role was more modest, including organization of the large existing data set, a limited preliminary analysis of this data and contribution to the writing of the manuscript.

The research technician in Dr. Ward's laboratory, Nathalie Martel, gave technical advice and suggestions for many of the assays used in this thesis.

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CHAPTER 1

INTRODUCTION

1.1 MEASLES VIRUS

Measles is an acute, monophasic illness characterised by fever, conjunctivitis and a maculopapular rash. Despite the availability of a vaccine for over 30 years, measles remains a major cause of mortality in children in the developing world and continues to cause outbreaks in developed countries. The etiologic agent of measles is a *Morbillivirus* of the Paramyxovirus family. Other *Morbilliviridae* include Rinderpest virus, pest de petit ruminants, and canine, phocid and cetacean distemper. Although other primates can be infected with measles virus experimentally, humans are the only known reservoir for this organism (Norrby E & Oxman MN 1990).

1.1.1 Morphology and Genetics

The measles virion is a pleomorphic, enveloped particle, 120 - 250 nm in diameter with a nucleocapsid consisting of RNA and protein (Bellini WJ et al 1994). The nucleocapsid is helical with a diameter of 17 - 18 nm and a total length of approximately 1μm. Two transmembrane glycoproteins project from the envelope surface: the hemagglutinin (H) and the (F) proteins. One, or both, of the cytoplasmic portions of these glycoproteins interact with the matrix (M) protein, which in turn is believed to interact with the nucleocapsid (Bellini WJ et al 1994).

The measles virus genome consists of a single-stranded, non-segmented RNA molecule of negative polarity. The entire genome, (15,940 nucleotides with a molecular mass of 4.5 kDa) has been sequenced (Baczko K et al 1983). The genome contains 6 non-overlapping genes encoding 8 major proteins: N (nucleocapsid; 60 kDa), the tricistronic gene P/V/C (phosphoprotein; 72 kDa), M (matrix; 37 kDa), F (fusion; 60 kDa single polypeptide which is subsequently cleaved into 41 and 18 kDa peptides), H, (hemagglutinin; 78 - 80 kDa) and L (large polymerase protein; 210 kDa) (Baczko K et al 1983).

The nucleocapsid protein (N) binds the phosphoprotein (P) and large protein (L) to form the replication complex. This complex first transcribes mRNA for protein synthesis, after which full length (antigenomic) RNA is made for genomic RNA transcription (Bellini WJ et al 1994). The functions of the V and C proteins remain undetermined although they may play a role in the regulation of transcription and viral replication (Cattaneo R et al 1989). The hemagglutinin (H) and fusion (F) glycoproteins control viral adhesion, fusion and entry into the host cell. The H protein binds specifically to CD46, a complement regulatory protein with wide distribution on human cells (Dorig RE et al 1993). Once the virion is bound, the 41 kDa hydrophobic fragment of the F protein mediates fusion (Tyrrell DJ & Ehrnst A 1979). Although antibodies directed against either F or H can protect against measles virus infection, antibodies to the H protein alone are capable of neutralizing the virus (Varsanyi TM et al 1987). The matrix protein is located at the inner surface of the viral envelope where it plays a key role in virus maturation and budding (Bellini WJ et al 1994).

Although measles was long considered to be a monotypic virus, partial sequencing of many wild type isolates has revealed at least 15 genotypes (Xu WB et al 1998). This classification of the different genotypes was based on sequence differences primarily in the coding regions of the hemagglutinin and nucleoprotein genes. However, a new wild type strain with a fusion protein unrecognizable by available monoclonal antibodies has recently been identified (Jin L et al 1998). Measles heterogeneity therefore appears to be greater than previously described (Schrag SJ et al 1999).

1.1.2 Clinical Measles

Clinical measles is characterized by fever, malaise, coryza, and conjunctivitis. These symptoms typically occur between the eighth and twelfth day after exposure and constitute the viral prodrome. After 2 - 3 days, measles-specific signs and symptoms appear: Koplik's spots (red spots on the buccal mucosa) and the typical maculopapular skin

rash between day 3 - 6 (Denton J 1925). In immunocompetent hosts, the virus is cleared within 5 - 7 days of the onset of the rash and immunity after natural infection is thought to be life-long (Panum P 1938, Sergiev PG et al 1960).

Complications following infection are common. Secondary viral or bacterial pneumonias occur in 1% - 7% of measles cases, and account for almost all of the mortality associated with this disease (Gellin BG & Katz SL 1994). Neurologic complications include post-infectious encephalomyelitis (an autoimmune disease) and subacute sclerosing panencephalomyelitis (a rare, progressive and fatal syndrome due to persistence of heavily mutated virus) (Rammonhan KW et al 1982). Measles can be a progressive and fatal disease (giant cell encephalitis or pneumonitis) in children with T cell disfunction (Griffin DE & Bellini WJ 1996).

1.1.3 Pathogenesis

Measles virus initially replicates in the epithelial cells of the upper respiratory tract. After 2 - 3 days, virus spreads to the draining lymph nodes in a primary viremia (Sherman FE & Ruckle G 1958, Sakaguchi M et al 1986). An augmented second viremia then disseminates the infection to sites throughout the reticuloendothelial system (Sergiev PG et al 1960, Kamahora J & Ni S 1961). Both primary and secondary viremias are cell-associated. In addition to epithelial and endothelial tissues, measles virus has been shown to infect B cells, T cells and monocytes *in vitro* (Joseph BS et al 1975, Sullivan JL et al 1975, Vladimarsson H et al 1975, Huddlestone JR et al 1980, Osunkoya BC et al 1990, Esolen LM et al 1993). During the acute illness, characteristic multinucleated giant cells can be found in many epithelial, endothelial and lymphoid tissues (Warthin AS 1931, Griffin DE & Bellini WJ 1996). Direct infection and sequestration of lymphocytes is though to be the cause of the profound lymphopenia that occurs at this stage of the infection. The onset of the typical measles rash heralds the production of measles-specific cellular and humoral responses. Immune effector mechanisms rapidly control the virus

which is eliminated from host tissues in a matter of days in most children (Bellini WJ et al 1994).

1.1.4 Epidemiology

Measles is among the most contagious infections of man (Hope-Simpson RE 1952, Gellin BG & Katz SL 1994b). In the last millenium, this Old World virus has used major population centers and human migration to become endemic throughout the world. Although vaccines have been remarkably effective in controlling measles, the World Health Organization (WHO) estimates that over 50 million measles cases continue to occur every year, resulting in ~ 1 million deaths (WHO 1998). Measles incidence varies cyclically (Cutts FT et al 1994a). Epidemics are more frequent in the developing world where birth rates are higher and vaccine coverage is less complete than the developed world. The average age of infection is lowest in urban settings where population densities and transmission rates are highest. This is important since measles mortality and morbidity increase with early infection and intense exposure (Griffin DE & Bellini WJ 1996). In the developing world, the majority of measles deaths occur in children < 5 years of age (Gellin BG & Katz SL 1994a).

1.2 MEASLES VACCINATION

In 1991, the WHO Expanded Program on Immunization (EPI) set the goals of reducing measles incidence by 90% and mortality by 95% from pre-vaccination levels before the end of 1995 (WHO: Wkly Epi Rec 1992). With some exceptions, these goals were largely achieved (EPI: Wkly Epi Rec 1997). The EPI reviewed and revised its goals in 1994 and began to seriously consider mobilization for complete erradication of this virus by 2005. Although this goal may have been too optimistic, there is now a world-wide commitment to interrupt transmission of measles virus and to work towards erradication as early as possible including a joint effort by WHO and Pan American Health Organization (PAHO) to eradicate measles from the western hemisphere by 2000 (PAHO 1999).

Large scale measles vaccination programs aim to control infection by establishing herd immunity which is achieved when a large enough proportion of the population is protected to break transmission (Fine PM 1993). Since measles is so highly contagious, it is estimated that at least 98% antibody seropositivity is required to achieve herd immunity and prevent major epidemics (Fine PM 1993).

1.2.1 Measles Vaccines

Measles virus was first isolated in 1954 by Enders and Peebles from an infected patient, David Edmonston (Enders JF & Peebles TC 1954). This isolation allowed for the development of both inactivated and live virus vaccines in 1960 and 1963 respectively (Enders JF & Peebles TC 1954, Warren J & Gailian MJ 1962). Although the formalin-inactivated, alum-precipitated vaccines elicited an excellent humoral response, measles-specific antibodies waned rapidly after vaccination. Futhermore, a small number of those who received the inactivated vaccine were at risk for developing atypical and unusually severe measles upon exposure to wild-type virus (Rauh LW & Schmidt R 1965, Nader PR et al 1968, Rod T et al 1970, Brodsky AL 1972). Consequently, in 1967 inactivated

measles vaccines were discontinued, and live attenuated vaccines became the tools of choice for protection against measles. The first attenuated measles virus vaccine, Edmonston B, was licensed in 1963 (Enders JF & Peebles TC 1954). This vaccine was moderately reactogenic, producing fever and rash in an unacceptably large proportion of immunized children. Further tissue culture passage of the Edmonston B virus led to the Schwarz strain (1965) that is still used throughout much of the world, and the Moraten strain (1968) which is used in Canada and the United States (Katz SL et al 1995, Redd SC et al 1999).

Attenuation of the wild type isolates was achieved by serial passage through various cell lines, such as human diploid cells and chick embryo fibroblasts (Redd SC et al 1999). Although the exact mutations responsible for attenuation have not been identified, recent evidence suggests that nucleic acid changes in genes contribute: amino acid substitutions in the polymerase and V and C proteins, viral transcriptional impediments caused by a faulty virion-associated RNA polymerase, and mutations in the 5' untranslated region of the F gene (Takeda M et al 1998, Valsamakis A et al 1998).

1.2.2 Optimal Age for Measles Vaccination

Coordinating the timing of vaccine delivery with the waning of maternal antibodies is crucial in measles vaccination. For example, seroconversion rates in Haitian children vaccinated at 9 months of age were ~ 84%, but increases to 100% when vaccination was delayed until 12 months (Halsey NA et al 1985). In the absence of maternal antibodies, 95% - 100% of children as young as 3 months of age develop protective levels of antibodies in response to vaccination (Cutts FT et al 1994b). The difference in seroconversion rates is primarily due to the presence of maternal antibodies in the younger children (Cutts FT & Markowitz LE 1994a).

Although maternal antibodies can persist up to and beyond 12 months of age (Redd SC et al 1999), many children in the developing world lose these passively acquired

antibodies as early as 3 - 6 months of age (Redd SC et al 1999). Many factors contribute to the waning of maternal antibodies: gestational age, antibody levels in the mother and catabolism of maternal antibodies in the child during acute infections (Norrby E 1995, Wesumperuma HL et al 1999). The variability with which the levels of maternal antibodies decline makes it difficult to determine the optimal age for vaccination in any given child. In countries where measles is not endemic, vaccination can be delayed safely until 12 - 15 months of age at which time 95 - 100% seroconversion rates can be anticipated. However, in measles endemic areas, such as the developing world, vaccination must be carried out as early as possible. The WHO currently recommends that routine vaccination take place at 9 months of age in the developing world (EPI: Wkly Epi Rec 1990) but as early as 6 months of age in the setting of a measles epidemic. These recommendations reflect a compromise between the risk of vaccine failure due to persistent maternal antibody and the risk of severe disease or death with early infection. Even with vaccination at 9 months of age, a "window of susceptibility" exists for measles in many children who have lost their maternal antibodies at an earlier age. As noted above, the severity of measles is greatest in the youngest children, and up to 30% of the deaths associated with measles occur in children under 9 months of age (EPI: Wkly Epi Rec 1979).

1.3 HIGH TITER MEASLES VACCINES

1.3.1 Development

High titer (HT) measles vaccines were developed to address this "window of susceptibility". While standard measles vaccines contain 10^3-10^4 plaque forming units (pfu), the so-called HT vaccines contained between $10^{4.7}$ - $10^{5.6}$ pfu (Markowitz LE et al 1990b, Whittle HC et al 1988). To be effective, live virus vaccines need to replicate in the host. In many young children, maternal antibody titers are sufficient to neutralize all of the viral particles in the standard vaccines before replication can occur (Osterhaus A et al 1998, Redd SC et al 1999). By increasing the number of viral particles, it was reasoned that the HT vaccines could "saturate" the maternal antibodies and leave at least some vaccine-strain virus in a viable state. These residual viral particles could then replicate and induce a protective immune response.

Several studies to test this hypothesis were initiated in the late 1980's. Edmonston-Zagreb, AIK-C, BIKEN-CAM, Connaught and Schwarz vaccine strains at doses $\geq 10^{4.7}$ pfu were used to vaccinate 3 - 6 month old children in Gambia, Sudan, Haiti, Mexico, Guinea-Bissau, and other sites (Whittle HC et al 1988, Tidjani O et al 1989, Job JS et al 1991, Whittle HC et al 1990, Markowitz LE et 1990b, Berry S et al 1992, Jensen TG et al 1994). The results were very encouraging, with high seroconversion rates even in the presence of maternal antibodies. For example, in a study carried out in Zaire, seroconversion rates in 4 - 6 month old children receiving HT Edmonston Zagreb and AIK-C strains were 94% and 96% respectively (Cutts FT et al 1994b). In Mexico, 98% of 6-month olds seroconverted when vaccinated with the HT Edmonston Zagreb strain, compared to 87% in 9-month olds receiving Schwarz standard vaccine (Diaz-Ortega JC et al 1992, Markowitz LE et al 1990b). Even more encouraging, the incidence of short-term side effects such as fever and rash following HT vaccine administration was similar to that observed following standard vaccination (Markowitz LE et al 1990b, Tidjani O et al 1989

and Whittle HC et al 1990). In view of these excellent early results, the Global Advisory Committee of the WHO met in 1989 and recommended the use of measles HT vaccines wherever measles was a serious cause of morbidity and mortality in children < 9 months of age (EPI: Wkly Epi Rec 1990).

1.3.2 Excess Mortality Following High Titer Vaccination

Eighteen months later, serious concerns were raised by Aaby et al who reported an increase in mortality associated with the use of HT vaccines (Aaby P 1991 - personal communication referenced in Halsey NA 1993). An urgent review of all of the HT trials was initiated and a similar effect on overall mortality was observed in three of the largest studies (Senegal, Guinea-Bissau and Haiti) (Aaby P et al 1993c, Aaby P et al 1993b, Holt EA et al 1993). The increased mortality had three unusual characteristics: i) deaths accumulated slowly over two to three years after vaccination; ii) the increase in mortality was attributed to a variety of non-measles causes such as respiratory infections, gastrointestinal infections, malaria, diarrhea and malnutrition; iii) most difficult to explain, the excess mortality was gender biased, with female HT recipients at greater risk of death than their male peers (relative risk $\approx$ 1.6) (Holt EA et al 1993, Aaby P et al 1993b, Garenne M 1991, Aaby P et al 1993c).

In an effort to address these peculiar observations, many aspects of the HT studies were reviewed. A 24 - 39 month follow-up of 1266 Senegalese children examined social, family and general health characteristics but found no explanation for the increase in mortality (Aaby P et al 1991). Similarly, investigators in Guinea-Bissau revisited 384 children who had received standard or HT vaccines but found no obvious cause for the excess mortality (Aaby P et al 1993b). In Peru, anthropometric and socio-economic parameters were compared in the HT and standard vaccine groups without discovering a plausible explanation for the excess mortality (Berry S et al 1992). Faced with these troubling but consistent data, the WHO recommended the discontinuation of HT

vaccination in 1991 pending further studies regarding their safety (EPI: Wkly Epi Rec 1992).

The first section of this thesis deals with immunologic and epidemiologic follow-up studies performed in Haiti and the Sudan. The central hypothesis underpinning these investigations was that HT vaccination may have contributed to increased infant mortality by disrupting normal immune cell function (Ward BJ et al 1993b). This hypothesis was both biologically plausible and attractive because of the wide range of immunologic abnormalities known to be associated with natural measles infection and measles vaccination (discussed below, Section 1.3.3).

1.3.3 A Common Thread Between Natural Disease and HT Vaccination

Several lines of evidence support the biologic plausibility of the observations made following HT vaccination. First, both wild-type and vaccine strain measles virus have the capacity to infect a wide range of immune cells and potently disrupt immune cell function (see sections 1.4.1 and 1.4.2 for full discussion). Following routine measles vaccination, *in vitro* lymphoproliferation to phytohemagglutinin (PHA) and non-measles antigens as well as delayed-type-hypersensitivity (DTH) responses to cutaneous antigens are all reduced (Hirsch RL et al 1981, Fireman P et al 1969, Munyer TP et al 1975, Nakayama T et al 1988). Recent evidence suggests that aspects of measles-induced immune disturbance persist for up to 6 months after standard titer measles vaccination (Bautista-Lopez NL et al submitted). Immune disruption following natural infection is thought to be responsible for the increased susceptibility to secondary infections and high mortality rates (1 - 15% in many regions of the developing world) (Norrby E 1995). The period of increased risk for death after natural measles infection may be quite long (2 - 3 years), well after apparent recovery from the acute illness (Aaby P et al 1993a) and at least one investigator has observed a striking female bias in the mortality following natural measles (Garenne M 1994a). Finally, the plausibility that HT vaccines can cause long-term excess mortality

without pronounced reactogenicity at the time of vaccination is most strongly supported by two studies performed in West Africa (Aaby P et al 1990a, Aaby P et al 1993a). In the first study carried out in Guinea-Bissau, 276 children were closely followed for five years after a community measles epidemic (Aaby P et al 1990a). Seventy-one children had been exposed to measles prior to 6 months of age without developing clinical illness (ie: they were presumed to have been "protected' by maternal antibodies). These exposed children were matched with 205 children taken from neighboring districts who had had no exposure to measles. The risk of death in the exposed group over the 5 year follow-up period was 34% compared to 11% in children not exposed to measles. The difference in mortality was maintained after correction for possible confounders such as socio-economic status, maternal education, and domestic crowding. Very similar findings were reported in the second study (Aaby P et al 1993b). Like the HT vaccine experience, the causes of death in these "exposure" studies were typical for the developing world (e.g. diarrhea, fever), and mortality accumulated slowly for 2 - 3 years after exposure. However, it is interesting that there was no bias towards female mortality in these studies.

Together, these observations strongly support the existance of a common immunologic thread between the protracted risk of death after natural measles and the excess mortality associated with HT vaccination.

1.4 IMMUNITY AND IMMUNOPATHOGENESIS

Despite progress in recent years, both the pathology and immunopathogenesis of natural measles virus infection remain poorly understood. Although the majority of patients recover uneventfully from acute measles, secondary infections are common and account for much of the mortality associated with this disease (Beckford AP & Kaschula RO 1985). As noted above, the increased susceptibility to secondary infections is due to measles-induced immune suppression which is characterized by lymphopenia and globally impaired lymphocyte function (Wesley A et al 1978, Whittle HC et al 1978, Coovadia HM et al 1981, Joffe MF et al 1983, Alpert G et al 1984, Dagan R et al 1987). It is interesting that this period of intense immune suppression does not inhibit the induction of a vigorous measles-specific immune response (Griffin DE & Bellini WJ 1996).

1.4.1 Measles Immune Response

Natural measles infection is associated with strong humoral and cellular responses (Griffin DE & Bellini WJ 1996). In addition to polyclonal B cell proliferation (Griffin DE et al 1985) there exists a measles-specific humoral response characterized by an initial burst of IgM, and followed rapidly by the production of large amounts of by IgG and IgA (Schluederberg A 1965, Ehrnst A 1978, Mathiesen T et al 1990). Although transient, measles virus infection also induces a strong IgE response (Griffin DE & Bellini WJ 1996, Imani F et al 1999). Western blot analysis has identified antibodies directed against almost all of the viral proteins, with a predominance of anti-N, F and H (Norrby E & Collmar Y 1972, Trudgett A et al 1980, Graves M et al 1984). The antibodies have a range of specificities and can fix complement, but anti-H antibodies neutralize the virus most efficiently, followed by anti-F antibodies (Giraudon P & Wild FT 1981, Giraudon P & Wild FT 1985). Four antigenic sites have been identified on the H protein that are highly immunogenic and account for the bulk of the anti-H neutralizing ability (Deroo S et al

1998). The development of measles-specific, antibody-dependant cellular cytotoxicity (ADCC) is likely to be important in natural disease, since the appearance of this capacity is closely correlated with the elimination of the cell-associated viremia (Forthal DN et al 1993, Forthal DN et al 1994).

Although classical measures of measles virus-specific cell mediated immunity (DTH, lymphoproliferation) have historically been difficult to elicit in acute infection, circulating T cells express high levels of activation markers (Griffin DE et al 1986a, Griffin DE et al 1990a, Furukawa S et al 1992) and are actively proliferating for 5 - 7 days after the onset of the rash (Ward BJ et al 1990). *In vivo* and *in vitro* cytokine studies suggest that the initial response to infection is Th_0/Th_1-like, with massive production of interferon-gamma (IFN-γ) and interleukin-2 (IL-2) (Ward BJ et al 1991, Griffin DE et al 1991). This cytokine profile would be expected to support a strong CTL response which is, indeed, readily detectable at this time (Kreth HW et al 1979, Sissons JGP et al 1985, Van Binnendijk RS et al 1990). This cytotoxic-T-lymphocyte (CTL) activity is initially due to $CD8^+$ T cells but subsequently involves $CD4^+$ CTLs as well (Griffin DE & Bellini WJ 1996, McFarland HF & McFarlin DE 1979). The period of most active $CD8^+$ T cell involvement (as evidenced by serum levels of soluble CD8 and β2-microglobulin) is the first week after the onset of the rash while serum levels of soluble CD4 peak much later and persist for up to 3 - 4 weeks (Griffin DE et al 1992, Griffin DE et al 1993). Serum and *ex-vivo* cytokine studies suggest that this later phase of $CD4^+$ T cell activity is predominantly Th_2-like with massive production of IL-4 and IL-5 (Ward BJ et al 1991, Ward BJ & Griffin DE 1993b). Similar observations (e.g. early Th_0/Th_1 evolving to Th_2) have been made in children after primary vaccination (Gans HA et al 1999, Pabst HF et al 1999b, Bautista-Lopez NL et al submitted) and adults undergoing revaccination (Ward BJ et al 1995). Athough the mechanism that underlies the $Th_1 \Rightarrow Th_2$ shift induced by measles is not yet fully understood, down-regulation of the Th_1 "master" cytokine, IL-12, has recently been described following measles virus-induced cross-linking of its cellular

receptor (CD46) (Karp CL et al 1996). Given the potent activation of T cells in acute measles virus infection, the rapid shift to a Th_2-biased response may also be adaptive to avoid aberrant T cell responses such as post-infectious encephalomyelitis (Griffen DE & Bellini WJ 1996).

1.4.2. Characteristics of Measles Immune Suppression

As noted above, measles virus has the capacity to infect several immune cell types including macrophages, T and B lymphocytes , both *in vitro* and *in vivo* (Joseph BS et al 1975, Sullivan JL et al 1975, Vladimarsson H et al 1975, Huddlestone JR et al 1980, Osunkoya BC et al 1990, Esolen LM et al 1993). The evidence that measles is a potently immunosuppressive virus is overwhelming. Delayed-type hypersensitivity responses to cutaneous recall antigens essentially dissappear at the time of rash and only reappear weeks-to-months later (Von Piquet C 1908, Starr S & Berkovich S 1964, Wesley A et al 1978, Tamashiro VG et al 1987). Specific humoral and cellular responses to non-measles antigens are compromised resulting in impaired recognition of novel antigens (Coovadia HM et al 1974). Although total serum Ig concentration is not affected during measles infection, *de novo* antibody responses to *Salmonella typhi* and diphtheria toxoid are depressed (Whittle HC et al 1973). T-cell and B-cell responses to soluble antigens as determined by cytotoxic function and antibody production are also suppressed (Galama JMD et al 1980, Casali P 1984a, McChesney 1986). Despite initial enhancement of activity (Casali P & Oldstone MBA 1982, Casali P & Trinchieri G 1984b), *ex-vivo* activity of natural killer (NK) cells is markedly reduced, both during the course of infection, and after recovery (Casali P & Trinchieri G 1984b, Griffin DE et al 1990b). Indeed, measles-induced immune suppression is sufficiently intense that children with pre-existing autoimmune or inflammatory conditions such as juvenile rheumatoid arthritis, idiopathic thrombocytopenic purpura and nephrotic syndrome, can sometimes recover for variable periods of time after acute infection (Blumberg RW & Cassady HA 1974, Yoshioka K et al

1981, Lin CW & Hsu HC 1986, Lin CW et al 1988, Kiepeila P et al 1987). Monocytes isolated form patients with natural infection produce more IL-1, less TNF-α and have increased expression of ICAM-1 (Lucas CJ et al 1977, Griffin DE et al 1987, Sanchez-Lanier M et al 1988, Ward BJ et al 1991). T cells produce low IL-2 and IFN-γ and higher than normal levels of IL-4. These abnormalities are thought to contribute to the suppression of lymphoproliferation to mitogenic stimuli such as PHA, pokeweed mitogen (PWM) and concavalin A (Con A) (Kantor FS 1975, Arneborn B & Biberfeld G 1983, Wesley A et al 1978, Whittle HC et al 1978, Hirsch RL et al 1981).

As previously mentioned, evidence of immune suppression also follows measles vaccination. Although this suppression is less intense than that observed in natural disease, it is similar in character, including measureable reductions of DTH to cutaneous antigens and lymphoproliferative responses to mitogens and antigens (Fireman P et al 1969, Munyer TP et al 1975, Hirsch RL et al 1981, Nakayama T et al 1988, Ward BJ et al 1991).

1.4.3 Mechanims of Measles-Induced Immune Suppression.

The mechanisms by which measles virus induces immune suppression are not yet fully understood but several contributing factors have been described including apoptosis, immune deviation (e.g. Th_1 shift to Th_2), T cell anergy and the production of soluble suppressive factors (Bell AF et al 1997, Fugier-Vivier I et al 1997, Ravanel K et al 1997, Schnorr JJ et al 1997, Sun XM et al 1998). Apoptosis is an important adaptive mechanism of eukaryotic cells to control many intracellular infections (Balachandran S et al 1998, Edwards KM et al 1999, Jaworowski A & Crowe SM 1999, Mortola E et al 1998, Wang LO et al 1999) and its probable role in the lymphopenia and immunologic abnormalities associated with measles virus infection will be discussed in Section 1.4.5.

Although measles is a cytolytic virus with a proven capacity to infect a wide range of immune cells, the influence of this virus on the human immune system is far more subtle

and interesting than simple destruction of effector cells. For example, the binding of measles virus to its cellular receptor, CD46, on monocytes causes a profound downregulation of IL-12, hence decreased IFN-γ production and reduced CTL induction (Karp CL et al 1996). Even more impressive is the viral manipulation that occurs when dendritic cells interact with infected T cells, leading to both an increase in measles virus replication and a reduction in IL-12 production (Fugier-Vivier I et al 1997). Simple surface contact with the F and H glycoproteins on a small number of measles-infected PBMC can induce a mitogen non-responsive state in a whole population of uninfected PBMC by as yet uncharacterized pathways (Schlender J et al 1996). Other immune disruptions reported include decreased antibody production when neucleoprotein (NP) binds to the FcγRII receptor (Ravanel K et al 1997) as well as the production of a novel 100 Kda soluble factor by infected T cells that appears to inhibit lymphocyte proliferation (Fujinami RS et al 1998, Sun XM et al 1998). The assault of measles virus on the human immune system is not limited to mature immune cells. Virus-induced inhibition of terminal differentiation of thymic-epithelial cells leads to the generation of incompetent T cells (Valentin H et al 1999). Subsequent interaction between infected thymic-epithelial cells and T cells results in the uncoupling of the activation of T cells rendering them incapable of progressing from the G_0 to G_1 stage during cell cycle (Schnorr JJ et al 1997). These last effects of measles virus are particularly potent and may plausibly contribute to immunologic abnormalities persisting for months to years after the last virus particle has been destroyed.

1.4.4 Apoptosis

Apoptosis, or programmed cell death, is a normal physiologic process essential for healthy embryonic development and the maintenance of tissue homeostasis (Kerr JFR et al 1972, Wyllie AH et al 1980). Apoptosis is crucial for the consolidation of the developing neural network and the education of lymphocytes in the thymus - specifically the deletion of thymocytes during positive and negative selection (Samali A et al 1996). The apoptotic

process is characterized by morphological changes in the plasma membrane (e.g. loss of asymmetry and attachment), condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA (White E 1996, Thompson EB 1998). In the final stages, the dying cell becomes fragmented into "apoptotic bodies" that are rapidly eliminated by phagocytic cells without causing significant damage to surrounding cells (White E 1996, Thompson EB 1997). Although apoptosis was described in the early 1970's (Kerr JFR et al 1972), the importance of this process in protecting against viral infections has only recently been appreciated (Teodoro JG & Branton PE 1997). Indeed apoptosis is probably a key host defense against all intracellular pathogens and these organisms have evolved a wide range of mechanisms for manipulating this process. Measles virus is no exception.

1.4.5 Apoptosis in Measles

Evidence of measles-induced apoptosis has recently been reported in *in vitro, ex-vivo* and *in vivo* studies. *In vitro,* green monkey kidney cells, human monocytic cell lines, and human dendritic cells all undergo apoptosis following experimental measles virus infection (Esolen LM et al 1995, Fugier-Vivier I et al 1997). Recently, it has been demonstrated that an elevated rate of spontaneous PBMC apoptosis occurs in patients suffering from measles infection that persists for up to 6 months after the acute illness (Pignata C et al 1998). In a hu-SCID (human-severe-combined-immunodeficiency) mouse model, Auwater et al observed that thymic epithelial and myelomonocytic cells support vigorous measles virus replication while adjacent thymocytes rapidly die by apoptosis (Auwaerter PG et al 1996b). Neurons, oligdendrocytes and microglia undergo apoptosis as part of the neuropathogensis of SSPE, a rare and fatal complication of mealses (Mcquaid S et al 1997). In the second part of this thesis, we describe the development of an *in vitro* measles infection model that was used to study the extent and kinetics of measles-induced apoptosis in PBMC (Manuscript VI).

1.4.6 Measles, Vitamin A and Immune Function.

The mortality and morbidity associated with measles are significantly influenced by nutritional status, especially body stores of vitamin A (terMeulen V & Billeter MA 1995). Supplementation of measles patients with vitamin A can reduce morbidity and mortality by as much as 50% (Barclay AJ et al 1987, Hussey GD & Klein M 1990). Although vitamin A is widely acknowledged to be an "anti-infective" vitamin, the precise mechanism(s) of benefit remain unknown (Semba RD 1994, Beaton WJ et al 1992, Fawzi WW et al 1993, and Glasziou PP & Mackerras DEM 1993).

Studying vitamin A deficiency in animal models has increased our understanding of the immunologic effects of vitamin A (Smith SM et al 1987). Hyporetinemia is associated with a reduction of lymphoid organ size, changes in immune cell morphology and aberrant lymphocyte function. In particular, T cell activation, cytokine production, B cell growth and immunoglobulin production are reduced in vitamin A deficient animals (Smith SM et al 1987, Sijtsma SR et al 1990, Yamamoto M et al 1988). All of these effects are rapidly reversible with vitamin A supplementation (Bowman TA et al 1990). *In vitro* exposure of human immune cell lines or human T cells to vitamin A upregulates expression of surface activation markers and adhesion molecules such as CD38, CD43 and ICAM-1; all critical for the effector functions of lymphocytes (Mehta K et al 1997, Babina 1997, Auodjit F et al 1995). Similar, rapidly reversible changes in immune cell phenotype and function have been demonstrated in vitamin A deficient children (Semba RD et al 1993). Vitamin A supplementation in children with acute measles has been shown to increase total lymphocyte counts as well as measles-specific IgG levels (Coutsoudis A et al 1992). Antibody production to tetanus toxoid has also been shown to increase following vitamin A repletion (Semba RD 1994). The profound effect of vitamin A on children with measles prompted an examination of the role of retinoids in measles virus-induced apoptosis (Section 4.3.2.1 - Appendix I)

1.4.7 Measles and Autoimmunity

Autoimmune diseases may be grouped according to the immunopathologic mechanism implicated: i) humoral autoimmunity in which autoreactive B cells produce antibodies that damage self tissues through complement fixation or antigen-antibody complex deposition; or ii) cell-mediated autoimmunity, in which autoreactive T-cells recognize self antigens and cause tissue damage through cytokine production and/or CTL induction (Steinman L 1993, Klinman DM & Steinberg AD 1987). Viral infections can initiate autoimmune processes by either mechanism (Zinkernagel RM & Stauffacher W 1993).

Acute measles virus infection is characterized by a period of vigorous immune activity manifested by both polyclonal B cell activation and increased T cell activity (Griffin DE et al 1989, Ward BJ & Griffin DE 1993a). The scope and intensity of this immune activity raise the possibility that exposure to measles virus antigens may initiate autoimmune processes in some individuals. Natural measles is known to induce post-infectious encephalomyelitis at a rate of approximately 1:1000 (Miller DL 1964). In this complication, autoimmune T cells with reactivity to myelin basic protein (MBP) are thought to attack brain tissue (Johnson RT et al 1984). Although controversy remains, sequence homologies between MBP and measles virus proteins raises the possiblility that molecular mimicry may underly this rare complication (Jahnke U et al 1985). Although several investigators have tried to link measles virus infection with a wide range of other poorly understood autoimmune, inflammatory and idiopathic conditions including multiple sclerosis, Paget's disease of the bone, chronic hepatitis, glomurelonephritis, Crohns disease, autism and lupus erythematosus, a causal role for measles has not yet been established for any of these conditions (Johnson RT et al 1984, Basle MF et al 1986, Robertson DF et al 1987, Andjaparidze OG et al 1989, Cosby SL et al 1989, McKenna MJ & Mills BG 1989, Wakefield AJ et al 1993, Singh VK et al 1998).

Two of the most striking features of the excess mortality observed after measles HT vaccination were (i) deaths accumulated slowly over a 2 - 3 year period and (ii) girls were at greater risk than boys. Many autoimmune processes are gender biased; women are >10-fold more likely to develop some autoimmune diseases than men (Steinman 1993). Futhermore, autoimmune processes frequently evolve over periods of months and years rather than days and weeks (Oldstone MBA 1987, Steinman L1993). The presence of autoantibodies after acute measles virus infection was reported by Haire et al in 1972 but no subsequent investigations have been published (Haire M 1972). We reasoned that increased autoantibody production might be a subtle and long-term marker for immunologic damage/disturbance caused by HT measles vaccination. An evaluation of autoantibody production in the children recruited to the Haiti HT vaccine trial is presented in Manuscript V. (Some aspects of this work are still in progress. As a result, this manuscript is not the final draft, and may be modified before submission).

1.4.8 Summary of Measles Immunopathology

The immunology and immunopathology of measles virus infections remain incompletely understood despite more than 3 decades of intense scientific scrutiny. Although measles vaccine development represents one of the greatest accomplishments of medical science, this effort has been marred by two unfortunate events. 1) Formalin-inactivated measles vaccine introduced in the early 1960's primed a small number of recipients for severe and atypical measles disease upon subsequent exposure to the wild-type virus. 2) More recently, HT vaccines appear to have placed a large number of children at risk for, as yet, unexplained excess mortality.

In part I of the thesis, two immunolgic studies of HT vaccine recipients are presented (Manuscripts II and III). These studies were performed between 4 - 5 years after measles vaccination in children recruited to the original Haitian and Sudanese HT trials. This section also includes a prospective, long-term evaluation of the impact of morbidity in

HT vaccine recipients carried out in the Sudan (Manuscript IV). In part II, a combination of clinical material and an *in vitro* infection model have been used to address questions related to measles virus immunopathogenesis. The potential value of autoantibodies as markers for HT vaccine-induced immunologic disfunction is explored in Manuscript V. The kinetics and magnitude of measles virus-induced apoptosis in PBMC and the role of retinoids in this process are presented in Manuscript VI and its accompanying Appendix.

This thesis work stimulated a wide range of questions related to measles, but peripheral to the original study goals. These included an evaluation of the tests used to measure measles virus-specific neutralizing antibodies (Manuscript I) and an assessment of the potential for early measles virus antigen exposure to prime the immune system for a particular pattern of response. This last study was made possible by the unique design of the Sudanese HT vaccine trial study and is presented in Manuscript VI (Appendix II).

CHAPTER 2

SUMMARY OF AIMS AND OBJECTIVES

2.1 GENERAL AIM

To better understand the pathology of measles virus infection with a particular emphasis on immunopathogenesis.

2.2 SPECIFIC AIMS

Part I:

2.2.1 Immunologic Investigations of High Titer Vaccine Recipients

To describe the immune status of Haitian and Sudanese HT measles vaccine recipients 4 - 5 years after vaccination. To compare a wide range of immunologic parameters in standard titer and HT vaccine recipients including:

(i) Measles virus-specific responses;

(ii) Responses to relevant non-measles antigens;

(iii) Activation and phenotypic markers on PBMC.

2.2.2 Evaluation of Morbidity Following High Titer Vaccination

To determine if HT measles vaccination influenced morbidity patterns in Sudanese children.

Part II

2.2.3 Mechanisms of Measles Immunopathogenesis

(i) To measure reactivity to a panel of autoantigens as potential markers for subtle immune effects following HT measles vaccine.

(ii) To develop an *in vitro* model for the study of measles virus-induced apoptosis in PBMC and to evaluate the role of virus strain in this process.

(iii) To examine the impact of vitamin A on measles virus-induced apoptosis of PBMC.

Part III

2.2.4 Immune Priming by Early Measles Vaccination

To determine if early exposure to measles virus antigens biases the immune system for subsequent responses to measles antigen.

CHAPTER 3

MATERIALS AND METHODS

Each manuscript/chapter includes a "Materials and Methods" section with the reagents and procedures specific to that manuscript. All of the reagents and techniques used to complete the entire work are listed alphabetically and described in detail in this chapter. Also included in this chapter is Manuscript I that describes a comprehensive evaluation of the Syncytium Inhibition Assay (SIA) for the detection of measles neutralizing antibodies. This assay was used in the immunologic investigations described in Manuscripts II, III and VI.

3.1 ALPHABETICAL LIST OF MATERIALS AND METHODS

3.1.1 Antigen preparations

3.1.2 Differential leukocyte count

3.1.3 Delayed type hypersensitivity (DTH)

3.1.4 Enzyme-linked immunosorbent assay (ELISA)

3.1.5 Flow cytometry (FACS)

3.1.6 Hematocrit

3.1.7 *In vitro* model of measles infection

3.1.8 Lymphoproliferation assay (antigen specific)

3.1.9 PBMC isolation and cryopreservation

3.1.10 Plaque assay

3.1.11 Plaque reduction neutralization assay (PRN)

3.1.12 Retinoid preparations

3.1.13 Syncytium inhibition assay (SIA)

3.1.14 Spontaneous proliferation assay

3.1.15 Statistics

3.1.16 Study design and subjects

3.1.17 Terminal uridine nick-end-labelling assay (TUNEL)

3.1.18 Virus preparations

3.1.19 White blood cell (WBC) count

3.1.1 ***Antigen Preparations:*** The following antigens were used for ELISA tests and/or lymphoproliferation assays: tetanus toxoid (TT; gift from B Latham, Massachussetts Laboratories, Boston, MA), *Corynebacterium diptheriae* toxoid (DT), *Bordatella pertussis* whole cell lysate (PT) and *Haemophilus influenza* type B unconjugated polysaccharide (HI) (all gifts from R Wittes, Connaught Laboratories Ltd, Willowdale, ON), myelin basic protein (MBP) (gift from T Owens, Montreal, QC), Hela cervical carcinoma cell lysate (gift from M Newkirk, Montreal, QC), and human Fc (Jackson Research Laboratories, Inc., West Grove, PA). Measles antigens were prepared from Vero cell monolayers infected with vaccine-strain (Edmonston B and Edmonston Enders, gifts from M. Hilleman; Merck Research, West Point, PA and Connaught Laboratories Ltd respectively) or CHI-1 wild-type virus (gift from W Bellini, Atlanta, GA) at a multiplicity of infection (m.o.i.) 0.01. Virus was harvested at 90% cytopathic effect after one freeze-thaw cycle, centrifuged for 10 minutes at 500 x g and filtered through a 0.22 μM filter (Falcon, Franklin, NJ). Filtrates were pooled and centrifuged at 20000 x g for 2 hours at 4°C and sonicated before resuspension in PBS and determination of antigen concentrations by modified Bradford Assay (Biorad, Hercules, CA). Proteins derived from uninfected vero cell monolayers processed in an identical fashion served as control antigen in most assays.

3.1.2 ***Differential Leukocyte Count*:** Differential counts were performed on Haitian and Sudanese whole blood samples using the following protocol: 10 μL of unheparinzed whole blood was placed at one end of a glass slide (Fisherbrand®, Fisher Scientific, Oakville, ON). Using a clean slide, a "feather-edged" smear was made on the original glass slide and allowed to air dry. Slides were subsequently stained with Difquik (Sigma, Ottawa, ON) and 100 WBC were identified and counted using a manual cell counter. All smears were read by a single microscopist who was blinded to the origin of the specimens.

3.1.3 ***Delayed Type Hypersensitivity (DTH):*** DTH is a recall or "memory" immune response thought to be effected by the cellular arm of the immune system. A positive (≥ 2 mm) DTH response indicates previous exposure to an antigen. DTH testing was performed in the Haiti study using a commercial multi-test (Institut Merieux, Lyon, France), that included a control antigen (glycerin) and seven test antigens (tetanus toxoid, diphtheria toxoid, proteus, candida, trichophyton, streptococcus, and tuberculin). The test was applied to the left forearm of each child at the time of recruitment according to manufacturer's instructions and the induration caused by each antigen was measured 48 hours later using calipers and recorded as the mean reactivity index.

3.1.4 ***Enzyme-Linked Immunosorbent Assay (ELISA):*** The ELISA is a technique used to quantify antibodies of a given specificity. Several in house ELISA tests were used (anti-measles) or developed (anti-tetanus toxoid, anti-diphtheria toxoid, anti-*B. pertussis,* anti-*H.Influenza,* anti-rheumatoid factor, anti-myelin basic protein, and anti-Hela cell lysate) during the course of this thesis work. The basic ELISA protocol for all tests was similar: 96-well plates were coated overnight with antigen (0.5 - 2 μg/mL) in carbonate-bicarbonate buffer (pH 9.6). After blocking with 5% goat serum (Life Technologies, Grand Island, NY), plasma samples were typically diluted at 1:200 in phosphate buffered saline (PBS) and incubated overnight at 4°C. Assays were completed with mouse monoclonal anti-human IgG (ATCC#1757) followed by incubations with biotinylated goat anti-mouse IgG (Fab_2; Boehringer Mannheim, Laval, QC) for 1 hour at 37°C, HRP-conjugated streptavidin (Boehringer Mannheim) for 30 minutes at 37°C and ABTS (Boerhinger Mannheim) with 0.4 μL/mL hydrogen peroxide (Sigma) for 10 - 30 minutes at room temperature. Optical density was determined by spectrophotometry (Titertek Multiskan® MCC/340, Flow Laboratories, Mississauga, ON) at 405 nm. Specific IgG concentrations were estimated by extrapolating from standard curves generated using serial dilutions of international standard sera (available for measles, TT,

DT; World Health Organization for Biological Standards, Hertfordshire, UK) or local standards and results are expressed either as milli-international units or arbitrary OD units.

3.1.5 ***Flow cytometry (FACS):*** Flow cytometry was used to detect surface expression of phenotypic markers, activation markers and co-stimulatory markers on PBMC (see Table 1). PBMC were quick-thawed in a water bath at 37°C, washed once in PBS and resuspended in PBS containing 5% bovine serum albumin (Life Technologies). PBMC were triple stained with various combinations of fluorescein isothiocyanate (FITC), phycoerytherin (PE) and peridinin chlorophyll protein (PerCP) conjugated monoclonal antibodies (2 - 5 $\mu L/10^5$ cells; Becton Dickinson, San Jose, CA or Dako, Toronto, ON) (see Table 1) according to manufacturer's instructions. Labelled cells were washed with ice cold PBS and resuspended in PBS containing 1% paraformaldehyde and .05% sodium azide (both American Chemicals, Montreal, QC). Data were acquired by FACScan (Becton Dickinson) within 24 hours of staining and were analyzed using CellQuest software (Becton Dickinson). FITC and PE conjugated mouse IgG_1/IgG_{2a} were used as negative controls. No negative control was available for PerCP. Results are expressed as absolute cell counts, ratios (e.g. CD4/CD8), or as percentages of given PBMC subsets expressing various markers (e.g. $CD8^+$ T cells expressing CD69).

3.1.6 ***Hematocrit:*** Hematocrits were performed in the Haiti study to evaluate anemia and to provide some immediate feedback to the parents of the study children. Heparinized blood was drawn up into a capillary tube, spun in a microcrit centrifuge for 60 seconds and the hematocrit was determined using the attached gauge.

3.1.7 ***In Vitro Infection Model:*** PBMC were isolated from healthy adults as described in Section 3.1.9. Each experiment began when 1 x 10^6 PBMC in cRPMI (cRPMI: RPMI 1640, with 10 mM HEPES, 50 µg/mL gentamicin and glutamine; all Life Technologies) were pelleted (300 x g for 10 minutes) in a 15 ml polypropylene tube. After discarding the supernatant the cells were resuspended by strong agitation. The PBMC were infected with either vaccine strain or wild-type virus at a m.o.i of 0.1 or 1.0. Virus was allowed to attach for 1.5 hours at 37°C and 5% CO_2. Mock-infected controls with an Vero cell lysate were used in each experiment. Infected cells were then washed 1 x in cRPMI. The pelleted cells were then resuspended in cRPMI + 5% heat inactivated (56°C for 30 min) FBS (Life Technologies) + PHA (2.5µg/mL, Sigma Fine Chemicals, NJ) and incubated at 37°C and 5% CO_2 for a maximun of 5 days.

3.1.8 ***Lymphoproliferation:*** We assessed lymphoproliferative responses to vaccine-strain and wild-type measles antigens as well as tetanus toxoid and control (Vero cell) antigens. Lymphoproliferation assays were performed as previously described (Ward BJ et al 1995). Briefly, antigens (10 µg/mL) were pre-coated onto 96-well plates in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C and washed twice with sterile Hanks buffered salt solution (HBSS; Life Technologies) immediately before use. Cryopreserved PBMC were quick-thawed, washed once in HBSS and resuspended at 1 x 10^6/mL in cRPMI with 5% heat-inactivated autologous plasma. Cells were distributed at 2 x 10^5/well in triplicate wells for each antigen and incubated for 6 days at 37°C in 5% CO_2 before 1 µCi of ^{3}H-thymidine (ICN, Radiochemicals, Irvine, CA) was added to each well. Control and test antigens were coated onto the same plates to ensure similar culture conditions. Cellular DNA was harvested onto glass-fiber filters 24 hours later after one freeze-thaw cycle and incorporated ^{3}H-thymidine was estimated by beta-emission. Lymphoproliferative results are expressed as stimulation indices (SI = cpm of antigen-stimulated wells/cpm of control wells). A stimulation index (SI) ≥ 3 was considered to indicate a significant response.

3.1.9 ***PBMC Isolation and Cryopreservation :*** A single heparinized tube of blood (5 mL) was obtained by venipuncture from each child in the Haitian and Sudanese studies. All samples were processed within 6 hours of collection. The blood was centrifuged at 300 x g for 10 minutes and plasma was aliquoted and frozen at -20°C until used in assays. After resuspension of the blood cells in twice the initial volume of HBSS, the PBMC were isolated using Ficoll-Paque (Pharmacia, Uppsala, Sweden) and counted by trypan blue exclusion. Cells were resuspended in freezing media (92.5% FBS + 7.5% DMSO; American Chemicals Ltd) aliquoted (~ 3 - 5 x 10^6 /Nunc vial; Nunc, Roskilde, Denmark) and frozen overnight in a methanol bath at -70°C. The following morning the cryopreservation vials were transferred to liquid nitrogen holding tanks where they were stored until use. For international field work, specimens were transfered to a nitrogen vapour "Dry Shipper" and transported to Canada.

3.1.10 ***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, Cambridge, MA) were washed with HBSS and infected with serial 10-fold dilutions of culture supernatant for 90 minutes at 37°C in 5% CO_2 (duplicate wells, 100 µL/well diluted in HBSS). A 16% methylcellulose (Life Technologies) overlay was applied to each well (200 µL) and allowed to gel prior to incubation 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 (Life Technologies) and visible plaques in duplicate wells were counted. Each plaque represents 1 viral particle, and results are expressed as pfu/mL (plaque forming units).

3.1.11 ***PRN:*** The PRN test was carried out as previously described (Ward BJ et al 1995). Briefly, serum or plasma specimens were heat-inactivated (56°C for 30 minutes) and serially diluted starting at 1:4 in Joklik's modified minimum essential media (SMEM; Bio-Whittaker, Walkersville, MD) in 96-well plates (120 µL/well). Samples were diluted

either two-fold or four-fold depending upon the expected titer range: for example two-fold dilutions were used initially for pre-vaccination samples and four-fold dilutions were used for samples obtained months after natural infection. A standard inoculum of Edmonston vaccine-strain measles virus was added to each well (120 μL containing 50-70 pfu) and incubated at 37°C in 5% CO_2. After 100 minutes, the inoculum-plasma/serum mixtures were added to duplicate Vero cell monolayers at ~95% confluency in 24-well plates (100 μL/well) and incubated at 37°C in 5% CO_2. After 75 minutes, the inocula were removed and the monolayers were covered with an overlay of 1.6% methyl cellulose in Leibovitz's L-15 media (Life Technologies; 500 μL/well). The plates were incubated for 4 days at 37°C in 5% CO_2 before the monolayers were stained with neutral red and the plaques were counted visually. The PRN values were determined using the Karber formula (Ballew 1986) to estimate the serum/plasma titer required to reduce the number of input pfu by 50%. PRN values < 8 are generally considered to be negative. Values ≥ 120 are thought to indicate protection from symptomatic disease while values > 800 indicate sterile immunity (ie: no antibody boosting upon exposure to wild-type virus) (Chen 1989).

3.1.12 ***Retinoids:*** Retinol (ROH) (Sigma Fine Chemicals, Ottawa, ON) was prepared by solubilizing in DMSO and diluting to 10^{-3} M in ethanol. All stocks were aliquoted and stored in opaque bottles in the dark at -70°C until used. Stock solutions were further diluted with RPMI 1640 (Life Technologies) immediately prior to addition to PBMC. The final ethanol concentration in PBMC cultures was $\leq 0.1\%$ v/v which had no effect on either PBMC viability or measles virus replication (unpublished data).

3.1.13 ***SIA (Syncytium Inhibition Assay):*** The SIA is a simple and reliable assay for measuring measles-specific neutralizing antibodies and the assay was performed as detailed in Manuscript I. Briefly, serial dilutions of individual plasma specimens in a 96-well plate were incubated with ~ 125 pfu of CHI-1 measles virus for 2 hours at 37°C and

5% CO_2. Following this period of neutralization, measles sensitive B-95.8 (ATCC# 1612, Rockville, MD) cells were added to each well and incubated for a further 72 - 96 hours at 37°C and 5% CO_2. The presence of syncitia was determined microscopically, and results are expressed as the reciprocal of the highest dilution capable of inhibiting the formation of syncytia.

3.1.14 ***Spontaneous Proliferation:*** Spontaneous proliferation of PBMC is a non-specific marker of immune system activation and was measured as previously described (Ward BJ et al 1990). Briefly, PBMC were suspended at 1 x 10^6/mL in cRPMI with 5% FBS and 1 µCi of ^{3}H-thymidine. Cells were distributed in triplicate in 96-well plates at 200 µL/well. Twenty-four hours later, plates were frozen at -20°C and stored until the DNA could be collected on glass-fiber filters for evaluation of ^{3}H-thymidine incorporation. Results are expressed as counts per minute (cpm).

3.1.15 ***Statistics:*** Both Statview™ (Cary, NC) and Statistical Package for Social Sciences (SPSS) software (Cary, NC) were used for the analyses presented in this thesis. The tests used are clearly explained in each manuscript. Parametric tests were used for comparsions of parameters known or demonstrated to be normally distributed. Occassionally, data were log-transformed to approximate the normal distribution. Otherwise, non-parametric tests were used. Data are expressed either as mean ± SD (standard deviation) or SEM (standard error of the mean) unless otherwise indicated. For all statistical tests, $p < 0.05$ was considered to be significant.

3.1.16 *Study Design and Sample Populations:*

Original Haitian High Titre (HT) Study: The original HT study was carried out in 1987 in a peri-urban district of Port-au-Prince (Cité Soleil) Haiti (Job JS et al 1991). Between 1987 and 1988, children 6 - 11 months of age attending the free, community-based, well-baby clinic were block randomized to receive one of four vaccines: Schwarz medium titre (n = 489), Schwarz high titre (n = 494), Edmonston-Zagreb medium titre (n = 489) or Edmonston-Zagreb high titre (n = 500). A control group received standard titre Schwarz vaccine (n = 371) at 9 months of age. All children participating in the original study resided in two boroughs of Cité Soleil; Boston or Brooklyn. There were no statistically significant differences in sex, socioeconomic status, (e.g. cement floor in the house), nutritional status (e.g. anthropometrics) or crowding between the vaccine groups at the time of randomization for the original study (Holt EA et al 1993).

Immunologic Follow-Up Study in Haiti: Increased mortality associated with the use of HT vaccines was reported in the Haitian HT vaccine study (Holt EA et al 1993). In 1993, a follow-up study was initiated to determine the immunologic status of the participants of this trial. It should be emphasized that the need for such a follow-up study was not anticipated at the original trial's inception. Community health workers attempted to locate parents or guardians of all the original vaccine trial children by using the last known addresses and interviewing family members and neighbours. The names of children not initially located were circulated to all of the community health workers in an effort to maximize recapture for the follow-up study. All of the children recaptured were offered nutritional support, but for logistical reasons (ready access to a small clinic and laboratory) and poor co-operation of the Brooklyn community workers, the immunologic follow-up study was conducted in the Boston population. The children selected for the immunologic study were representative of the original study population (Table 2).

Original Sudanese HT Trial: The original Sudanese measles HT trial was conducted in 1990. Children were recruited from 14 villages of the Umdawanband and Essailat rural councils, ~ 50 kilometers from Khartoum. These villages ranged in size from 800 to 5900 residents and there were no significant socio-economic, environmental or nutritional differences between the children in the different villages (Hoskins EW 1993). Over a ten-month period, 510 five-month old infants were enrolled and block randomized to receive one of three vaccine regimens: Edmonston-Zagreb HT at 5 months followed by meningococcal A + C vaccine at 9 months (EZ-Mg); Connaught HT at 5 months followed by Schwarz standard titer vaccine at 9 months (CN-SCH); or meningococcal A + C vaccine at 5 months followed by Schwarz standard titer vaccine at 9 months (Mg-SCH). There were no significant differences between the vaccine groups. In contrast to the Haiti study, long-term follow-up of the Sudanese children was an integral part in the original study design. Bi-monthly morbidity and mortality data were collected by the village mid-wives throughout the study. The Sudan study is the only HT trial in which morbidity data was prospectively collected. A trend towards increased mortality in the Sudanese HT recipients was reported (Hoskins EW 1993).

Immunologic Follow-Up Study in the Sudan: For the immunologic study, 6 of the 14 villages were chosen at random (Table 3), and an attempt was made to recruit all of the children in these villages. This was possible because, as previously mentioned, there were no socio-economic, environmental or nutritional status differences amongst the 14 villages (Hoskins EW 1993). One-hundred-ninety-three children (mean age 5.1 years; range 4.6 - 5.6) from the 280 enrolled in the original vaccine trial (69%), were recruited to the immunologic follow-up study. All of the vaccine groups are well represented (Table 4).

3.1.17 ***TUNEL (Terminal Uridine Nick-End-Labelling) Assay:*** The TUNEL assay was used to detect cells undergoing apoptosis. Cells undergoing apoptosis activate several endonucleases which cut DNA and expose 3'-OH terminals, to which labelled dUTP can be enzymatically added and detected. The incorporation of dUTP inside the cell is therefore a reflection of apoptosis. To detect apoptosis, PBMC were washed, permeabilized with 0.1% Triton X-100 (American Chemicals) in 0.1% sodium citrate (Fisher Scientific) for 2 minutes on ice. Once permeabilized, the cells were washed twice in PBS and resuspended in TUNEL reaction mixture: (TUNEL reaction buffer: 200 mM potassium cacodylate, 25 mM Tris-HCL, 1 mM $CoCl_2$ (Pharmagen) dUTP-Biotin (Boerhinger Mannheim) and terminal-deoxytransferase (TdT) (Pharmagen)) for 1 hour at 37°C in the dark. Cells were then blocked with PBS + 5% FBS for 15 minutes, washed and incubated with 5µL of PE-conjugated streptavidin (Dako) for 30 minutes at 37°C in the dark. Cells were washed and dUTP incorporation was measured by flow cytometry (FACScan, Becton Dickenson). Results are expressed as either a percentage of cells expressing biotinylated-dUTP or as mean flourescence intensity of cells.

3.1.18 ***Virus Stocks:*** A total of 12 different measles virus strains were used in the experiments described in this thesis. Four vaccine strains: Edmonston B (EdB; gift from M Hilleman, Merck Research), Connaught (CN; gift from R Wittes, Pasteur Merieux Connaught), Edmonston Zagreb and Schwarz (EZ & SCH; gifts from W Bellini, Atlanta, GA), and 8 wild-type strains; Bilthoven (Bilt; gift from A Osterhaus; Rotterdam, Holland), Chicago-1 (CHI-1; gift from W Bellini; Atlanta, GA), Massachussetts, Minnesota, Utah, Hawaii, Gambia, and Guam (gifts from P Rota, Atlanta, GA). Viruses were plaque purified using Vero cells and re-seeded on Vero cell monolayers in MEM (Life Technologies) + 5% FBS (Life Technologies) for approximately 48 - 72 hours to ensure maximal cytopathic effect. Cell-free supernatants were harvested at 80 % cytopathic effect

and titered by plaque assay (Section 3.1.10). Viral stocks were aliquoted and maintained frozen at -70°C until used. For inactivated measles virus studies, virus was thawed and immediately inactivated by heat (56°C for 30 minutes) or UV-irradiation (120mJ/cm^2 for 4 minutes) using a Spectrolinker XL-1000 UV-crosslinker (Spectronics Corporation; New York, NY).

3.1.19 WBC count: WBC counts were performed manually by operators blinded to the origin of the specimens using a WBC diluting pipette and hemocytometer.

Table 1 - Antibodies Used for FACS Analysis: Cell Type and CD Designation

Cell Type	Surface Marker	Function
CD4+ T cell	CD45RA	Naive T cells
	CD45RO	Memory T cells
	CD45RB	Low on mature T cells
	CD30	Possible marker for Th-2 phenotype
	CD25	Low affinity IL-2R
	CD75	High affinity IL-2R
	CD28	Co-stimulatory molecule
	HLA-DR	MHC II
	LFA-1	Integrin adhesion molecule
	CD71	Transferrin receptor
CD8+ T cells	CD25; CD75; HLA-DR; LFA-1	As above
	CD69	Early activation marker
	CD38	Early activation marker
	CD71	Transferrin receptor (activation marker)
B cells (CD19)	CD80	Ligand for CD28/CTLA-4
	CD86	Ligand for CD28/CTLA-4
	CD23	Activation marker
	CD25	As above
	CD38	As above
NK cells (CD16)	CD69	As above
Macrophages (CD14)	CD80	As above
	CD86	As above
	CD54	Intercellular adhesion molecule (ICAM-1)

Table 2 - Comparison of Haitian Children Included or Excluded in 1993 Immunologic Follow-Up Study

	Original Participants Not Included	Original Participants Included	P value
% Male % Female	47.3% (n=247) 51.5% (n=269)	50.7% (n=228) 49.3% (n=222)	
Age at Vaccination (months)	8.6 ± 2.0* (5.8 - 19.4)	8.8 ± 2.4 (5.9 - 23.4)	0.15
Weight at Vaccination (grams)	7489.9 ± 1203.7 (4000 - 11 000)	7624.7 ± 1253.3 (3800 - 16 400)	0.12
Crowding (mean # of persons per room/house)	4.7 ± 2.0 (0.10 - 15.0)	4.83 ± 2.0 (0 - 12)	0.37
% with cement floors in home	96.4%	97.2%	

*mean ± SD (range).

Table 3 - Village Size and Participants in Original and Follow-Up Studies in Sudan

Village	Population	#Children in Original Study	#Children in Immunologic Study	% of Original Vaccinees
Umdawanban	4800	96	--------	
Hawiela	800	11	--------	
Essailat	5900	106	88	83%
Hasonab	1250	15	--------	
Hamda West	800	14	--------	
El Hashid/ Abuzayd	1350	41	23	57%
Abugroon	1550	41	24	58%
Baknab	1000	22	--------	
Fadnia	950	27	19	70%
Sheikh Mustafa	1800	37	27	73%
Bambonad	1700	28	12	43%
Mahab Kutrang	1700	46	--------	
Kutang	2250	22	--------	
Mahab Tayba	550	4	--------	
Total	**26 400**	**510**	**193**	**69%**

Table 4 - Distribution of Sudanese Children in Immunologic Follow-Up Study by Vaccine Group and Gender

Group	First Dose (5 months)	Second Dose (9 months)	# Original Study	# Follow-up Study	
				Male / Female	Total
Mg-SCH*	Mg	SCH	170	28 / 33	61
EZ-Mg	EZ	Mg	170	41 / 32	73
CN-SCH	CN	SCH	170	33 / 26	59
Total			510	102 / 91	193

*Mg = Meningococcal (placebo)
SCH = Schwarz ($10^{3.9}$ pfu)
EZ = Edmonston Zagreb ($10^{4.7}$ pfu)
CN = Connaught ($10^{4.7}$ pfu)

3.2 MANUSCRIPT I

Measurement of Measles Virus-Specific Neutralizing Antibodies: Evaluation of the Syncytium Inhibition Assay (SIA) in Comparison with the Plaque Reduction Neutralization Test (PRN)

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Running Head: Assays for measles neutralizing antibodies

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Abstract

Plaque reduction neutralization (PRN) is the 'gold-standard' for the measurement of measles-specific neutralizing antibodies. However, it is a complicated assay and tends to be operator-dependent. It has been suggested that the simpler syncytium inhibition assay (SIA) can give results comparable to the PRN test. We compared these two assays using 594 serum or plasma samples obtained from children at various times after natural infection, primary measles immunization and measles revaccination. The results of the two assays correlated well overall ($r = .86$; $p<.0001$). The strain of challenge virus (wild-type vs. vaccine strain) did not significantly influence SIA titers and the assay performed equally well with serum and plasma. PRN titers ≥ 120 and > 800 are thought to indicate protection against clinical illness and infection respectively. The equivalent SIA cut-off values using 125 plaque-forming units (pfu) as the challenge inoculum were ≥ 16 and > 128 respectively. At low PRN titers (< 200), the correlation between PRN and SIA values was reasonable ($r = .60$; $p = .001$) when a challenge inoculum of 12.5 pfu was used. At the lowest PRN titers (< 100), 15% of the samples gave divergent results. These data confirm the utility of the SIA in the determination of measles-specific neutralizing antibodies when antibody titers are high. However, the PRN assay remains the test of choice when maximum sensitivity at low titers is required.

Introduction

A number of serologic tests are available for the measurement of measles-specific humoral immunity. These include hemagglutination inhibition, complement fixation, IgG and IgM enzyme immunoassays (EIA), the plaque reduction neutralization test (PRN) and most recently, the syncytium inhibition assay (SIA) (Albrecht P et al 1981, Forthal DN et al 1994, Markowitz LE & Katz SL 1994, Ratnam S et al 1995). Hemagglutination inhibition and complement fixation have been widely used in the past but these tests are relatively insensitive and the requirement for red blood cell reagents (eg: monkey, sheep) makes them difficult to standardize. Commercial EIA tests are simple, sensitive and specific (Ratnam S et al 1995) but give limited quantitative (Albrecht P et al 1981) information, particularly at low antibody titers. The PRN test developed by Albrecht is a highly sensitive and specific assay of neutralizing antibodies and is widely considered to be the 'gold-standard' in measles serology. This test requires the incubation of a standard virus inoculum with dilutions of patient serum followed by infection of Vero cell monolayers. The monolayers are stained 4 - 5 days later and viral plaques are counted manually. The titer of the serum dilution which reduces the number of plaque-forming units (pfu) in the standard inoculum by 50% is the PRN value. This assay is labor-intensive and is sufficiently technically demanding that only a small number of laboratories throughout the world perform it as a reference service. The syncytium inhibition assay (SIA) also measures neutralizing antibodies but uses the measles-susceptible B cell line, B95-8, in an end-point dilution format (Forthal DN et al 1994). In this assay, serial dilutions of patient serum are incubated with a standard virus inoculum and then added directly to B95-8 cells in 96 well plates. Syncytia (giant cells) are easily detected in infected wells 72 - 96 hours later by scanning the plates with an inverted microscope. The SIA value is the titer of the highest serum dilution at which syncytium formation is completely inhibited. The SIA is much simpler to learn and to perform than the PRN test. One small

study of 25 subjects has suggested that SIA and PRN results are highly correlated (Forthal DN et al 1994).

The circulation of wild-type measles virus has been interrupted in many regions of the world. As a result, a steadily increasing proportion of the world's population is protected by measles vaccination alone (ie: no natural boosting). The precise nature and duration of vaccine-induced immunity remain the subjects of active research. The availability of a relatively simple, quantitative test for the measurement of measles neutralizing antibodies would be of considerable interest for vaccine-related research as well as for the epidemiologic evaluation of sporadic cases and outbreaks. To investigate the utility of the SIA, we measured SIA titers in 594 samples from 331 children at various times after natural infection, measles vaccination or measles revaccination and compared these results with PRN values. We also evaluated the influence of the challenge virus strain on the SIA and the use of plasma rather than serum in this assay.

Materials and Methods

Serum/Plasma Test Panels. Three plasma/serum panels were available for testing: 31 plasma samples obtained at various times after natural infection (range 2 - 380 days) from outbreaks in Philadelphia (1990; mean age 5.1 ± 1.3 years) and Ontario (1994; mean age 16 ± 1 years); 383 plasma or serum samples obtained from 240 children in Montreal and Newfoundland at various times after routine vaccination with MMR (range 6 days to 15 years); 180 plasma and serum samples obtained from 60 children in Quebec City before and at 1 and 6 months after measles revaccination (mean age 9.4 ± 3.7 years).

Measles Virus Stocks. A wild-type measles virus (CHI-1, gift from W Bellini, Atlanta, GA) and Edmonston vaccine-strain virus (American Type Tissue Collection (ATCC), Rockville, MD) were seeded at low multiplicity of infection (m.o.i. = .001) on ~85% confluent Vero cell monolayers. Culture supernatants collected at peak cytopathic effect were centrifuged (1000 x g for 10 minutes at 4°C) and filtered (0.2 mm) to remove cellular debris. Viral stock titers were determined by plaque assay (generally .75 - 1.5 x 10^6 pfu/mL) and aliquots were frozen at -70°C until used.

Plaque Reduction Neutralization Test. The PRN test was carried out as previously described (Ratnam DN et al 1995). Briefly, serum or plasma specimens were heat-inactivated (56°C for 30 minutes) and serially diluted starting at 1:4 in Joklik's modified minimum essential media (SMEM; Bio-Whittaker, Walkersville, MD) in 96-well plates (120 μL/well). Samples were diluted either two-fold or four-fold depending upon the expected titer range: for example two-fold dilutions were used initially for pre-vaccination samples and four-fold dilutions were used for samples obtained months after natural infection. A standard inoculum of Edmonston vaccine-strain measles virus was added to each well (120 μL containing 50 - 70 pfu) and incubated at 37°C in 5% CO_2. After 100 minutes, the inoculum-plasma/serum mixtures were added to duplicate Vero cell

monolayers at ~95% confluency in 24-well plates (100 μL/well) and incubated at 37°C in 5% CO_2. After 75 minutes, the inocula were removed and the monolayers were covered with an overlay of 1.6% methyl cellulose in Leibovitz's L-15 media (Life Technologies, Grand Island, NY; 500 μL/well). The plates were incubated for 4 days at 37°C in 5% CO_2 before the monolayers were stained with neutral red and the plaques were counted visually. The PRN values were determined using the Karber formula (Ballew HC 1986) to estimate the serum/plasma titer required to reduce the number of input pfu by 50%. PRN values < 8 are generally considered to be negative. Values ≥ 120 are thought to indicate protection from symptomatic disease while values > 800 indicate sterile immunity (ie: no antibody boosting upon exposure to wild-type virus) (Chen RT et al 1989).

Syncytium Inhibition Assay. The SIA was slightly modified from that described by Forthal et al (Forthal DN et al 1994). Briefly, heat-inactivated serum/plasma specimens were serially diluted (two- or four-fold) starting at 1:4 in 96-well plates with Hanks buffered salt solution (HBSS; Life Technologies) containing 0.5% fetal bovine serum, 50 μg/mL gentamicin and 10 mM HEPES (Life Technologies; 100 μL/well). A challenge inoculum (125 pfu of either wild-type or vaccine-strain virus) was added to each well and incubated at 36°C in 5% CO_2. After 2 hours, 2 x 10^5 measles-susceptible B95.8 cells (ATCC #CRL-1612) diluted in acid RPMI 1640 with 50 μg/mL gentamicin and 30 mM HEPES and 20% fetal bovine serum (FBS) were added to each well (100 μL/well). Acid RPMI 1640 is prepared by adjusting RPMI 1640 (Life Technologies) to pH 6.8 with HCl. The plates were then incubated at 37°C in 5% CO_2 and giant cells (syncytia) were identified microscopically 72 hours later. SIA values were recorded as the titer of the highest dilution of plasma/serum at which syncytium formation was completely inhibited.

Specimens with low PRN values (PRN < 300) were tested repeatedly with slight modifications to the SIA test to increase the discriminatory power of the syncytium assay at low titers. Modifications included protein pre-coating the assay wells (eg: 10% FBS or

bovine serum albumin (BSA)), agitation of the assay plates and reduction of the challenge inoculum (50, 25 or 12.5 pfu/well). Both serum and plasma samples were available from 180 individuals which were tested in parallel using our standard SIA (125 pfu/well; $SIA_{(125)}$). Sixty-eight plasma samples were tested in the $SIA_{(125)}$ using either the wild-type or vaccine-strain virus as the challenge inoculum. Intra-assay variability was assessed by measuring $SIA_{(125)}$ titers in 90 specimens three times in different 96-well plates on the same day. Inter-assay variability was assessed by determining $SIA_{(125)}$ titers in 33 samples on three separate occasions.

Statistical Evaluation. Since both PRN and SIA results are bimodal, correlations between the different tests were evaluated using the Spearman rank-order coefficient of correlation. Correlation coefficients between the replicate experiments (a, b and c) are expressed as r_{ab}, r_{ac}, r_{bc}.

Results

Measles-specific neutralizing antibody titers ranged from < 4 to 14,000 in the PRN test and from < 8 to 1024 in the $SIA_{(125)}$. The overall correlation between these two tests was excellent ($r = 0.86$; $p < .0001$; Figure 1) confirming the results of the much smaller comparison performed by Forthal (Forthal DN et al 1994). Both the PRN test and $SIA_{(125)}$ performed equally well using serum or plasma (data not shown) although non-specific 'clumping' of cells occurred in the $SIA_{(125)}$ at serum and plasma dilutions lower than 1:8. The $SIA_{(125)}$ also performed well when either wild-type or vaccine-strain measles was used as the challenge virus and the results were highly correlated ($r = .80$, $p < .001$) (Figure 2).

The capacity of the 'standard' $SIA_{(125)}$ to discriminate between low antibody titers was limited. Most subjects with PRN values < 200 had $SIA_{(125)}$ values at or below the lowest dilution which could be interpreted in the SIA (≤ 8). We tried a number of modifications to increase the discriminatory power of the SIA at low antibody titers such as continual agitation of the assay plates, protein pre-coating of test wells (to reduce non-specific inactivation of antibody or virus) and reduction of the challenge inoculum (50, 25 or 12.5 pfu/well). The assay conditions which yielded SIA values which most closely paralleled the titers determined by PRN were 10% BSA pre-coating of the test wells and a challenge inoculum of 12.5 pfu ($SIA_{(12.5)}$; Figure 3). Using these conditions, the correlation between the $SIA_{(12.5)}$ and PRN results was reasonable ($n = 108$; $r = .60$; $p < .001$) although a subset of children (15%) with very low PRN titers (< 100) had divergent results. More experience with the SIA will be needed to fully understand the implications of this divergence for the interpretation of SIA results in this range. Such experience may also permit the selection of either $SIA_{(125)}$ or $SIA_{(12.5)}$ on the basis of a screening ELISA test. The intra-assay variability of the $SIA_{(125)}$ was acceptable at both high (r_{ab}, r_{ac}, r_{bc} = .92, .89, .93 respectively; all $p < .001$) and low antibody titers (r_{ab}, r_{ac}, r_{bc} = .80, .89, .92

respectively; all $p < .001$). The inter-assay variability of the $SIA_{(12.5)}$ was also acceptable (r_{ab}, r_{ac}, r_{bc} = .87, .91, .93 respectively; all $p < .001$).

Discussion

Although the correlates of immunity against measles are not yet fully understood, a limited amount of evidence suggests that PRN titers may in some circumstances be used to classify children as fully susceptible (PRN < 120), susceptible to modified or asymptomatic infection (PRN between 120 and 800) or immune (PRN > 800) (Chen RT et al 1989). In our standard assay conditions (eg: $SIA_{(125)}$ using CHI-1 virus), the equivalent $SIA_{(125)}$ values would be < 16 (fully susceptible), between 16 and 128 (susceptible to mild or asymptomatic infection) and ≥ 128 (completely immune). Using 12.5 pfu of CHI-1 as the challenge inoculum ($SIA_{(12.5)}$), the value predicting partial or full immunity against measles (ie: PRN ≥ 120) was $SIA_{(12.5)} \geq 256$. The differences we observed with varying the size of the challenge inoculum and the discrepancies between our cut-off values and those reported by Forthal (Forthal DN et al 1994) suggest that the conditions for this test will need to be standardized before SIA values generated in different laboratories can be compared.

Our data confirm the utility of the SIA for the determination of measles-specific neutralizing antibodies. SIA values were highly correlated with PRN values overall. This correlation is weakest in samples with the lowest anti-measles antibody titers (PRN < 100). The SIA and PRN assays use different target cell lines and may not be evaluating precisely the same antibody populations. More experience with the SIA will be needed before the discrepancy at low titers can be fully understood. For example, Samb B and colleagues (Samb B et al 1995a) have recently demonstrated that many previously vaccinated but PRN seronegative children are protected from natural infection. Clearly much remains to be learned about the correlates of immunity in measles.

Although the SIA test still requires a tissue culture facility and takes three days to perform, this assay can be learned rapidly by individuals with even limited technical skills and requires less than 25% of the time needed for the PRN test. In practise, a screening ELISA might be used to descriminate between higher titer samples which could be tested

with the $SIA_{(125)}$ and lower titer samples which would be better evaluated using the $SIA_{(12.5)}$. Although it is unlikely that even large hospital laboratories will be tempted to offer either of these tests, the SIA may be an attractive alternative to the complicated PRN in state, provincial and research laboratories.

Certainly quantitative tests for measles serology will gain in importance as measles becomes an even rarer disease; both to evaluate the durability of vaccination-induced protection and to investigate sporadic cases and small outbreaks. Since B95-8 cells are also useful for the isolation of measles virus (Forthal DN et al 1992), wider use of the SIA could have the additional advantage of an improved capacity to recover wild-type virus from sporadic cases.

Until more data are available using the SIA under a wide variety of conditions (eg: revaccination studies, natural disease outbreaks), the apparent limitations of this test at very low antibody titers suggest that the PRN test should remain the 'gold standard' for measles serology.

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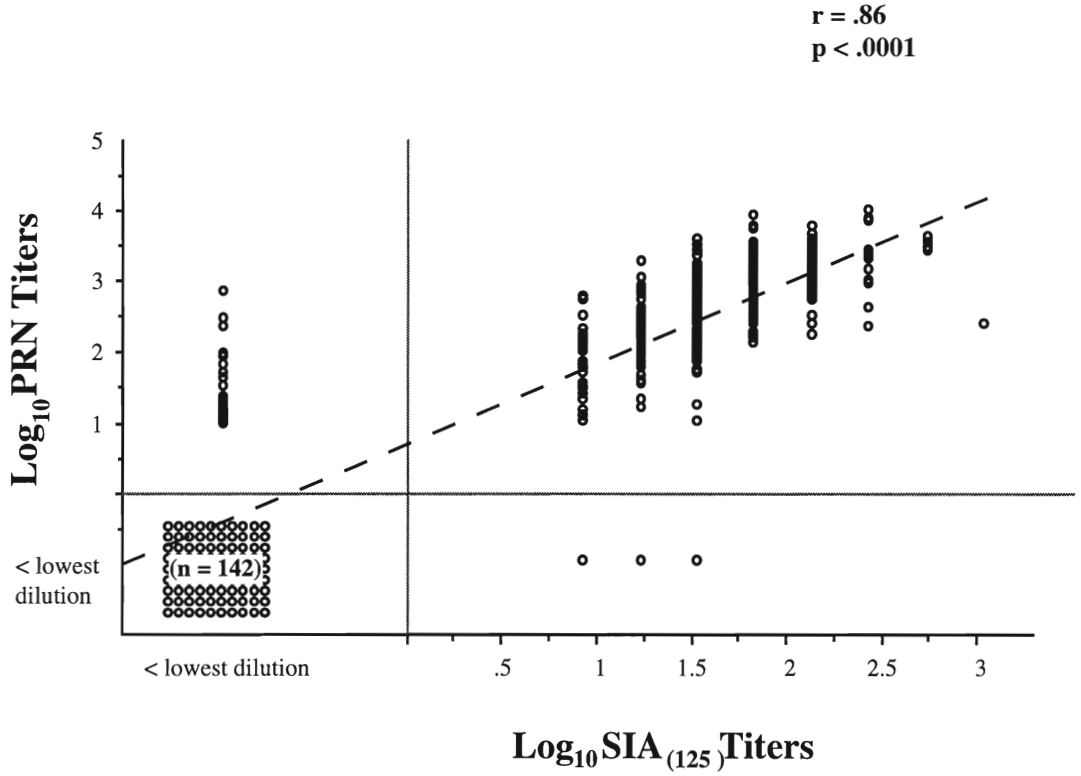
Figure 1 - Comparison of PRN and Standard SIA_{125} Tests



Measles-specific neutralizing antibodies were measured by the plaque reduction neutralization test (PRN) and the syncytium inhibition assay (SIA) in 594 plasma or serum specimens from children following standard measles vaccination, revaccination or natural measles infection. The challenge inoculum was 125 pfu/well of CHI-1 virus. Each point represents a single result.

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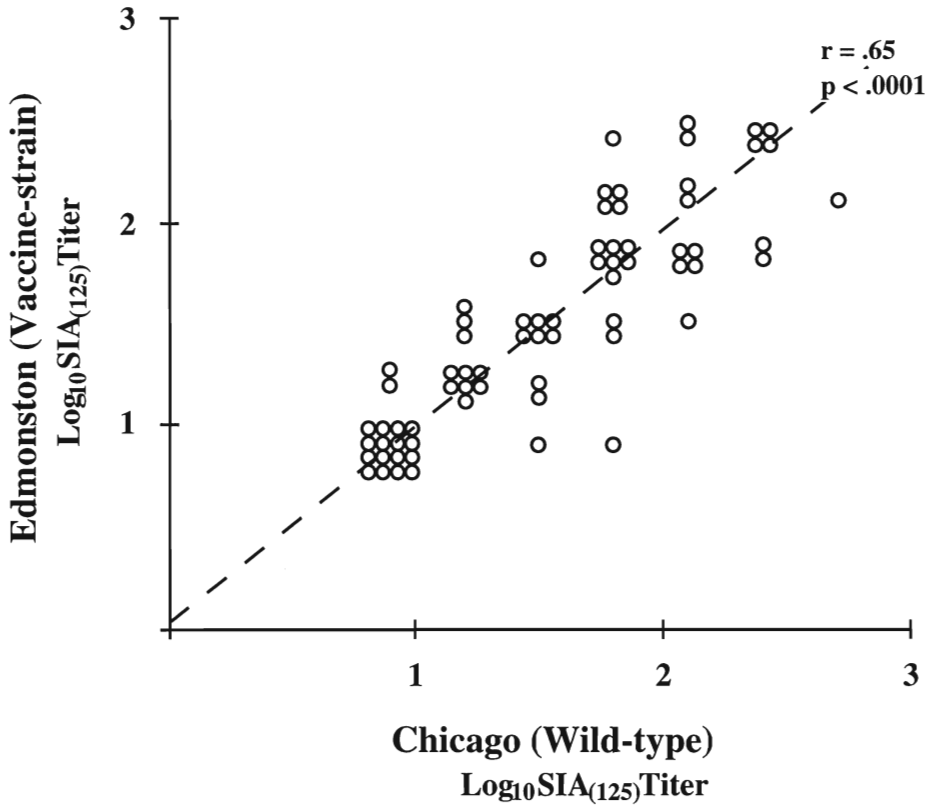
Figure 2 - Comparison of SIA(CHI-1) with SIA(EdB)



Comparison of syncytium inhibition assay (SIA with 125 pfu/well) performed using either a wild-type (CHI-1) or vaccine-strain measles virus (Edmonston) as the challenge inoculum (n=68). Each point represents a single result.

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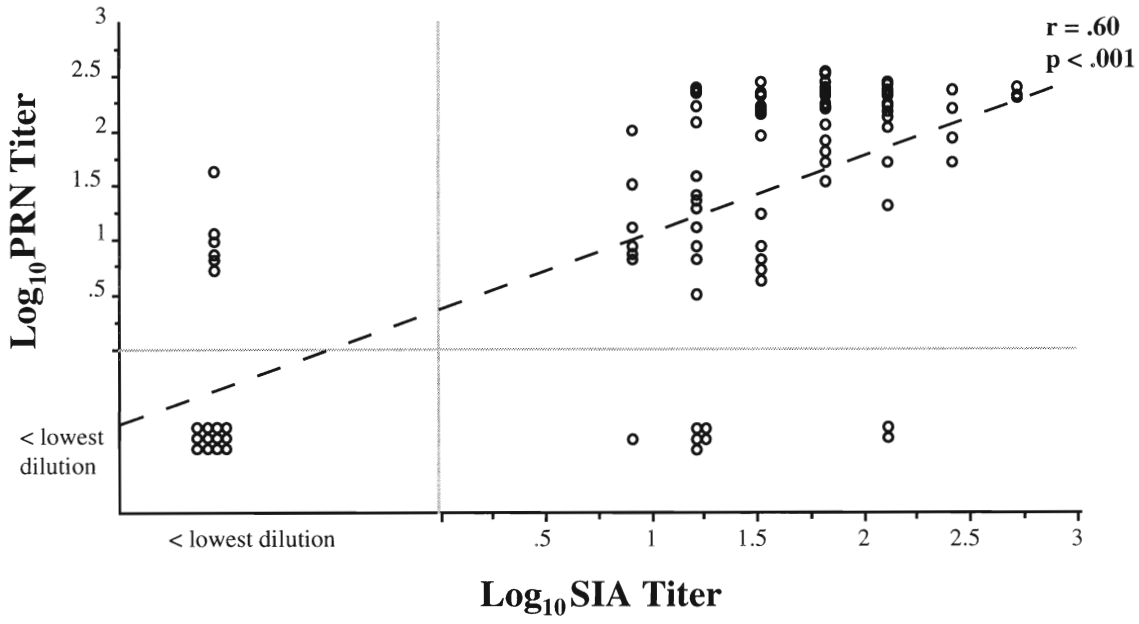
Figure 3 - Comparison of PRN with $SIA_{12.5}$ for Low Titer Sera



Comparison of the plaque reduction neutralization tests (PRN) and a modified syncytium inhibition assays (SIA) in 108 specimens with low titers of neutralizing antibodies. The standard SIA was modified by pre-coating the assay wells with 10% BSA and the use of a smaller challenge inoculum (12.5 pfu/well). Each point represents a single result.

CHAPTER 4

SPECIFIC AIMS

4.1. SPECIFIC AIM # 1a:

Immunologic Investigations of High Titer Vaccine Recipients

HT measles vaccines were introduced in the developing world to address the problem of increased susceptibly to severe disease and death in very young children (Halsey NA 1993). The association of these vaccines with increased mortality gave rise to the 1991 WHO recommendation that their use be discontinued pending further investigation (EPI: Wkly Epi Rec 1992). Attempts to identify specific causes of the "excess" mortality (e.g. study design errors, problems with randomization, social factors, and specific pathogens) have been uninformative. To date, the hypothesis that these vaccines may have subtly damaged or altered the immune system has received only limited attention (Leon ME et al 1993, Lisse IM et al 1994).

The biological plausibility that measles HT vaccines act as an immunologic insult is supported by several observations including the profound and prolonged immune suppression that follows natural infection and the immune cell tropism of the virus (reviewed in detail in Sections 1.4.2, 1.4.3). However, the best support comes from the striking epidemiologic observation that mortality rates in children exposed to measles virus early in life are higher than those of unexposed children (reviewed in Section 1.3.3). Like the HT vaccine experience, the causes of death in these natural exposure studies were typical for the developing world (e.g. diarrhea and fever) and deaths accumulated slowly for follow-up periods of several years.

This chapter contains two reports of long-term immunologic follow-up studies examining measles specific and non-specific immunity in HT vaccine recipients. Manuscript II describes the immunologic investigation of the Haiti HT measles trial. Manuscript III, reports a similar investigation which was carried out in a smaller HT measles vaccine trial in the Sudan.

4.1.1 MANUSCRIPT II

Long-Term Follow-up of Haitian High Titre Measles Vaccine Recipients: No Evidence of Immunologic Damage 4 - 5 Years After Vaccination.

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Running Head: High titer measles vaccine - immunologic follow-up
Key Words: Measles, measles vaccine, mortality, vaccine associated adverse event

Abstract

Background: High titer (HT) measles vaccines are associated with poorly understood excess mortality and subtle immunological changes that can be detected for 2 - 3 years after vaccination.

Methods: We performed a battery of tests of immunologic status on 450 Haitian children originally recruited to a trial of HT vaccines ~ 5 years earlier (24% of original study population). The children had received one of five vaccines: Edmonston Zagreb (EZ) high ($10^{5.6}$plaque forming units (pfu)) or medium ($10^{4.4}$ pfu) titer at ~ 8 months of age, Schwarz (SCH) high ($10^{5.4}$ pfu) or medium ($10^{4.6}$ pfu) titer at ~ 8 months of age or standard titer Schwarz vaccine (SCH $10^{3.0\text{-}3.7}$ pfu) at ~ 12 months of age. The battery of tests included delayed-type hypersensitivity (DTH), descriptive parameters (WBC and differential counts, spontaneous proliferation of PBMC, flow cytometric analysis of differentiation and activation marker expression), measures of measles-specific immunity (neutralizing antibodies, IgG subclass distribution, lymphoproliferation), and measures of immunity to non-measles pathogens (antibody titers for tetanus, diphtheria, *H. influenza* and *B. pertussis* specific antibodies, and lymphoproliferation to tetanus toxoid).

Results: There were no significant differences between children grouped by age at vaccination, sex, vaccine strain or vaccine titer. When data from all HT recipients (EZ and SCH) were pooled at 8 months and compared to lower titer (LT) recipients (standard or medium titer EZ or SCH at 8 or 12 months), minor differences were observed between groups but no consistent pattern could be identified.

Conclusion: There was no evidence of immunologic differences between Haitian HT and LT measles vaccine recipients 4 - 5 years after vaccination.

Introduction

In the 1980's, measles vaccines containing 10 - 100 fold more viral particles than standard vaccines were tested in the developing world in an attempt to reduce measles-associated morbidity and mortality in children less than 9 months of age (e.g. $10^{4.4-5.7}$ pfu versus standard titer at $10^{3-3.7}$ pfu) (Tidjani O et al 1989, Markowitz LE et al 1990b, Job JS et al 1991, Berry S et al 1992). It was reasoned that high titers of the commonly used vaccine-strain viruses might successfully induce seroconversion despite the presence of maternal antibodies. Early results from HT measles vaccine trials were very encouraging with seroconversion rates $\geq 80\%$ in children as young as 3 months of age (Tidjani O et al 1989, Markowitz LE et al 1990b, Whittle HC et al 1990, Job JS et al 1991, Berry S et al 1992). Indeed, the results were so encouraging that in 1989 the WHO recommended the use of HT vaccines in regions of the world where children younger than the age of routine vaccination suffered significant measles-associated morbidity/mortality (EPI: Wkly Epi Rec 1990). Enthusiasm for these vaccines was quickly tempered, however, when increased mortality was reported in HT recipients in a large trial in Guinea Bissau (Aaby P 1991 personal communication - reported in Halsey NA 1993). This observation triggered an urgent re-evaluation of children who had been enrolled in other HT trials throughout the world. Increased mortality in HT recipients was identified in 3 of the 4 largest studies and a similar trend was noted in some of the smaller studies (Garenne M et al 1991, Aaby P et al 1991, Aaby P et al 1993b, Holt EA et al 1993). The increased mortality in HT recipients had several unusual characteristics: deaths accumulated slowly after vaccination, and the "excess mortality" occurred predominantly in females (Aaby P et al 1993b, Holt EA et al 1993). Although none of the original studies had been designed to rigorously evaluate cause of death over a prolonged follow-up period, the HT recipients appeared to have died from a variety of non-measles causes (eg: diarrhea, malnutrition, malaria) (Garenne M et al 1991, Aaby P et al 1991, Aaby P et al 1993b, Holt EA et al 1993).

Since exposure to both wild-type and vaccine-strain measles viruses can result in a wide range of immunologic abnormalities (Munyer TP et al 1975, Wesley A et al 1978, Hirsch RL et al 1981, Tamashiro VG et al 1987, Griffin DE et al 1990a,b), it was plausible that HT vaccination may have acted as an immunological "insult" (Ward BJ et al 1993b). Studies performed between 2 - 3 years after vaccination found subtle immunologic differences between HT and standard titer vaccine recipients (Leon ME et al 1993, Lisse IM et al 1994). Compared with standard titer vaccine recipients, Lisse et al reported that HT vaccinees in Guinea Bissau had slightly higher $CD8^+$ T cell counts and lower $CD4^+/CD8^+$ ratios and Leon et al found that HT vaccinees in Peru had slightly lower DTH responses, mitogen-induced lymphoproliferation and $CD4^+$ T cell numbers (Leon ME et al 1993, Lisse IM et al 1994). These results prompted us to initiate an immunologic follow-up study of children originally enrolled in a large HT trial in Haiti (Job JS et al 1991).

Materials and Methods

Subjects: The original Haitian HT vaccine trial and the follow-up study documenting increased mortality have been described in detail elsewhere (Job JS et al 1991, Holt EA et al 1993). Briefly, children between the ages of 5 - 11 months were recruited between 1987 - 1988 in Cité de Soleil, a disadvantaged sector of Port-au-Prince subject to high measles attack rates and significant measles-associated morbidity. A total of 1972 children were block randomized to receive one of four vaccines at 5 - 11 months of age: i) Edmonston-Zagreb high titer (EZ HT; $10^{5.6}$) ii) Schwarz high titer (SCH HT; $10^{5.4}$) iii) EZ medium titer (EZ MT; $10^{4.4}$) iv) SCH medium titer (SCH MT; $10^{4.6}$). A fifth group of 371 children recieved standard low titer Schwarz vaccine (SCH LT; $\sim 10^{3.0-3.7}$) at ~ 12 months of age. The initial evaluation of vaccine effectiveness demonstrated excellent seroconversion rates for the HT vaccines (84% and 92% for the EZ and SCH HT vaccines respectively vs 81% for SCH LT) (Job JS et al 1991). A follow-up study performed 2 - 3 years after vaccination suggested excess female mortality following administration of either of the HT vaccines compared with low or medium titer vaccination (overall risk ratio 1.71, 95% confidence interval = 0.91 - 3.24) (Holt EA et al 1993). Similar to the findings in other large HT trials, the deaths in Haitian children had accumulated slowly over the 2 - 3 year period following vaccination and were attributable to a range of non-measles causes. For logistical reasons, the 450 children included in the present study were randomly selected from one of the two recruitment sites used for the inital trial (98 EZ HT, 90 SCH HT, 108 EZ MT, 91 SCH MT; mean age 8.1 years, and 63 SCH LT; mean age 12.2). Based upon socio-economic parameters and anthropometric data collected both at the time of the original study and the immunologic follow-up, these children were representative of the original study population (Table 1).

Evaluation of Delayed-type Hypersensitivity: A commercial multi-test (Institut Merieux, Lyon, France) including a control antigen (glycerin) and seven test antigens

(tetanus toxoid, diphtheria toxoid, proteus, candida, trichophyton, streptococcus, and tuberculin) was applied to the left forearm of each child at the time of recruitment. The induration caused by each antigen was measured 48 hours later by a single member of the research team blinded to the vaccine assignment of the children (RM). Indurations larger than 2 mm were scored as positive. Results are reported either as mm induration size or negative/positive.

Sample Handling: A single heparinized blood sample (1 - 5 mL) was obtained by veni-puncture from each child. All samples were processed within 6 hours of collection. A small aliquot from each sample was used immediately to make blood smears for manual WBC and differential counts. Differential counts were performed by a single member of the research team (BJW) and WBC counts were performed by the hematology technicians of l'Hôspital Ste-Catherine, Port-au-Prince, Haiti. Operators were blinded to group assignment. The remaining blood was centrifuged (300 x g for 10 minutes) and plasma was aliquoted and frozen at -20°C until used in assays. After resuspension in 2 x the original volume of HBSS (Life Technologies, Grand Island, NY) the peripheral blood monuclear cells (PBMC) were isolated using Ficoll-Paque (Pharmacia, Uppsala, Sweden) and counted by trypan blue exclusion. A small aliquot of PBMC (6 x 10^5) was used immediately to determine spontaneous lymphoproliferative activity (see below) and the remainder were re-suspended in freezing media (92.5% fetal bovine serum, Life Technologies; 7.5% DMSO; American Chemicals Ltd, Montreal, QC) aliquoted (~ 3 - 5 x 10^6 /Nunc vial; Nunc, Roskilde, Denmark) and frozen overnight in a methanol bath at -70°C. The following morning the cryopreservation vials were transferred to liquid nitrogen holding tanks. The samples were batched and shipped to North America on dry ice (plasma) or in nitrogen vapour 'dry-shippers' (PBMC).

Antigen Preparations: Measles antigens were prepared as previously described (Ward BJ et al 1995) from Vero cell monolayers infected with vaccine-strain (Edmonston B and Edmonston Enders, gifts from M Hilleman; Merck Research, West Point, PA and R Wittes; Connaught Laboratories LTD, Willodale, ON respectively) or CHI-1 wild-type virus (gift from W Bellini, Atlanta, GA) at a multiplicity of infection of 0.01. Culture supernatants were harvested at 90% cytopathic effect after one freeze-thaw cycle, centrifuged for 10 minutes at 1000 x g and filtered (0.22 μM) (Falcon, Franklin, NJ). The filtrate was then centrifuged (20000 x g) for 2 hours at 4°C, sonicated and the viral pellet was resuspended in PBS. Similarly processed uninfected vero cell lysate antigen served as a control antigen. Antigen concentration was determined by modified Bradford Assay. The other antigens used for ELISA tests and/or lymphoproliferation assays were tetanus toxoid (TT; gift from B Latham, Massachussetts Laboratories, Boston, PA), *Corynebacterium diphtheriae* toxoid (DT), *B. pertussis* whole cell lysate (PT) and *H. influenza* type B unconjugated polysaccharide (HI) (all gift from R Wittes, Connaught Laboratories Ltd).

Spontaneous Proliferation: Spontaneous proliferation of PBMC is a non-specific marker of immune system activation and was measured as previously described (Ward BJ et al 1990). Briefly, PBMC were suspended at 1 x 10^6/mL in cRPMI (cRPMI: RPMI 1640 containing 10 mM HEPES, 50μg/mL gentamicin and glutamine (all Life Technologies) with 5% heat-inactivated fetal bovine serum (FBS, Life Technologies) and 1 μCi of ^{3}H-thymidine (ICN, Radiochemicals, Irvine, CA). Cells were distributed in triplicate in 96-well plates at 200 μL/well. Plates were frozen at -20°C twenty-four hours later and stored until the DNA could be collected on glass-fiber filters for evaluation of ^{3}H-thymidine incorporation. Results are expressed as counts per minute (cpm).

Tests of Humoral Immunity: Measles-specific neutralizing antibodies were measured by the syncytium inhibition assay (SIA) as previously described (Ward BJ et al 1999;

Manuscript I). SIA results are expressed as the reciprocal of the lowest plasma dilution capable of inhibiting syncytium formation in measles-susceptible B95-8 cells (ATCC # 1612, Rockville, MD) upon challenge with a standard virus inoculum. In our assay, a SIA titer of 16 is equivalent to a "protective" PRN value of 120 (Ward BJ et al 1999; Manuscript I). The measles-specific IgG subclass distribution was determined by ELISA using monoclonal antibodies directed against human IgG and IgG_{1-4} (ATCC #1757, 1755, 1788, 1756, 1776 respectively). Subclass results are expressed as percentages of the total optical density observed. Antibody responses to non-measles antigens (TT, DT, PT and HI) were also determied by ELISA. Specific IgG concentrations were estimated by extrapolating from curves generated using international standard sera (anti-TT and anti-DT; National Institute for Biological Standards and Controls, Hertfordshire, UK) or a local standard serum including in each assay. All ELISA tests were performed by coating 96-well plates overnight with antigen (0.5-2 μg/mL) in carbonate-bicarbonate buffer (pH 9.6). After blocking with 5% goat serum (Life Technologies), plasma samples were typically diluted at 1:200 in phosphate buffered saline (PBS) and incubated overnight at 4°C. Assays were completed with mouse monoclonal anti-human IgG or IgG_{1-4} followed by incubations with biotinylated goat anti-mouse IgG (Fab_2; Boehringer Mannheim, Laval, QC) for 1 hour at 37°C, HRP-conjugated streptavidin (Boehringer Mannheim) for 30 minutes at 37°C and ABTS (Boerhinger Mannheim) with 0.4 μL/mL hydrogen peroxide (Sigma, Ottawa, ON) for 20 minutes at room temperature. The optical density was determined by spectrophotometry at 405 nm. Results are expressed as milli-international units (mIU; α-tetanus and α-diphtheria responses) or as optical density values for anti-PT and anti-HI responses. For tetanus and diphtheria, titers > 10 mIU are thought to indicate protection.

Lymphoproliferation Assay: We assessed the lymphoproliferative responses to both vaccine-strain and wild-type measles antigens as well as tetanus toxoid and control

antigens. Lymphoproliferation assays were performed as previously described (Ward BJ et al 1995). Briefly, antigens (10 μg/mL) were pre-coated onto 96-well plates in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C and washed twice with sterile HBSS immediately before use. Cryopreserved PBMC were quick-thawed, washed once in HBSS and resuspended at 1 x 10^6/mL in cRPMI with 5% heat-inactivated autologous plasma (56°C for 30 min). Cells were distributed at 2 x 10^5/well in triplicate for each antigen and incubated for 6 days at 37°C in 5% CO_2 before 1 μCi of ^{3}H-thymidine was added to each well. Control and test antigens were coated unto the same plates to ensure similar culture conditions. Cellular DNA was harvested onto glass-fiber filters 24 hours later after one freeze-thaw cycle and incorporated radioactivity was estimated by beta-emission. Lymphoproliferative results are expressed as stimulation indices (SI = cpm of antigen-stimulated wells/cpm of control wells). A stimulation index (SI) ≥ 3 was considered to indicate a significant response.

PBMC Phenotype and Activation State: Cryopreserved PBMC were quick-thawed, washed with HBSS and resuspended in PBS containing 5% bovine serum albumin (Life Technologies). A panel of 23 FITC-, PE- or PerCP-conjugated monoclonal antibodies (Becton Dickenson, San Jose, CA or Dako, Toronto, ON) (Table 2) was used to stain aliquots of the PBMC for three-color analysis according to manufacturers' recommendations. Labelled cells were washed with ice cold PBS and resuspended in PBS containing 1% paraformaldehyde and .05% sodium azide (American Chemicals). Data was acquired by FACScan (Becton Dickinson) within 24 hours of staining and were analyzed using CellQuest software (Becton Dickinson). Results are expressed as absolute cell counts, ratios (eg: CD4/CD8), percentages of a given PBMC subset expressing a marker (eg: $CD8^+$ T cells expressing CD69), or as mean intensity of staining for constitutively expressed markers (eg: monocyte ICAM-1).

Statistical Analysis:

Results were analysed using Statview™ (Cary, NC) or Statistical Package for Social Sciences (SPSS) software (Cary, NC). The data were first analyzed by individual vaccine group (EZ HT, EZ MT, SCH HT, SCH MT, SCH LT) and by sex. Subsequent analyses pooled the HT vaccine recipients (EZ HT + EZ MT) and all of the lower titer vaccine recipients (SCH HT + SCH MT + SCH LT). Student's t test was used for comparsions of data found to be normally distributed. Otherwise, the Mann Whitney U test was used. The HIV status of these children was determined by ELISA and HIV^+ subjects (n = 31) were removed from the analysis. Due to the limited blood volume collected from each child (1 - 5 ml), not all tests could be performed on all samples.

Results

WBC and Differential Counts. WBC and differential counts were available for 425 and 418 children respectively (Table 3). Female EZ HT recipients had slightly higher monocyte counts than females in other groups ($p < .05$). There were no other significant differences in any parameter between the vaccine groups or between the sexes.

Delayed-type Hypersensitivity. The cutananeous multi-test was placed on 432 children (97%) and 357 children (83%) had the skin test read within 48-72 hours. Overall, 76 (21%) of the 357 children who had a skin test read were anergic. There was no statistical relationship between anergy and sex, vaccine titer or vaccine titer by sex. Most children had positive responses to tetanus (76%) and diphtheria (72%) antigens. Approximately one-half exhibited positive responses to candida antigen (52%) and tuberculin (50%). Fewer children responded to the other antigens. Only 1 child had a positive response to the glycerin control. No statistically significant association was found between sex, vaccine titer or vaccine titer by sex and a positive response, except for candida antigen. More HT than MT or standard titer recipients responded to candida antigen ($p=0.007$) and these differences were most prominent in males (HT males vs. males receiving lower titer vaccines ($p=0.02$)). Of the males and females with positive responses, the greatest mean induration was 10.86 mm (range 2.00 - 26.06 mm) and the mean reactivity index was 2.98 (range 2.00 - 8.69 mm). We found no statistical association between these measures of delayed hypersensitivity and sex, vaccine titer or vaccine titer by sex.

Spontaneous Proliferation. Spontaneous proliferation of PBMC is seen in a wide variety of infectious, inflammatory and neoplastic conditions. Spontaneous proliferation data was available for 284 children evenly distributed between the vaccine groups (Figure 1). The range in spontaneous proliferation was 16 - 1130 cpm (mean; 277± 12) There were

no consistent differences between the high and lower titer recipients or between sexes within each vaccine subgroup. Analysis of pooled data suggested that the HT females had slightly lower spontaneous proliferation than the HT males (226 ± 28 cpm vs. 303 ± 29 cpm; $p < .02$) (Figure 1).

Measles-specific Antibody Responses. As noted above, measles seroconversion rates determined 18 - 24 months after enrollment in the initial vaccination trail exceeded 85% in all groups. Although the excess mortality in the HT recipients was attributed to non-measles causes, natural measles is often modified in subjects who are partially immune and can be difficult to recognize clinically. We measured measles-specific neutralizing antibody titers by syncytium inhibition in 431 children. The mean titer of neutralizing antibodies in these children was 125 ± 10 and all, (100 %), of the study children had antibody levels thought to be protective (SIA ≥ 16) (Figure 2). The lowest titers were observed in the standard titer recipients (SIA 71 ± 17) largely as a result of very low titers in males (SIA 44 ± 12 vs. female LT recipients 103 ± 33; $p = .08$). There was no difference between the SIA values of pooled HT vs. LT recipients (192 ± 15 vs. 133 ± 12; $p = .37$ respectively) (Figure 2). These findings strongly suggest that wild-type measles was ciruculating in the study community in the 4 years between the initial vaccine trial and the follow-up immunologic study.

Since IgG subclass distribution can give insight into the quality of the immune response to vaccine antigens, we measured measles-specific IgG_{1-4} in a subset of the children (n = 219). All groups showed the same pattern of IgG subclass distribution with a preponderance of measles-specific IgG_1 (72-79%), lesser amounts of IgG_3 (10-20%) and barely detectable amounts of IgG_2 (3-5%) and IgG_4 (1.5-4%) (Figure 3). Overall, the pooled female HT recipients had significantly more measles-specific IgG_3 and less IgG_4 than LT recipients (IgG_3: 20% ± 4% vs. 10% ± 2%, $p < .03$ and IgG_4: 3% ± .9% vs. 6 ±

.9%, $p < .01$, respectively). There were no other differences between the vaccine groups or between the sexes.

Measles-Specific Cellular Responses. A growing body of epidemiologic and laboratory evidence supports the contention that cellular responses to measles virus infection are essential for viral clearance and sufficient for the maintenance of long-term immunity (Griffin DE & Bellini WJ 1996). We measured measles-specific lympho-proliferative responses to wild-type (CHI-1) and both partially and fully attenuated vaccine-strain measles antigens (Edmonston B and Edmonston Enders respectively) in a subset of 119 children evenly distributed across the vaccine groups. The overall SI to the CHI-1 antigen was 4.9 ± 1.9 and 19% of the study children had $SI \geq 3$. Responses to wild-type and vaccine-strain antigens were very similar (data not shown) and there were no significant differences between children grouped either according to vaccine titer or sex (Table 4).

Humoral and Cellular Responses to Non-Measles Pathogens. Since the excess mortality associated with HT vaccination was thought to be the result of infection with non-measles pathogens, we assessed humoral and cellular responses to several common pathogens. Antibodies for TT, DT, PT, and HI were measured in 392 children and lymphoproliferative responses to TT were measured in 112 children. The overall mean antibody titers/values were 32.5 ± 3.7 mIU (TT), 6.8 ± 1 mIU (DT), 1220 ± 412 OD (PT) units and HI 840 ± 355 OD units (HI) (Data not shown). There were no differences between children grouped either by vaccine titer or sex. The lymphoproliferative response to TT was low overall (S.I. $= 2.7 \pm .7$) and no differences were found between children grouped according to vaccine titer or sex (Table 4).

FACS Analysis of PBMC Phenotype and Activation State. As noted above, two prior studies demonstrated subtle changes in either absolute or relative T lymphocyte

numbers, 2 - 3 years after HT vaccination (Leon ME et al 1993, Lisse IM et al 1994). We performed an expanded analysis of PBMC phenotype with samples from 125 children, focussing primarily on activation and co-stimulatory molecule expression (Table 1). Overall, PBMC ($CD4^+$, $CD8^+$ T lymphocytes, B lymphocytes, natural killer cells, monocytes) percentages, and $CD4^+/CD8^+$ ratios were within the expected ranges for developing world children. Although several minor differences were found when the children were grouped by vaccine type, vaccine titer or sex, all of the differences were of borderline significance and there was no pattern of 'abnormality' in any group (Table 4). For example $CD8^+$ expression of CD69 and CD75, NK expression of CD69 and B cell expression of CD23 were all slightly reduced in pooled HT females compared with the pooled LT recipients ($p < 0.03$, $p < 0.05$, $p < 0.01$ and $p < 0.06$ respectively, data not shown), but the opposite trend was observed in males.

Discussion

The mortality associated with HT measles vaccination has proved to be enigmatic. The fact that such mortality occurred is difficult to dispute since an effect was seen in three of the four largest trials (Aaby P et al 1993b, Aaby P et al 1993c, Holt EA et al 1993), and a similar trend was observed in several of the smaller studies (Diaz-Ortega JL et al 1992, Hoskins EW 1993). Although only the HT trial in the Sudan included long-term morbidity and mortality as an original study outcome (Hoskins EW 1993), the *post-facto* meta-analysis performed by Knudsen and Fine is unambiguous in its conclusions: (i) that increased mortality occurred in children who received a HT vaccine in areas with high background infant mortality rates overall (RR = 1.23; p = 0.05) and girls were more affected than boys (RR = 1.95; p = 0.027) (Knudsen KM et al 1996). The "excess" mortality accumulated slowly over a period of 2 - 3 years after vaccination and deaths appeared to have been caused by a wide variety of non-measles infectious agents (Aaby P et al 1994b, Holt EA et al 1993).

Serious vaccine-associated adverse events are very rare and the vast majority are thought to occur within a short period of time after vaccination (eg: anaphylaxis) (Stratton KR et al 1994). However, very late complications can certainly occur after some vaccines. Examples of late events for which causality has been clearly established include deaths from live attenuated organisms in immunocompromised hosts many months after vaccine exposure (eg: measles, polio, BCG) (Stratton KR et al 1994, Cohen AD & Shoenfeld Y 1996, Nieminen U et al 1993) and atypical or enhanced pathology upon exposure to wild-type organisms many years after vaccination (e.g. measles, RSV) (Rauh LW & Schmidt R 1965, Rod T et al 1970, Brodsky AL 1972). Although a small number of investigators believe that certain chronic illnesses (eg: diabetes, autism, multiple sclerosis, Crohns Disease) (Cosby SL et al 1989, Singh VK et al 1998, Wakefield AJ et al 1993) are also late complications of individual vaccines or patterns of vaccination, there is to date, no conclusive proof for any such association. The excess mortality following HT measles

vaccine exposure does not appear to have been due to either measles virus infection or enhanced pathology following exposure to wild-type virus (Halsey NA 1993). As a result, more subtle explanations were sought: for example that exposure to HT formulations had somehow damaged the immune systems of the vaccine recipients reducing their capacity to respond to subsequent (non-measles) challenges (Ward BJ et al 1993b). Both wild-type and vaccine-strain measles have long been known as potent modulators of immune cell function (Starr S & Berkovich S 1964, Nakayama T et al 1988, Ward BJ et al 1991, Hussey GD et al 1996, Karp CL et al 1996). Aaby and co-workers have reported delayed and poorly understood "excess mortality" following exposure to natural measles virus early in life and even *in utero* (Aaby P et al 1993a, Aaby P et al 1990a,c). The biologic plausibility of the observed HT effect is further supported by the observation that mortality after natural measles appears to be greater in females than males in a wide range of societies (Garenne M 1994a).

Early field evaluations of the immunologic damage hypothesis were tantalizing, but inconclusive. Auwater et al found no gross immunologic damage after HT vaccination in South African children but described striking changes in Vβ T cell receptor usage (Auwaerter PG et al 1996a). Although alterations in Vβ usage could plausibly influence long-term immune response capabilities (ie: a superantigen-like effect), T cell receptor profiles returned to normal within 3 - 4 months of vaccination in this study (Auwaerter PG et al 1996a); an effect too transient to explain the slow accumulation of deaths for 2 - 3 years after HT vaccine exposure. An immunologic survey performed ~ 2 years after vaccine exposure demonstrated that Peruvian HT recipients had decreased proliferative responses to PHA, decreased reaction to cutaneous recall antigens, and lower percentages of $CD4^+$ T cells compared with standard vaccine recipients (Leon ME et al 1993). A similar survey of immunologic parameters in Guinea Bissau 3 - 4 years after vaccination found small but significant differences in $CD4^+$ T cell percentages and $CD4^+/CD8^+$ ratios between HT and standard titer recipients (Lisse IM et al 1994).

Subsequent evaluations of children exposed to the HT vaccines in the Senegal and Guinea Bissau studies have found no immunological differences between HT and standard titer recipients ~ 4 years after vaccination (Samb B et al 1995b, Aaby P et al 1996). The present work extends these reassuring immunologic findings to the Haitian HT vaccine recipients who were recruited between 4 and 5 years post-vaccination. Although a small number of borderline differences were found between males and females and between high and low titer vaccine recipients, a large number of immunologic parameters was measured (n = 66) suggesting that these observations may have occurred by chance alone. Furthermore, there was no consistent overall pattern of immunologic difference between high and lower titer recipients in the current study (or males vs females). Similar, essentially negative, immunologic findings have also recently been reported for Sudanese children studies 4 - 5 years after HT vaccine exposure (Bertley FMN et al Manuscript III). Even in aggregate, these immunologic follow-up studies cannot reliably distinguish between dissipation of a general immunologic effect over time and a "survivor" effect (ie: all of those adversely affected by the HT vaccines have died by 4 - 5 years after vaccination). On the one hand, the changes in Vβ receptor usage were a consistent feature of HT vaccination in South African children (Auwaerter PG et al 1996a) and the immunologic differences observed in HT recipients from Peru and Guinea Bissau, 2 - 3 years after HT exposure were not the result of skewing by a small number of outliers (ie: supporting a small but general effect of HT vaccination) (Leon ME et al 1993, Lisse IM et al 1994). On the other hand, a recent analysis of ~ 40,000 prospective follow-up visits in the Sudanese trial demonstrates that exposure to HT vaccination had no measurable impact on several general measures of morbidity such as episodes of fever or diarrhea (ie: supporting an idiosyncratic effect of HT vaccination) (Libman M et al Manuscript IV). The Sudanese trial had a relatively small number of particitpants however (n = 510), and there was no definitive evidence of an increase in mortality in HT recipients (unpublished

results). Furthermore, the measles vaccines used in this trial were at the lower limit of those classified as high titer ($10^{4.7}$) (Hoskins EW 1993).

HT measles vaccines were introduced in the late 1980's to overcome the twin problems of neutralizing maternal antibodies and high measles mortality in very young children (Halsey NA 1993). At that time, more than a million children were dying every year from measles. A little more that a decade later, the situation is beginning to change favorably. Second dose strategies and mass vaccination campaigns have had a huge impact on measles incidence in many parts of the world (Davidkin I & Valle M 1998, Mansoor O et al 1998). Antibody titers in women of child-bearing age are declining in both the developed and the developing world due to increased vaccine coverage and decreased wild-type virus circulation (ie: decreased natural boosting) (Pabst HF et al 1999a). Many authorities now believe that measles eradication is possible with the currently available vaccines and the year 2005 has been set as a goal by the EPI. As a result, there is relatively little pressure to introduce new measles vaccines especially while medium titer formulations appear to have an excellent record in terms of both efficacy and safety in young children (Job JS et al 1991, Markowitz LE 1990b, Hoskins EW 1993, Nkrumah FK et al 1998, Pabst HF et al 1999a,b). Given that the effect of HT measles vaccines appears to have dissipated and the associated "excess" mortality has stopped, there is also little pressure to fully understand the mechanism(s) by which these vaccines had their deleterious effect. Although this situation may be unavoidable in the absence of a low-cost animal model for measles (Griffin DE & Bellini WJ 1996), as a large number of closely related viruses are currently targeted for human and veterinary vaccine development (e.g. parainfluenza 1 - 4, respiratory syncytial virus, and human, porcine and equine morbilliviruses) (Redd SC et al 1999). Full support for the drive to eradicate measles and enhanced vigilance for similar effects with new vaccines directed against other paramyxoviridae may be the only options open for the near future.

Table 1 - Comparison of Haitian Children Included or Excluded in 1993 Immunologic Follow-Up Study

	Original Participants Not Included	Original Participants Included	P value
% Male % Female	47.3% (n=247) 51.5% (n=269)	50.7% (n=228) 49.3% (n=222)	
Age at Vaccination (months)	8.6 ± 2.0* (5.8 - 19.4)	8.8 ± 2.4 (5.9 - 23.4)	0.15
Weight at Vaccination (grams)	7489.9 ± 1203.7 (4000 - 11 000)	7624.7 ± 1253.3 (3800 - 16 400)	0.12
Crowding (mean # of persons per room/house)	4.7 ± 2.0 (0.10 - 15.0)	4.83 ± 2.0 (0 - 12)	0.37
% with cement floors in home	96.4%	97.2%	

*mean ± SD (range).

Table 2 - Antibodies Used for FACS Analysis: Cell Type and CD Designation

Cell Type	Surface Marker	Function
CD4+ T cell	CD45RA	Naive T cells
	CD45RO	Memory T cells
	CD45RB	Low on mature T cells
	CD30	Possible marker for Th-2 phenotype
	CD25	Low affinity IL-2R
	CD75	High affinity IL-2R
	CD28	Co-stimulatory molecule
	HLA-DR	MHC II
	LFA-1	Integrin adhesion molecule
	CD71	Transferrin receptor
CD8+ T cells	CD25; CD75; HLA-DR; LFA-1	As above
	CD69	Early activation marker
	CD38	Early activation marker
	CD71	Transferrin receptor (activation marker)
B cells (CD19)	CD80	Ligand for CD28/CTLA-4
	CD86	Ligand for CD28/CTLA-4
	CD23	Activation marker
	CD25	As above
	CD38	As above
NK cells (CD16)	CD69	As above
Macrophages (CD14)	CD80	As above
	CD86	As above
	CD54	Intercellular adhesion molecule (ICAM-1)

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Table 3 - WBC and Differential Counts

	All		Female		Male	
	LT	HT	LT	HT	LT	HT
WBC	6498±131	6317±167	6207±194	6380±222	6677±178	6234±244
(n)	(247)	(178)	(111)	(80)	(136)	(98)
%Neutrophils	36.7±.7	37.9±.96	35.3±1.1	37.7±1.5	37.9±1.1	38.2±1.2
(n)	(241)	(177)	(108)	(80)	(133)	(97)
%Lymphocytes	43.4±.9	42.6±1.1	45.5±1.2	43.3±1.7	41.8±1.3	42.0±1.4
%Eosinophils	10.7±.6	10.4±.6	10.4±.8	10.1±.9	11.0±.8	10.6±.8
%Basophils	.16±.03	.11± .03	.20±.06	.10±.04	.13±.03	.11±.04
%Monocytes	6.4±.2	6.7±.4	6.1±.4	6.3±.3	6.5±.3	7.2±.5
%Atypical lymphocytes	7.9±.3	7.7±.4	8.0±.5	8.5±.7	7.8±.5	6.9±.5

WBC and differential counts were carried out by individuals blinded to the specimen origin. Results are expressed as mean ± SEM.

Table 4 - Lymphoproliferative Responses to Measles and Tetanus Toxoid Antigens

	All		Female		Male	
	LT	HT	LT	HT	LT	HT
Antigen						
CHI	(71) 4.2±1.3	(48) 5.7±2.6	(43) 5.1±2.1	(28) 4.1±1.7	(28) 2.9±.8	(20) 8.1±5.8
TT	(68) 2.9±.8	(44) 2.5±.6	(40) 4.0±1.3	(27) 2.4±.7	(28) 1.3±.1	(17) 2.7±1.2

Lymphoproliferation to measles antigen (CHI-1: n = 119) tetanus antigen (TT: n = 112) by a solid-phase LPA assay. Results are expressed as stimulation indeces (SI) (mean ± SEM). SI > 3 is thought to indicate a significant response to the test antigen.

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Table 5 - PBMC Subsets by Vaccine Titer and Gender.

	All		Female		Male	
	LT (78)	HT (47)	LT (38)	HT (23)	LT (40)	HT (24)
% CD4+	23.2±1.6	20.5±2.4	22.0±2.5	21.0±2.2	23.4±2.2	25.0±2.6
%CD8+	27.8±1.7	25.4±2.5	27.9±2.0	30.4±2.6	27.7±2.7	27.0±2.1
% B-Cell	26.1±1.4	27.3±1.7	29.1±2.0	28.8±2.0	24.2±1.9	24.8±1.9
% NK	10.2±1.1	10.6±1.2	9.5±.9	9.4±.7	10.7±.9	11.1±1.0
% Monocyte	9.2±1.0	9.4±.9	8.6±.7	9.2±.8	10.1±1.1	9.7±.9
CD4/CD8	.86±.08	.8±.1	.8±.1	.7±.1	.9±.1	.9±.11

PBMC subsets (CD4+, CD8+, B-cell, NK and monocytes) and CD4/CD8 ratios were measured by flow cytometry in 125 children. Results are expressed as mean ± SEM percentage of total PBMC population or ratio between populations

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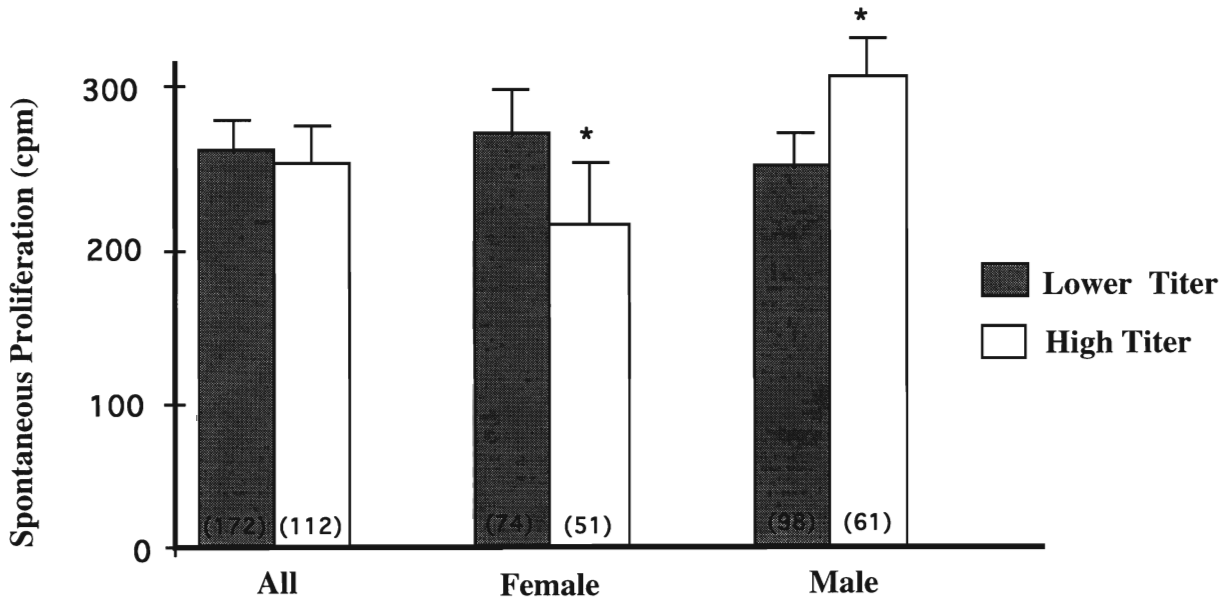
Figure 1 - Spontaneous Proliferation



Spontaneous proliferation of PBMC isolated from 284 children. Results are expressed as mean counts per minute (cpm) of ^{3}H-thymidine incorporation $\pm$ SEM. * $p < 0.02$, female vs. male.

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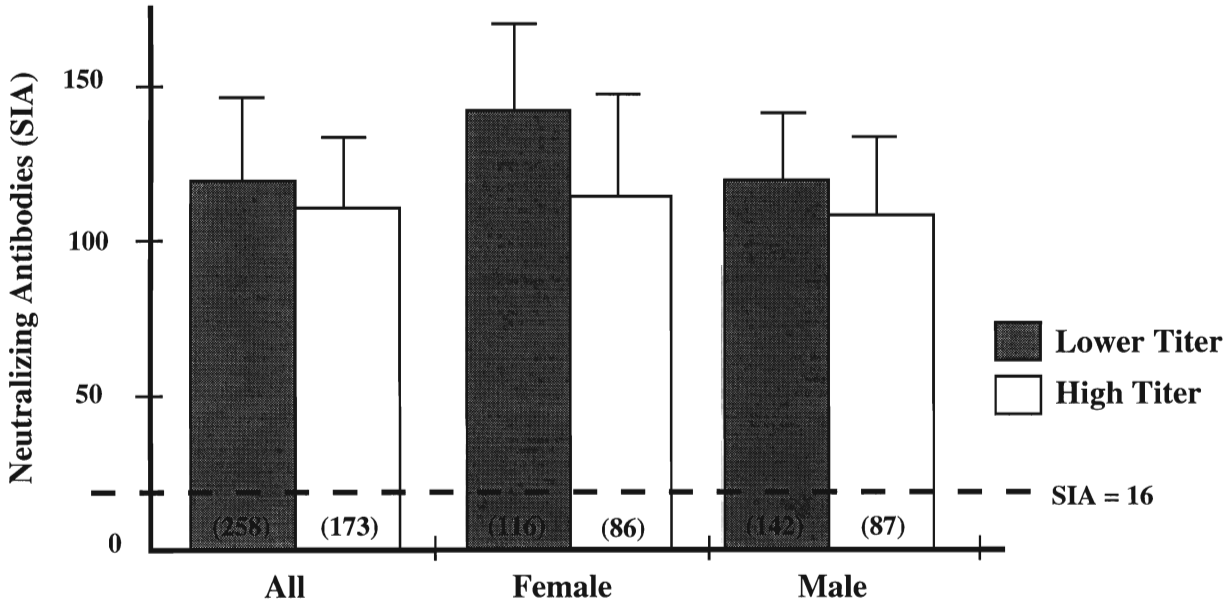
Figure 2 - Humoral Response: Measles Neutralizing Antibodies



Measles neutralizing antibodies measured by Syncytium Inhibition Assay (SIA) in 431 children. SIA values > 16 are thought to indicate protection from infection.

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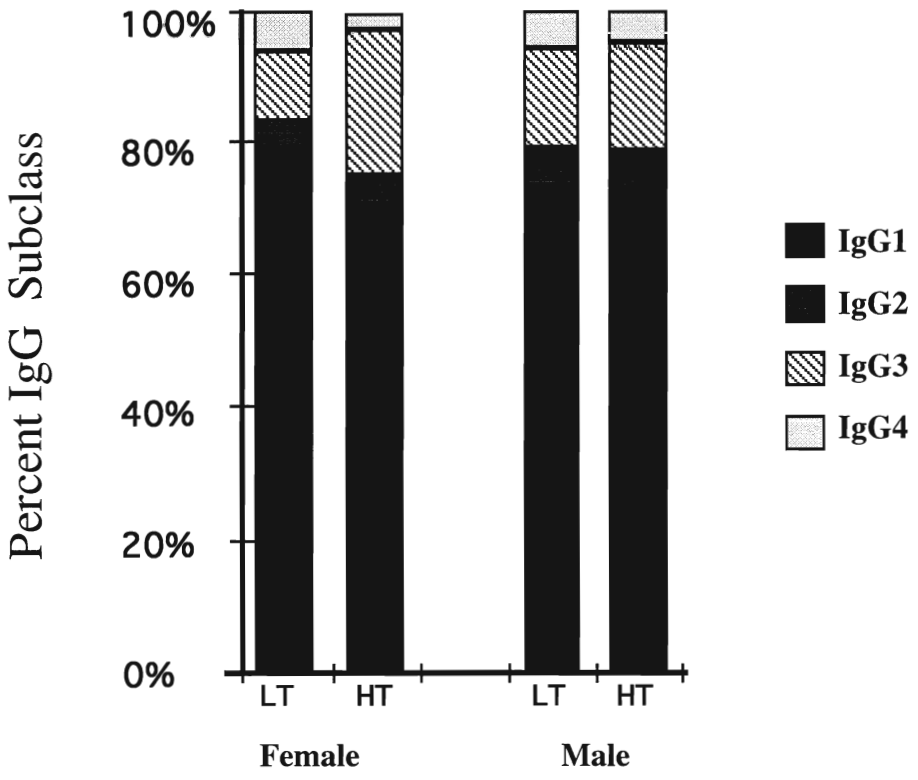
Figure 3 - Humoral Response: Measles-Specific Antibody Subclass Distribution



Measles specific IgG subclass antibodies were detected by ELISA using mouse-anti-human monoclonal IgG_{1-4}, in 219 children. Data are presented as percentages (%) of total measles specific antibody.

4.1.2. MANUSCRIPT III

Absence of Immunologic Injury Following High Titer Measles Vaccination in the Sudan.

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Running Head: High titer measles vaccine: Sudanese follow-up
Key Words: Measles, measles vaccine, mortality, vaccine-associated adverse event

Abstract

Background: High Titer (HT) measles vaccines are associated with poorly understood excess mortality and subtle immunologic changes that can be detected for 2 - 3 years after vaccination.

Methods: We performed a late immunologic follow-up study on samples obtained from 193 children who had received either HT Connaught ($10^{4.7}$ pfu/dose), HT Edmonston Zagreb ($10^{4.7}$ pfu/dose) or standard Schwarz measles vaccine ($10^{3.9}$ pfu/dose) in a randomized, placebo controlled trial of measles HT vaccination in the Sudan. The immunologic tests were performed ~ 5 years after vaccination and included flow cytometry (e.g. phenotypic description, activation marker and co-stimulatory molecule expression), measures of measles-specific immunity (e.g. neutralizing antibodies and lympho-proliferation to measles antigens) and measures of immunity to non-measles pathogens (e.g. antibody titers for tetanus, diptheria, *H. infuenza* and *B. pertussis* and lymphoproliferation to tetanus toxoid).

Results. No immunologic differences between children grouped by gender, vaccine strain or vaccine titer were identified.

Conclusions. There was no evidence of immunologic injury ~ 5 years after HT measles vaccination in the Sudan.

Introduction

In an attempt to reduce measles-associated morbidity and mortality in children less than 9 months of age, vaccines containing 10 - 100 fold more viral particles than standard vaccines (e.g. $10^{4.4-5.7}$pfu vs, standard titer at $10^{3-3.7}$pfu) were tested in the late 1980's (Tidjani O et al 1989, Markowitz LE et al 1990b, Job JS et al 1991, Berry S et al 1992). It was reasoned that high titers of the standard vaccine-strain viruses might effectively induce seroconversion in the presence of maternal antibodies (Halsey NA 1993). Preliminary results from HT measles vaccine trials were very encouraging. The vaccines achieved seroconversion rates $\geq$ 80% in children as young as 3 months of age (Markowitz LE 1990b, Job JS et al 1991, Berry S et al 1992, Tidjani O et al 1989, Whittle HC et al 1990). In 1989, the success of the initial trials prompted the WHO to recommend the use of HT vaccines in regions of the world where children suffered significant morbidity and mortality before the age of routine vaccination (EPI: Wkly Epi Rec 1990). Concern over the use of these vaccines arose, however, when Aaby et al reported increased mortality in HT recipients from Guinea Bissau (Aaby P 1991 - personal communication, reported in Halsey NA 1993). This finding forced investigators to re-assess the possibility of late adverse effects in other HT trials throughout the world. The initial observation was supported when similar increases in delayed mortality were identified in HT recipients enrolled in 3 of the 4 largest studies (Aaby P et al 1993b, Aaby P et al 1993c, Holt EA et al 1993). The increased mortality in HT recipients had unusual characteristics: deaths accumulated slowly after vaccination and the "excess mortality" occurred predominantly in females (Aaby P et al 1994b, Holt EA et al 1993). Futhermore, the HT recipients appeared to have died from a variety of non-measles causes (eg: diarrhea, malnutrition, malaria) (Garenne M 1991, Holt EA et al 1993, Aaby P et al 1994).

Initial re-evaluations of children enrolled in these studies provided no satisfactory explanation for the excess mortality, but raised the possibility that the HT measles vaccines had somehow acted as an immunologic insult, leaving HT vaccine recipients susceptible to

secondary infections (Ward BJ et al 1993b). This hypothesis was biologically plausible because of the established wide range of immunologic abnormalities associated both with natural disease and (to a lesser extent) standard titer measles vaccination (Fireman P et al 1964, Hirsch RL et al 1981, Munyer TP et al 1975, Wesley A et al 1978, Tamashiro VG et al 1987, Griffin DE et al 1990a,b, Ward BJ et al 1991). Immunologic studies performed between 2 - 3 years after vaccination found subtle differences between HT and standard titer vaccine recipients including: (i) higher $CD8^+$ T cell counts and lower $CD4^+/CD8^+$ ratios, (ii) lower $CD4^+$ T cell numbers and (iii) lower DTH responses and mitogen-induced lymphoproliferation (Leon ME et al 1993, Lisse IM et al 1994). We report herein the findings of a late immunologic follow-up study of children who participated in a randomized, placebo controlled trial of HT measles vaccines initiated in the Sudan in 1989.

Materials and Methods

Study design. The original HT measles vaccine trial was carried out in 14 villages in the Umdawanban and Essailat regions neighboring Khartoum, Sudan (Hoskins EW 1993). Children (n = 510) born between January 1 and November 1, 1989 were block randomized to receive either HT Connaught (CN; Edmonston-Enders strain, $10^{4.7}$ plaque forming units (pfu)/dose, Connaught Ltd, Mississauga, ON), Edmonston-Zagreb vaccine (EZ; $10^{4.7}$ pfu/dose, Institute of Immunology, Zagreb, Yugoslavia) or placebo (Mg; meningococcal A + C vaccine, SmithKline Beecham Pharma, Oakville, ON) at 5 months of age. The CN and Mg vaccine recipients were revaccinated at 9 months of age with Schwarz standard titer vaccine (SCH: $10^{3.9}$ pfu/dose). Children who received EZ at 5 months received placebo (Mg vaccine) at 9 months of age. In 1994, 193 children (mean age 5.1 years; range 4.6 - 5.6) were recaptured for the current immunologic follow-up study from 6 of the original 14 villages. There were no socio-economic, environmental or nutritional status differences between the villages included or excluded from this follow-up study (Hoskins EW 1993). The children represent 69% of the children from the 6 villages enrolled in the original vaccine trial (193/280) and all vaccine groups were well represented (Table 1).

Sample Handling. After informed consent was obtained from the parents, a single heparinized blood sample (1 - 6 mL) was obtained from each child by venipuncture. Samples were processed within 6 hours of collection at the University of Khartoum, Khartoum, Sudan. Blood samples were centrifuged at 300 x g for 10 minutes and plasma was aliquotted and frozen at -70°C until used in assays. Peripheral blood mononuclear cells were isolated using Ficoll-Paque (Pharmacia, Uppsala, Sweden), re-suspended in aliquots in freezing media (92.5% heat-inactivated fetal bovine serum (FBS), Life Technologies, Grand Island, NY + 7.5% DMSO; American Chemicals Ltd, Montreal, QC) and frozen overnight in a methanol bath at -70°C. The following morning the

cryopreservation vials (Nunc, Roskilde, Denmark) were transferred to liquid nitrogen holding tanks. Specimens were transported to Canada in a nitrogen vapour "dry-shipper", where all of the immunologic assays were performed.

Antigen Preparation/Source. The following antigens were used for ELISA tests and/or lymphoproliferation assays: tetanus toxoid (TT; gift from B Latham, Massachussetts Laboratories, Boston, MA), *Corynebacterium diphtheriae* toxoid (DT), *Bordatella pertussis* whole cell lysate (PT) and *Haemophilus influenza* type B unconjugated polysaccharide (HI) (all gifts from R Wittes, Connaught Laboratories Ltd). Measles antigens were prepared as previously described (Ward BJ et al 1995) from Vero cell monolayers infected with vaccine-strain (Edmonston B; (EdB), gift from M. Hilleman, Merck Research, West Point, PA and Edmonston-Enders; (CN), Connaught Laboratories Ltd) or CHI-1 wild-type virus (gift from W Bellini, Atlanta, GA) at a multiplicity of infection of 0.01. Mock-infected Vero cells were cultured to provide a negative control. Culture supernatants were harvested at 90% cytopathic effect after one freeze-thaw cycle, centrifuged for 10 minutes at 500 x g and filtered (0.22 μM) (Falcon, Franklin, NJ). Filtrates were pooled and centrifuged (20000 x g) for 2 hours at 4°C and the viral pellet was lightly sonicated on ice and resuspended in PBS. Antigen concentration was determined by modified Bradford Assay (Biorad, Hercules, CA).

Tests of Humoral Immunity. Measles-specific neutralizing antibodies were measured by the syncytium inhibition assay (SIA) as previously described (Ward BJ et al 1999, Manuscript I). SIA results are expressed as the reciprocal of the lowest plasma dilution capable of inhibiting syncytium formation in measles-susceptible B95-8 cells (ATCC# 1612, Rockville, MD) upon challenge with a standard virus inoculum. In our assay, an SIA titer $\geq$ 16 is equivalent to a "protective" PRN value of 120 (Ward BJ et al 1999, Manuscript I). Antibody responses to non-measles antigens (TT, DT, PT and HI) were

determied by ELISA. Specific IgG concentrations were estimated by extrapolating from curves generated using international standard sera (anti-TT and anti-DT, National Institute for Biological Standards and Controls - World Health Organization for Biological Standards (NIBSC-WHO), Hertfordshire, UK) or local standard sera for PT and HI. All ELISA tests were performed by coating 96-well plates overnight with antigen (0.5-2 μg/mL) in carbonate-bicarbonate buffer (pH 9.6). After blocking with 5% goat serum (Life Technologies), plasma samples were typically diluted 1:200 in phosphate buffered saline (PBS) and incubated overnight at 4°C. Assays were completed with mouse monoclonal anti-human IgG (ATCC# 1757, Rockville, MD), followed by incubations with biotinylated goat anti-mouse IgG (Fab_2; Boehringer Mannheim, Laval, QC) for 1 hour at 37°C, HRP-conjugated streptavidin (Boehringer Mannheim) for 30 minutes at 37°C and ABTS (Boerhinger Mannheim) with 0.4μL/mL hydrogen peroxide (Sigma, Ottawa, ON) for 20 minutes at room temperature. The optical density (OD) was determined by spectrophotometry (Titertek Multiskan® MCC/340, Flow Laboratories, Mississauga, ON) at 405 nm. Results are expressed as milli-international units (mIU) for anti-tetanus and anti-diphtheria responses or as arbitrary OD units against local standards for anti-PT and anti-HI responses. For tetanus and diphtheria, titers > 10 mIU are thought to indicate protection.

Lymphoproliferation Assay. We assessed the lymphoproliferative responses to wild-type (CHI-1) and vaccine strain (CN and EdB) measles antigen as well as tetanus toxoid and control (Vero) antigens. Lymphoproliferation assays were performed as previously described (Ward BJ et al 1995). Briefly, antigens (10 μg/mL) were pre-coated onto 96-well plates in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C and washed twice with sterile HBSS (Life Technologies) immediately before use. Cryopreserved PBMC were quick-thawed, washed once in HBSS and resuspended at 1 x 10^6/mL in cRPMI (cRPMI: RPMI 1640 containing 10 mM HEPES, 50μg/mL gentamicin and glutamine (Life

Technologies) with 5% heat-inactivated autologous plasma (56°C for 30 min). Cells were distributed at 2 x 10^5/well in triplicate for each antigen and incubated for 6 days at 37°C in 5% CO_2 before 1 μCi of ^{3}H-thymidine (ICN, Radiochemicals, Irvine, CA) was added to each well. Control and test antigens were coated onto the same plates to ensure similar culture conditions. Cellular DNA was harvested onto glass-fiber filters 24 hours later after one freeze-thaw cycle and incorporated radioactivity was estimated by beta-emission. Lymphoproliferative results are expressed as stimulation indices (SI = cpm of antigen-stimulated wells/cpm of control wells). A stimulation index (SI) ≥ 3 was considered to indicate a significant response.

PBMC Phenotype and Activation State. Cryopreserved PBMC were quick-thawed, washed with HBSS and resuspended in PBS containing 5% bovine serum albumin (Life Technologies). A panel of 23 FITC-, PE- or PerCP-conjugated monoclonal antibodies (Becton Dickenson, San Jose, CA or Dako, Toronto, ON) (Table 2) was used to stain aliquots of the PBMC for three-color analysis according to manufacturers' recommendations. Labelled cells were washed with ice cold PBS and resuspended in PBS containing 1% paraformaldehyde and .05% sodium azide (American Chemicals). Data were acquired by FACScan (Becton Dickinson) within 24 hours of staining and were analyzed using CellQuest software (Becton Dickinson). Results are expressed as absolute cell counts, ratios (e.g. CD4/CD8), percentages of a given PBMC subset expressing a marker (e.g. $CD8^+$ T cells expressing CD69), or as mean intensity of staining for constitutively expressed markers (e.g. monocyte ICAM-1).

Statistical Analysis. Results were analysed using Statview™ software (Cary, NC). The data were first analyzed by individual vaccine group (Mg-SCH, EZ-Mg, CN-SCH) and by sex, then HT recipients were pooled (CN-SCH + EZ-Mg). Student's t test (two-tailed) was used for comparsions of data found to be normally distributed otherwise, the

Mann Whitney U test was used. Results are expressed throughout as mean ± standard error of the mean (SEM).

Results

Measles-Specific Neutralizing Antibodies. Neutralizing antibody titers were excellent (mean SIA = 248.0 ± 24.4; range 16 - 2565). All 187/193 children tested (6 plasma samples were unusable) had titers thought to indicate "protection" (SIA ≥ 16) (Table 3). Control children (Mg-SCH; Mg at 5 months - SCH at 9 months) tended to have higher neutralizing antibody titers (309.8 ± 55.7) than either the EZ-Mg group (210.2 ± 36.0) or the CN-SCH group (231.4 ± 31.5). Although these differences did not reach the $p \leq 0.05$ level, the difference in SIA between control and pooled HT vaccine recipients (EZ-Mg + CN-SCH) approached significance ($p = 0.08$). When the children were grouped by sex and vaccine assignment, control females were found to have significantly higher levels of neutralizing antibodies than both male controls (432.2 ± 101.8 vs. 183.2 ± 29.0; $p \leq 0.03$) and female HT (EZ-Mg + CN-SCH) recipients (199.7 ± 30.1; $p \leq 0.008$) (Table 3).

Measles-Specific Lymphoproliferation. Sufficient cells were available to perform lymphoproliferative assays on samples from 128 children. Lymphoproliferative responses to measles antigen were limited overall (mean SI to wild-type antigen; 1.8 ± .1) and significant responses (SI ≥ 3) were observed in only 19/124 children (15%) (SI = 4.5 ± .3) (Figure 1). Responses to vaccine strain antigen tended to be higher than those for wild-type antigen preparations (mean SI: CHI-1 = 1.8 ± .1, CN = 2.8 ± .4, EdB = 2.2 ± .5), but these differences did not reach statistical significance. Overall, lymphoproliferative responses tended to be highest in the CN-SCH group that had received two doses of measles vaccine (eg: 3.1 ± .7 vs. 1.1 ± .2 for EZ-Mg, in response to EdB antigen; $p < 0.005$). There were no other differences in measles-specific responses between vaccine groups.

Antibody Levels to Non-Measles Antigens. Since the excess mortality observed in the HT trials was attributed to a range of non-measles, infectious causes, we assessed IgG levels for tetanus toxoid, diphtheria toxoid, *B. pertussis* and *H. influenza.* Specific IgG against the four test organisms was readily detected by ELISA in all children (Table 4). Although no differences were found between vaccine groups, small differences (e.g. PT antibodies in EZ-Mg and CN-SCH groups) were identified between sexes. Titers for tetanus and diphtheria (for which international standard sera were available) were above the levels thought to confer protection (10 mIU).

Lymphoproliferation to Non-Measles Antigens. Results for lymphoproliferation to TT antigen were available for 120 children and were higher than responses to measles antigens overall (SI 3.9 ± .6). Almost 30% of the children (in all vaccine groups) had SI ≥ 3 (Figure 1). There were no significant differences when vaccine recipients were grouped by vaccine or sex.

Flow Cytometric Analysis. Sufficient cells were available to perform flow cytometric analysis on samples from 138 children. Overall, $CD4^+$, $CD8^+$ T lymphocyte percentages, absolute T cell counts and $CD4^+/CD8^+$ ratios were within the expected ranges for developing world children (Table 5). Although there were no differences between control children (Mg-SCH) and HT recipients overall, small differences were identified when the children were grouped by vaccine or sex. For example, $CD4^+$ expression of the transferrin receptor (CD71) was elevated in female Mg-SCH recipients compared to males in the same group ($p = 0.04$) and $CD8^+CD38^+$ cells were slightly reduced in pooled HT females compared with standard titer recipients ($p = 0.05$) (Data not shown). However, there was no consitant pattern of differences between the groups and, given the large number of comparisons performed ($n = 26$), the differences observed may be due to chance alone.

Discussion

Measles is still responsible for ~ 1 million deaths annually, with > 30% of these deaths occuring in children under the age of routine measles vaccination (WHO 1998). High titer measles vaccines ($\geq 10^{4.7}$ pfu/dose) were developed in an effort to address this problem of severe measles in very young children (Halsey NA 1993). Although HT vaccines were effective at inducing seroconversion in children as young as 3 months of age (Tidjani O et al 1989, Markowitz LE et al 1990b, Job JS et al 1991, Berry S et al 1992) the unexpected association of these vaccines with excess mortality (RR = 1.2 - 1.4), (Aaby P et al 1993b, Aaby P et al 1993c, Holt EA et al 1993, Knudsen KM et al 1996) prevented their widespread use.

Although no explanation for the increased mortality was obvious in initial investigations of demographic, nutritional and socio-economic factors, subtle differences in $CD4^+$ cell number and CD4/CD8 ratios between high and standard titer vaccine recipients were identified in follow-up studies performed in Senegal and Peru 2 - 3 years after vaccination (Leon ME et al 1993, Lisse IM et al 1994). Given the known immunomodulatory (McChesney MB et al 1988, Griffin DE et al 1992, Ward BJ et al 1991) and immunosuppressive (Starr S & Berkovich S 1964, Casali P & Oldstone MBA 1982, McChesney MB et al 1986, Tamashiro VG et al 1987) effects of measles virus, these observations prompted continued close follow-up of children enrolled in HT vaccine trials, including the current investigation.

To the relief of all concerned, late follow-up studies in Africa and Haiti suggested that, whatever the effect of high titer vaccines had been on infant survival, it had either disappeared by 4 - 6 years after vaccination or all of the children susceptible to the effect had died (Aaby P et al 1994a, Aaby P et al 1996). Late studies of immunologic parameters in Guinea Bissau, Senegal and Haiti performed ~ 4 - 6 years after vaccination found no evidence of a residual HT effect on $CD4^+$ T cell numbers, CD4/CD8 ratios or any other parameter measured (Samb B et al 1995b, Bertley FMN et al Manuscript II). The current

study, performed 4 - 5 years after vaccination provides further reassurance that whatever immunologic impact the HT vaccines may have had, it was no longer evident by 5 - 6 years of age in the Sudanese HT recipients. Although no earlier immunologic data were available in these Sudanese children (other than measles virus neutralization antibody titers) the absence of long-term immunologic impact in this study is particularly reassuring due to the design of the original Sudan HT vaccine trial. This study was unique among the HT trials both in the inclusion of a placebo at 5 months of age and the bi-monthly, prospective follow-up of all children for 4 years after vaccination (Hoskins EW 1993, Libman M et al Manuscript IV). The recent analysis of the prospective follow-up data involving almost 40,000 visits is also quite reassuring: neither of the HT vaccines used in the Sudan trial had a negative impact on any measures of morbidity (eg: cough, fever, diarrhea) at any time after vaccination (Libman M et al Manuscript IV).

The formulations used in the Sudanese trial were at the "low end" of vaccines considered to be "high titer" ($10^{4.7}$ pfu). All three of the studies in which mortality was observed included groups that received vaccines containing $\geq 10^5$ pfu (Aaby P et al 1993b, Aaby P et al 1993c, Holt EA et al 1993). Although a trend towards increased mortality in the Sudanese HT recipients has been reported (Hoskins EW 1993), the small overall size of the original study (n = 510) and the low background mortality observed (Libman M et al Manuscript IV) seriously limits its power to detect differences in mortality rates between groups. Many more children in the Haiti HT trial received formulations containing very similar titers ($10^{4.4}$-$10^{4.6}$ pfu; classified as medium titer; n = 978) with no evidence of increased mortality (Holt EA et al 1993). Together with the impressive seroconversion rates documented in young children immunized with formulations containing titers in the "high-medium" to "low-high" range ($10^{4.4}$ - $10^{4.7}$ pfu), (Job JS et al 1991, Berry S et al 1992, Markowitz LE et al 1990b, Cutts FT et al 1995), these data suggest the possibility that a "higher-than-standard-titer" measles vaccine could be developed with an attractive risk/benefit ratio. However, studies to evaluate such a vaccine would be ethically

defensible only in areas of the world with large and regular measles epidemics. Such areas are becoming gratifyingly rare in the face of the international drive to erradicate measles virus by the year 2005 (PAHO 1999). Furthermore, a large number of novel measles vaccines including ISCOM and DNA-based preparations show considerable promise for the induction of protective immunity, even in the presence of maternal antibodies (Hsu SC et al 1996, Siegrist CA et al 1998c, Kovarik J et al 1999). As a result, it is unlikely that there would be great enthusiasm for fine-tuning measles vaccine titers to optimize seroconversion rates in young children while avoiding the still poorly understood excess mortality associated with HT exposure.

Table 1 - Distribution of Children in Immunologic Follow-Up Study by Vaccine Group and Gender

Group	First Dose (5 months)	Second Dose (9 months)	# Original Study	# Follow-up Study	
				Male / Female	Total
Mg-SCH*	Mg	SCH	170	28 / 33	61
EZ-Mg	EZ	Mg	170	41 / 32	73
CN-SCH	CN	SCH	170	33 / 26	59
Total			510	102 / 91	193

*Mg = Meningococcal (placebo)
SCH = Schwarz ($10^{3.9}$ pfu)
EZ = Edmonston Zagreb ($10^{4.7}$ pfu)
CN = Connaught ($10^{4.7}$ pfu)

Table 2 - Antibodies Used for FACS Analysis: Cell Type and CD Designation

Cell Type	Surface Marker	Function
CD4+ T cell	CD45RA	Naive T cells
	CD45RO	Memory T cells
	CD45RB	Low on mature T cells
	CD30	Possible marker for Th-2 phenotype
	CD25	Low affinity IL-2R
	CD75	High affinity IL-2R
	CD28	Co-stimulatory molecule
	HLA-DR	MHC II
	LFA-1	Integrin adhesion molecule
	CD71	Transferrin receptor
CD8+ T cells	CD25; CD75; HLA-DR; LFA-1	As above
	CD69	Early activation marker
	CD38	Early activation marker
	CD71	Transferrin receptor (activation marker)
B cells (CD19)	CD80	Ligand for CD28/CTLA-4
	CD86	Ligand for CD28/CTLA-4
	CD23	Activation marker
	CD25	As above
	CD38	As above
NK cells (CD16)	CD69	As above
Macrophages (CD14)	CD80	As above
	CD86	As above
	CD54	Intercellular adhesion molecule (ICAM-1)

Table 3 - Measles-Specific Neutralizing Antibodies 4 - 5 Years After Vaccination

Vaccine type	All		Female		Male	
Mg-SCH	(59)	309.8 ±55.7	(30)	432.2 ±101.8 †	(29)	183.2 ±29.0 †
EZ-Mg	(72)	210.2 ±36.0	(31)	190.4 ±41.2 †	(41)	225.2 ±55.4
CN-SCH	(56)	231.4 ±31.5	(25)	211.2 ±44.8	(31)	247.7 ±44.4

Measles-specific neutralizing antibody titers measured by syncytium inhibition assay (SIA). Control group (Mg-SCH) received meningococcal vaccine at 5 months and standard titer ($10^{3.8}$ pfu) Schwarz vaccine at 9 months of age. EZ-Mg group received HT ($10^{4.7}$ pfu) Edmonston Zagreb vaccine at 5 months and meningococcal vaccine at 9 months of age. CN-SCH group received HT ($10^{4.7}$pfu) Connaught vaccine at 5 months and standard titer Schwarz vaccine at 9 months of age. SIA titers $\geq$ 16 are thought to indicate protection. † $p < 0.03$ vs. control females.

Table 4 - Antibody Titers to Non-Measles Antigens

	Vaccine Group					
	Mg-SCH		EZ-Mg		CN-SCH	
	male (n = 29)	female (n = 30)	male (n = 41)	female (n = 31)	male (n = 31)	female (n = 25)
Antigen						
TT(mIU)	74±9	54±10	64±8	62±7	71±9*	40±9*
DT(mIU)	15±2	14.5±2	13±2	14±2	13±2	11±2.5
PT(OD)	1270±450	1140±344	980±204†	1440±598†	1240±421¥	885±247¥
HI(OD)	840±270	930±310	910±350	1070±415	850±270	940±370

Antibody levels determined by ELISA using tetanus toxoid (TT), diphtheria toxoid (DT), whole cell *B. perstussis* lysate (PT) or *H. Influenza* (HI) unconjugated polysaccharide as antigens. For tetanus and diphtheria, titers > 10 mIU are thought to confer protection. In the absence of international standard sera, PT and HI expressed in arbitrary units by comparison to "in house" standards. *$p < 0.02$, †$p < 0.04$ and ¥$p < 0.01$ in male vs. female comparisons.

Table 5 - PBMC Subsets by Vaccine Titer and Gender.

	All		Female		Male	
	Mg-SCH (42)	HT (96)	Mg-SCH (23)	HT (51)	Mg-SCH (19)	HT (45)
% CD4+	27.2±2.5	26.5±2.4	26.0±2.3	28.0±2.7	29.4±2.2	26.0±2.4
%CD8+	23.8±2.6	21.4±2.5	25.9±2.1	25.4±2.2	22.7±2.1	22.0±2.1
% B-Cell	24.1±1.4	23.3±1.7	27.1±2.0	26.8±2.2	22.2±1.9	21.8±2.5
% NK	9.5±1.1	10.2±1.2	9.7±.9	9.1±.7	9.4±.6	10.9±1.0
% Monocyte	10.2±1.0	9.6±.9	9.6±.7	10.2±.8	10.6±1.0	9.5±.9
CD4/CD8	1.1±.1	1.2±.2	1.0±.1	1.1±.1	1.3±.1	1.2±.1

PBMC subsets (T cells; CD4+, CD8+, natural killer cells; NK, B-cells and monocytes) and T cell ratios (CD4/CD8) in control children (Mg-SCH) and HT vaccine groups combined (HT = EZ-Mg and CN-SCH) determined by flow cytometry.

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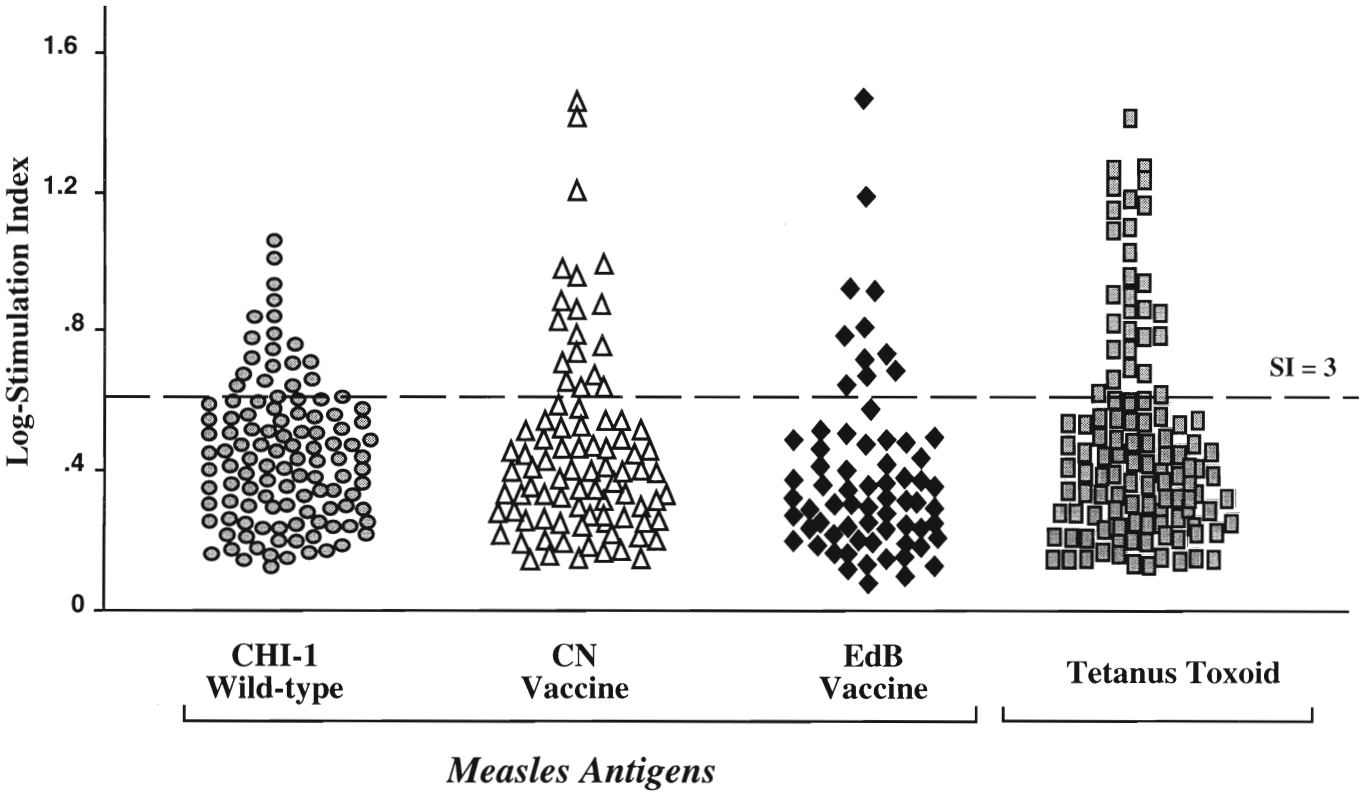
Figure 1 - Lymphoproliferative Responses to Specific Antigens



Lymphoproliferative responses to measles-specific antigens (wild-type (CHI-1) n = 124, CN; Connaught fully attenuated vaccine strain (CN) n = 88, and partially attenuated Edmonston B, (EdB) n = 69) and Tetanus Toxoid (n = 120). Results are expressed as the log (1 + X) of stimulation index (SI). SI $\geq$ 3 is thought to indicate a significant lymphoproliferative response.

4.2. SPECIFIC AIM # 1b:

Morbidity Investigation of High Titer Vaccine Recipients

To a great extent, investigators involved in HT vaccine trials were caught unprepared by the observations of increased late mortality. None of the initial trials had been designed to assess long-term morbidity let alone mortality. The exception to this rule was the study carried out in the Sudan which was not only placebo controlled but also incorporated a comprehensive prospective evaluation of morbidity and mortality for two years after vaccination in the original design. Is is ironic in retrospect that this study design was intended to document the expected *benefits* of HT vaccination. Another advantage of the Sudanese trial was that it had a date of initiation that was slightly later than most of the other studies. When excess mortality was reported by Aaby et al in 1991, support was secured not only to perform the immunologic study outlined in Manusctipt III, but also to extend the period of active, prospective follow-up to 4.5 years after vaccination (Manuscript IV).

4.2.1. MANUSCRIPT IV

No Evidence for Short- or Long-Term Morbidity After High Titer Measles Vaccination in the Sudan

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Running Head: High titer measles vaccine morbidity
Key Words: Measles, measles high titer vaccine, morbidity, mortality

Abstract

Background: Increased delayed mortality rates have been reported after high titer measles vaccination in several large studies in the developing world. Although poorly understood, the negative effect of the high titer vaccines on survival appears to last 2-3 years, and affects girls predominantly. A high titer measles vaccine study conducted in Sudan was unique in that it included a prospective evaluation of childhood morbidity after vaccination.

Methods: A total of 510 children were randomized to receive one of 3 regimens at 5 and 9 months of age. The placebo group received meningococcal vaccine, then standard titer Schwarz measles vaccine. The first high titer group received high titer ($10^{4.7}$ pfu) Edmonston-Zagreb measles vaccine followed by meningococcal vaccine. The second received high titer ($10^{4.7}$ pfu) Connaught vaccine followed by standard titer Schwarz measles vaccine.

Results: Information was available from 31,582 semi-monthly and monthly health worker visits over the 5 year follow-up period. Although no increase in infant mortality was observed in the Sudan trial, the statistical power to detect mortality differences was limited. There were no differences in duration or incidence of illness between the vaccine groups, either immediately after vaccination or during the 5 years after vaccination, despite good statistical power.

Interpretation: We were unable to document increased morbidity in recipients of the high titer measles vaccines used in this study. These data do not support previous suggestions that the apparent association between high titer vaccines and mortality is the result of increased and cumulative morbidity.

Introduction

In the developing world where measles still causes significant morbidity and mortality, there is a need for a measles vaccine which is effective as early in life as possible (Markowitz LE et al 1990b). In the late 1980s, formulations containing relatively high titers ($\geq 10^{4.7}$ plaque forming units (pfu)) of vaccine strain viruses showed sufficient promise (Job JS et al 1991, Berry S et al 1992, Markowitz LE et al 1990b, Tidjani O et al 1989, Whittle HC 1990, Cutts FT et al 1995) that the Global Advisory Group of the Extended Program on Immunization (EPI) recommended, in countries with a high incidence of measles before the age of routine vaccination (9 months), high titer Edmonston-Zagreb (EZ) vaccination at 6 months of age (Wkly Epi Rec 1990)

Not long after this recommendation was disseminated, there was first a preliminary (Garenne M et al 1991), then a more definitive report (Aaby P et al 1991) of increased mortality in children who had received the high titer vaccine in Guinea-Bissau. These reports prompted an urgent re-evaluation of high titer measles vaccine trials throughout the world. Evidence for increased mortality was found in several of the largest studies which had compared standard titer Schwarz (SWZ) vaccine ($10^{3.9}$ $TCID_{50}$) with high titer SWZ and EZ formulations ($10^{4.7}$ -$10^{5.5}$ pfu) (Aaby P et al 1993b, Holt EA et al 1993, Aaby P et al 1993c). The increased mortality was not measles related, was most prominent in females, and the 'excess' deaths accumulated slowly in the years after vaccination (Aaby P et al 1993b, Holt EA 1993, Aaby P et al 1994b, Halsey NA 1993, Knudsen KM et al 1996). These studies were not originally designed to look at an effect on survival and the evidence for increased mortality was gathered by retrospective re-analysis of data or re-initiating follow-up of study subjects. In 1992, the available data from all high titer measles vaccine trials was pooled and suggested an increased relative risk of mortality in high titer recipients (RR ~1.25) (Knudsen KM et al 1996). Subsequently, the

recommendation for the use of these vaccines was rescinded by the EPI (Wkly Epi Rec 1992).

In July, 1989, a randomized, blinded and placebo controlled study was initiated in The Sudan comparing high-titer EZ ($10^{4.7}$ pfu) and Connaught (CN; $10^{4.7}$ pfu) vaccines and standard titer SWZ vaccine ($10^{3.9}$ $TCID_{50}$) (Hoskins EW 1993). This trial was unique in that it was prospectively designed to look at long-term vaccine cost-effectiveness. As a result, infection-related morbidity, mortality and vaccine efficacy were all end-points *a priori*. At the time the Sudanese study was initiated, it was postulated that the high titer formulations would not only protect against measles but would also provide the reported non-specific benefits of measles vaccine at a younger age, resulting in marked increases in infant survival (Aaby P et al 1995b, Desgrees A et al 1995). The only modification made to the Sudan trial was to increase the duration of the study to ensure at least 4 years of follow-up after enrollment.

If the high titer vaccination was truly a cause of increased infant mortality and this increased mortality was the result of non-specific immunosuppression, we reasoned that it should be relatively easy to find an effect on morbidity. Conversely, failure to identify increased morbidity in the high titer vaccine recipients either immediately after vaccination (high titer vaccine vs. placebo for 4 months) or later after vaccination (high vs. standard titer vaccine) would cast doubt on the reported association between the high titer vaccines and infant/childhood mortality (Halsey NA 1993, Knudsen KM et al 1996)

Methods

Study Design: The study population consisted of 510 five-month old infants recruited from the Umdawanban and Essailat rural council regions in the peri-urban fringe of Khartoum, Sudan. Full details of the trial are reported elsewhere (Hoskins EW 1993). Briefly, fourteen of the villages in the area were selected, representing approximately one third of the total number of births in the region. Consecutive infant names were obtained from birth registers. Over 95% of births in these regions are attended by local midwives and recorded in the registers. Infants born between January 1 and October 1, 1989 were eligible for the study.

Infants were 18 - 22 weeks of age at the time of recruitment. They had to have been born in the study area and had to have been residing in the study area at the time of enrollment. If the families of infants enrolled indicated their intention to reside within the study area for at least the following 18 months, informed consent was obtained from the parent(s) of the infant. Exclusion criteria were infants believed to be immunocompromised, serious malnutrition (< 60% of expected weight for age), illness at the time of recruitment requiring hospitalization, previous history of measles or measles vaccination, and a known allergy to meningococcal vaccine. Infants were randomized to receive either standard titer SWZ vaccine ($10^{3.9}$ $TCID_{50}$) at 9 months of age or high titer ($10^{4.7}$ pfu) EZ (Institute of Immunology, Zagreb, Yugoslavia) or CN vaccine (Edmonston-Enders strain, Connaught Canada Ltd, Mississauga, Canada) at 5 months of age. To ensure masking of the study, the SWZ and EZ groups received meningococcal A+C vaccine (SmithKline Beecham Pharma, Oakville, ON) at 5 and 9 months of age respectively. Since little was known about the immune response to the CN formulation at the time, this group received standard titer SWZ vaccine at 9 months of age. Vaccine titers for the lots used in the study were measured by plaque assay at the London School of Hygiene and Tropical Medicine, London, England. Group assignment was by block randomization. Consecutive infants

were divided into groups of three and distributed to the three arms of the study using computer-generated random numbers.

Each of the 510 children was visited in their village every third day for the first 21 days after vaccination to monitor the immediate side effects of vaccination. Thereafter, they were visited every two weeks until 18 months of age and then every month until December 1994 (5 – 6 years of age). There were 20 trained interviewers, each assigned to a particular village and particular children. All interviewers and parents were masked to the vaccination group. At each visit, the child's family was questioned regarding any illness that had occurred since the previous visit. If any illness had occurred, the number of days of illness was recorded. The presence of any of the following symptoms during the interval was also noted: high fever, low fever, rash, diarrhea, vomiting, seizures, cough, tearing or red eyes. Any medical care was also noted, as were the costs to the family of medical consultation, medications and transportation to and from medical facilities. Data from missed visits during which no illness had occurred were simply recorded at the next visit. Illnesses which had begun or occurred during the interval prior to a missed visit were coded as 'missing data' unless the precise timing of events was available (eg: clinic notes or maternal diary).

Statistical Analysis: The available data for each child were stratified by age group: enrollment (5 months) to 1 year, 1 - 2 years, 2 - 3 years, 3 - 4 years, 4 - 5 years and 5 years to termination of the study which occurred during the sixth year of life. For each child and for each age group, we calculated the total number of days for which health status data was available. The total number of days of illness was then calculated in order to derive the mean number of days ill per month for each child. The number of visits during which an illness in the preceding period had occurred was calculated in order to derive the proportion of visits during which an illness was recorded. If there were fewer than three visits recorded for a given child in a given age stratum, that child's data for that age group

was excluded from analysis. If a child died, the visits for that age group were similarly excluded if fewer than three visits were recorded in the relevant age category prior to death.

Univariate analysis of baseline demographic variables was performed using the chi square test for categorical variables and ANOVA for continuous variables. All further analyses were stratified by sex and age. Statistical comparison of certain outcome variables required transformations in order to satisfy assumptions of normality of distribution and homogeneity of variances. These assumptions were verified by visual inspection of the transformed distributions and plots of residual values. The mean number of days ill per month was logarithmically transformed. The proportion of visits during which one or more symptoms were present was transformed using the formula $y'=2*\arcsin\sqrt{y}$ as has been recommended for variables of this nature (Neter J et al 1990) Study groups were compared by analysis of variance with repeated measures. The F values and associated p values were calculated using the general linear models procedure in SAS for Windows version 6.10 (SAS Institute Inc., Cary, NC). Estimates of power of the study to detect differences between groups also used these transformed distributions. Anthropometric parameters (z-scores) were calculated using the EPINUT module in Epiinfo (version 6.04a; Center for Disease Control and Prevention, Atlanta GA).

Results

A total of 35,386 visits was theoretically possible. The interviewers were unable to record information on 3,804 (10.8%) occasions. Of these, about 1,740 (46%) visits were missed because of the decision to reduce the scale of the study and not to follow the CN group systematically during the Gulf War (1990). One hundred and four visits (3%) did not take place because of deaths in the study population. The remainder (51%) of the missing visits account for 5.5% of the total number possible and arose due to inability to locate a family member during the interviewers' visit to the village. Data is shown in Table 1. Baseline demographic variables for the three vaccination groups are shown in Table 2. A total of 560 infants were eligible for the trial. Fifty infants were excluded from enrollment, almost all because of a failure to obtain consent. The male:female ratio among participants closely reflected the ratio for the six-year birth cohort of the entire region which was 1.15:1.

Thirty-seven infants were unavailable for the second vaccination. In seven cases, the child died before 9 months of age. Four children, all in the control group, were temporarily outside the study area at the time of the second vaccination and consent for the second vaccination was refused for 25 children (SWZ = 13, EZ = 8, CN = 4). All children in the control group who did not receive standard SWZ vaccine from study personnel received the vaccine in local clinics. One infant in the EZ group accidentally received an 'extra' dose of SWZ vaccine at 9 months of age.

There was a slight difference in the number of children who were ill on the day of the initial vaccination (CN = 27%, EZ = 35%, SWZ = 24%; SWZ vs. EZ $p<.05$) but no difference in the prevalence of illness during the preceding 2 weeks (CN = 30%, EZ = 30%, SWZ = 34%) or the percentage requiring medical attention (CN = 15%, EZ = 20%, SWZ 23%). There were also no differences among groups in vaccination status for BCG, DPT or polio vaccines. A variety of measures of socio-economic status including measures of family income, parental education level, type of employment, ownership of household

items and livestock, number of siblings, sharing of beds and access to health care were also similar among the three groups.

No difference was found in anthropometric parameters among the vaccine groups overall. When girls were analyzed separately, there were significant differences in mean weight-for-height z-scores at study entry (SWZ = 0.34, EZ = 0.10, CN = 0.20; p value for EZ vs. SWZ = 0.003) but not for height-for-age. This difference was no longer statistically significant at 9 months of age. When morbidity outcomes (below) were adjusted for weight-for-height differences at study entry, the results were unchanged (data not shown).

We were unable to demonstrate any significant differences in morbidity between vaccine groups either during the first 4 months after vaccination (high titer vaccine vs. placebo) or later in the study (high vs. standard titer vaccine). More importantly, stratification by sex and age did not reveal any suggestion of increased morbidity in either sex at any age attributable to the vaccine group. These negative results are illustrated in Figures 1 - 4 showing the mean number of days per month of illness for all subgroups as well as the mean proportion of visits during which any illness was documented. Results for individual symptoms and combinations of symptoms were similar.

The study had good power to detect differences between vaccination groups within subgroups of age, even when the results were stratified by sex. For each comparison within the 12 subgroups, the study had 80% power or more (at the 0.05 confidence level, two-tailed) to detect a difference of one day/month in measures of mean days of illness per month and a 12% difference in the proportion of visits with an illness or a symptom recorded. There were no significant differences seen in the mortality rates for the vaccine groups with 10 deaths in the CN group and 13 deaths in each of the other two groups (SWZ and EZ) (Table 3). The absolute number of deaths was small and the power to detect differences in mortality was limited. Nevertheless, there were slightly more deaths among females during the second and third years of life in the EZ high titer group.

The number of measles cases was also small with 5 cases in the SWZ group, 6 cases in the EZ group and 3 cases in the CN group (Table 4). There was only one case of measles in the control group which occurred in an unvaccinated child (ie: between recruitment and 9 months of age). When all groups were combined, there were 12 measles cases among boys and only 2 among girls ($p = 0.04$).

Discussion

Despite continued efforts to control measles, the World Health Organization estimates that this virus infects 40 - 60 million children every year accounting for 0.5 - 1 million deaths (World Health Report 1995). Measles remains uncontrolled in part due to high rates of transmission in young infants (Aaby P et al 1990b, Loening WE & Coovadia HM 1983, Taylor WR et al 1988). Most of the severe measles-associated morbidity and mortality occurs in children < 1 year of age. High titer measles vaccines were able to induce excellent seroconversion rates in children as early as 3 - 4 months of age, often despite the presence of maternal antibodies (Job JS et al 1991, Berry S et al 1992, Markowitz LE et al 1990b, Tidjani O et al 1989, Whittle HC et al 1990, Cutts FT et al 1995). The withdrawal of these vaccines has been a major setback in the effort to control measles.

It has been postulated that the high titer vaccines may increase a child's susceptibility to potentially lethal infections common in the local community without directly causing mortality. Such an impact would be predicted to be 'multiplicative' rather than 'additive' (Knudsen KM et al 1996, Wkly Epi Rec 1992). In other words, children in areas with high background mortality rates would be at particular risk from high titer vaccination. There is, as yet, no biologically plausible mechanism by which such an effect could be mediated. No increase in mortality attributable to high titer measles vaccines was reported in trials conducted in areas with relatively low background mortality rates (eg: Mexico (Diaz-Ortega JL et al 1992), Peru (Berry S et al 1992), The Philippines, USA (Markowitz LE et al 1990b)) (Knudsen KM et al 1996, Wkly Epi Rec 1992). In contrast, the background childhood mortality rates were much higher in the Senegal (38-48/10^3 person-years), (Garenne M et al 1991, Aaby P et al 1996) Haiti (12-22/10^3 person-years) (Holt EA et al 1993) and Guinea Bissau (30-32/10^3 person years) (Aaby P et al 1994b) where the association between high titer vaccines and increased mortality was observed. The overall mortality during the first three years of life in Sudan was ~19/10^3 person-years

suggesting that the children exposed to high titer vaccination in our study might have been at increased risk for death (Wkly Epi Rec 1992).

Several investigators have postulated that the high titer vaccines act as immunosuppressive or immunomodulatory agents (Ward BJ et al 1993b). Certainly, there is a wealth of data demonstrating the immunomodulatory potential of both wild-type and vaccine strain measles virus (Griffin DE et al 1994, Griffin DE et al 1995). Although Hussey et al. were unable to find dramatic immunologic perturbations immediately after high titer vaccination (Hussey GD et al 1996), Auwaerter et al. observed acute changes in T cell Vβ usage immediately after exposure to high titer vaccination (Auwaerter PG et al 1996a) and two groups have demonstrated subtle immunologic differences between standard and high titer vaccine recipients between 2 and 3 years after vaccination (Lisse IM et al 1994, Leon ME et al 1993). These differences did not persist to 4-5 years after vaccination (Samb B et al 1995b, Bertley FMN et al Manuscripts II and III), and none of the differences observed were large enough to suggest that the high titer recipients were 'immunocompromised' (Lisse IM et al 1994, Leon ME et al 1993). Furthermore, the proposed impact of high titer vaccination on mortality also appears to have disappeared by 3 - 5 years after vaccination (Halsey NA 1993, Aaby P et al 1996). Our data convincingly demonstrate that if high titer vaccine-induced immunomodulation lies at the root of the increased mortality, it does so with no discernable impact on infant morbidity. The absence of an impact on morbidity attributable to high titer vaccination is perhaps most striking during the first four months of the study, during which time we were able to compare the morbidity rates in 340 high titer recipients (EZ or CN) and 170 children who had received a non-measles, 'placebo' (meningococcal) vaccine (Hoskins EW 1993). While it remains possible to consider some sort of 'threshold' or idiosyncratic immunologic mechanism for high titer vaccine-induced mortality, the absence of an effect on morbidity, and the wide range of the causes of death in the high titer trials (Aaby P et al 1991, Aaby P et al 1993c, Aaby P et al 1994b) diminish the plausibility of the immunologic argument.

It is certainly possible that the high titer effect on mortality is seen only at the highest vaccine titers used. Intensity of exposure to natural measles virus is known to be a predictive factor in the development of severe and fatal measles (Aaby P et al 1986). Our study used vaccines containing $10^{4.7}$ pfu, the lowest dose still defined as 'high titer' by the WHO (Wkly Epi Rec 1992). In Guinea-Bissau, a study using $10^{5.1}$-$10^{5.3}$ pfu found evidence of sex-specific increased mortality attributable to the high titer vaccine (Aaby P et al 1993b) while a study using $10^{4.6}$ pfu did not (Aaby P et al 1994a. Very similar results were obtained in the Haitian study (Holt EA 1993). The last large study in which increased mortality was demonstrated (Senegal) also used high titer vaccines at the upper end of the titer range ($10^{5.0}$-$10^{5.5}$ pfu) (Aaby P et al 1991). Although differences in titer may help to explain why a significant increase in mortality was not observed in the Sudanese study, one is still left to invoke a 'threshold' effect to explain the absence of impact on morbidity. There is currently no biologic evidence that such a threshold exists. For example, a wide range of immunologic effects including suppression of cutaneous reactivity and depressed lymphoproliferative responses to antigens and mitogens are readily observed *in vivo* after standard titer (~10^3) measles vaccination (Griffin DE et al 1994, Griffin DE et al 1995). Although Aaby and others have suggested that the increase in mortality associated with the high titer vaccines would be better thought of as the absence of some 'non-specific' beneficial effect of standard titer vaccination (Aaby P et al 1984, Hull HF et al 1983, Aaby P et al 1989), the biological plausibility of this hypothesis is also tenuous (Aaby P et al 1995b).

The higher number of measles cases among boys has not been noted in other high titer vaccine trials and may have been a chance finding. As would be expected in a vaccinated population, the total number of cases was small. Although there are reports that females generate higher antibody titers in response to vaccination than males (Green MS et al 1994), such a difference was not observed in the Sudanese study (Hoskins NA 1993) and has not been reported in any of the other high titer trials (Job JS et al 1991, Berry S et

al 1992, Markowitz LE et al 1990b, Cutts FT et al 1994b, Aaby P et al 1991, Aaby P et al 1993b, Aaby P et al 1994b, Holt EA et al 1993).

The question of the increased mortality after high titer vaccines has been difficult to evaluate from the outset. With the exception of our study, none of the other high titer trials were designed to evaluate either morbidity or mortality prospectively. Nonetheless, the data suggesting an increased mortality related to the high titer vaccines is difficult to discount. An effect was seen in three different countries (Senegal, Guinea Bissau and Haiti) using different vaccines (EZ, SWZ, Biken-CAM) and different vaccine lots (Aaby P et al 1991, Aaby P et al 1993b, Aaby P et al 1994b, Holt EA et al 1993). Delayed mortality after natural measles infection (Aaby P et al 1993a) and even exposure to natural infection *in utero* (Aaby P et al 1990c) provides some biological plausibility for the observed high titer effect. Recent epidemiologic data suggests that the mortality attributable to natural measles is indeed higher in females of all ages than in males (Garenne M et al 1994a). It has been suggested that investigators were "too easily convinced of the safety of high titer vaccines" implying that harm may have been done by their use in large clinical trials (Halsey NA 1993). Paradoxically, others have argued that investigators were too easily convinced of the harm done by the high titer vaccines with the consequence that we now have little to offer populations in which measles morbidity and mortality is severe under the age of routine vaccination. Our data demonstrate that there is no deleterious effect of vaccines with titers in the range of $10^{4.7}$ per dose. This type of vaccine is known to elicit good antibody responses in children younger than 9 months of age (Job JS et al 1991, Berry S et al 1992, Markowitz LE et al 1990b, Cutts FT et al 1994b, Whittle HC et al 1990, Tidjani O et al 1989). With the proviso that appropriate, prospective studies of sufficient power are essential before the widespread re-introduction of 'higher titer' vaccines, the current moratorium on the use of this type of vaccine should be reevaluated.

Acknowledgements

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Table 1 - Number of Children Excluded from Analysis

	AGE											
	5-12 mo		12-24 mo		24-36 mo		36-48 mo		48-60 mo		60-72 mo	
	M	F	M	F	M	F	M	F	M	F	M	F
CN	0	0	32	22	3	3	0	1	0	0	9	6
EZ	0	0	4	3	3	1	1	0	1	0	5	4
SWZ	1	0	6	7	3	3	0	2	0	0	2	5

Number of children excluded from analysis of each stratum because <3 visits were recorded during the specified time interval. Exclusions due to death are not counted in this table.

Table 2 - Selectet Demographic Variables of Study Population

Vaccine group		Connaught	E-Z	SWZ
Male:female		102:68	90:80	87:83
Received 2nd vaccination Male:Female		100:65	85:75	73:75
Age (weeks ± sd)		20.2 ± 1.5	20.2 ± 1.5	20.1 ± 1.5
Weight (kg ± sd)		6.63 ± 0.83	6.52 ± 1.03	6.63 ± 0.97
Length (cm ± sd)		63.34 ± 2.41	62.95 ± 3.27	63.11 ± 2.77
Weight-for-height (z-score ± sd)	M	-0.03 ± .0.89	0.25 ± 1.01	0.01 ± 1.02
	F	0.20 ± 1.05	-0.10 ± 0.94	0.34 ± 0.97
Number of siblings		2.8 ± 2.4	3.0 ± 2.4	2.9 ± 2.3
% with annual family income:	<$100	12%	14%	14%
	$100-$400	67%	61%	67%
	>$400	21%	24%	19%
Mean persons per bed in household		1.0	1.0	1.0
Mean persons in same bed as infant		1.1	1.1	1.1
Mean distance to health post (minutes)		14.1	16.9	14.9

Table 3 - Number of Deaths by Age, Sex and Vaccine Group

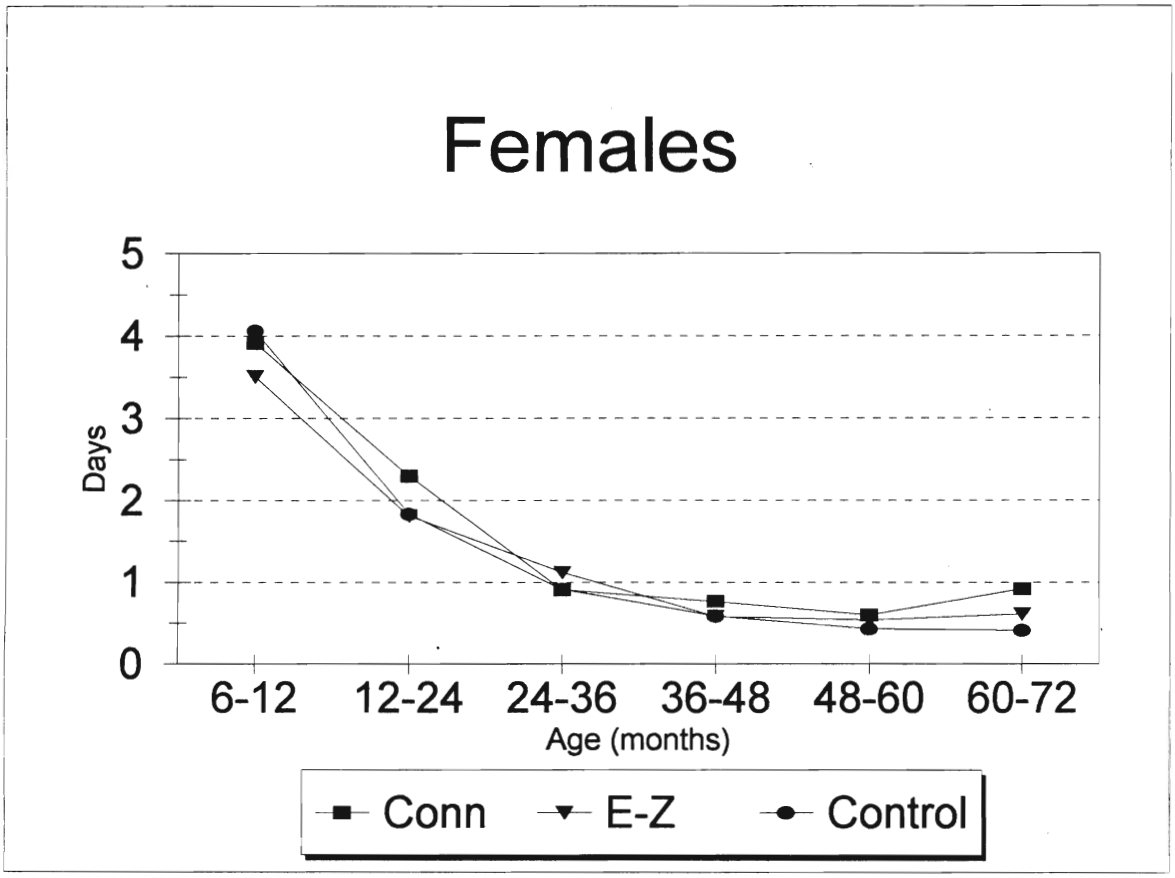
	AGE													
	5-12 mo		12-24 mo		24-36 mo		36-48 mo		48-60 mo		60-72 mo		Total	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
CN	2	1	2	1	1	1	0	0	0	0	1	1	6	4
EZ	2	1	0	5	1	3	1	0	0	0	0	0	4	9
SWZ	4	2	2	3	1	1	0	0	0	0	0	0	7	6

Table 3 - Number of Measles Cases by Age, Sex and Vaccine Group

	AGE													
	5-12 mo		12-24 mo		24-36 mo		36-48 mo		48-60 mo		60-72 mo		Total	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
CN	0	0	0	0	1	1	1	0	0	0	0	0	2	1
EZ	1	0	2	0	2	0	1	0	0	0	0	0	6	0
SWZ	1	0	1	0	1	0	1	1	0	0	0	0	4	1

Libman et al

Figure 1 - Mean Days of Illness Per Month (Females)



Libman et al

Figure 2 - Mean Days of Illness Per Month (Males)

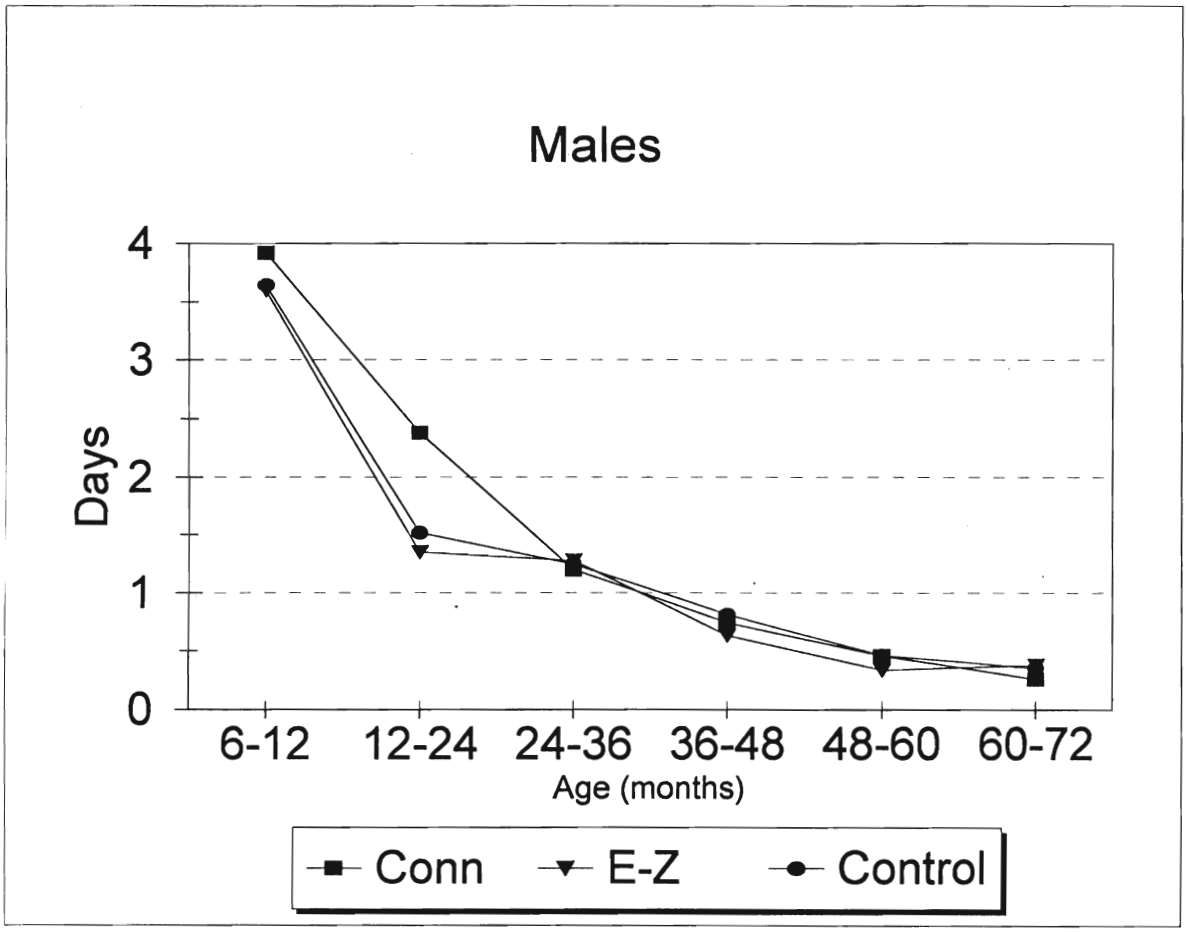


Figure 3 - Proportion of Visits with Illness (Females)

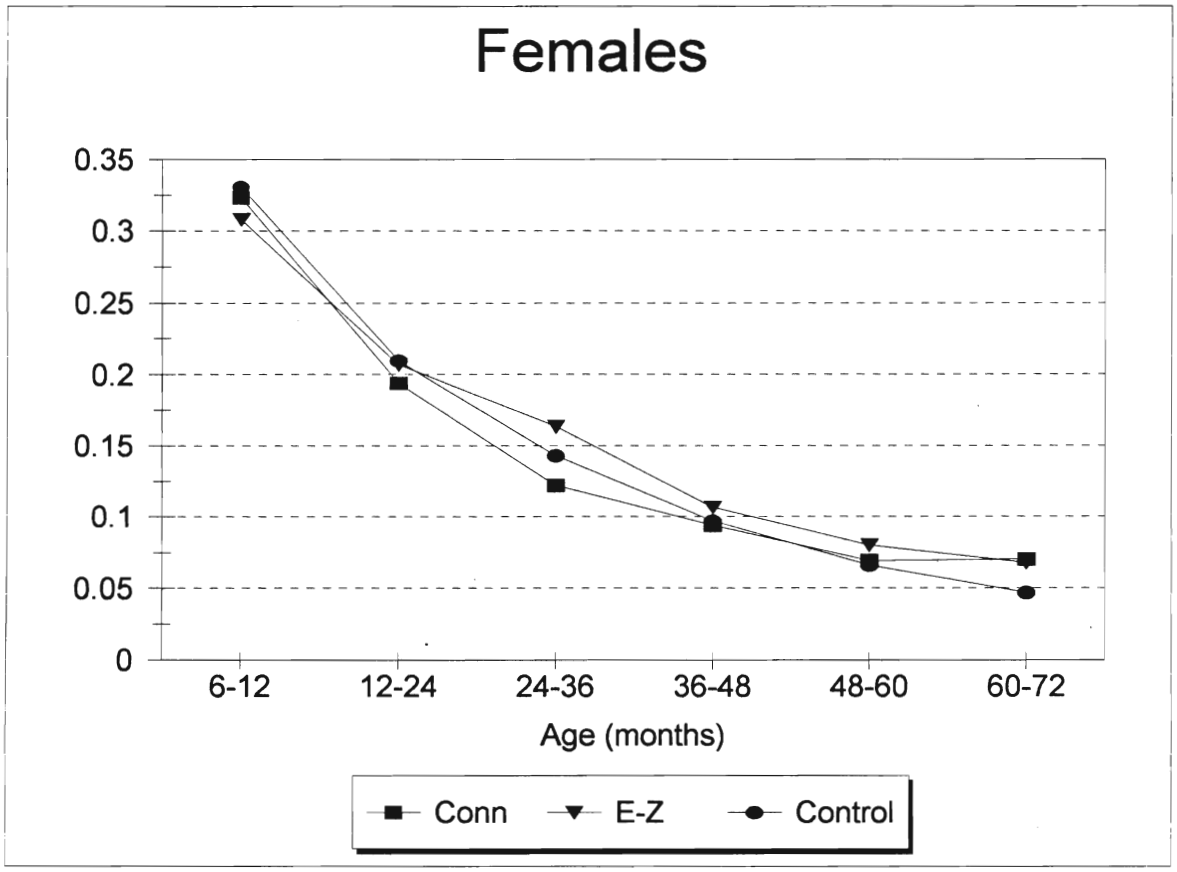
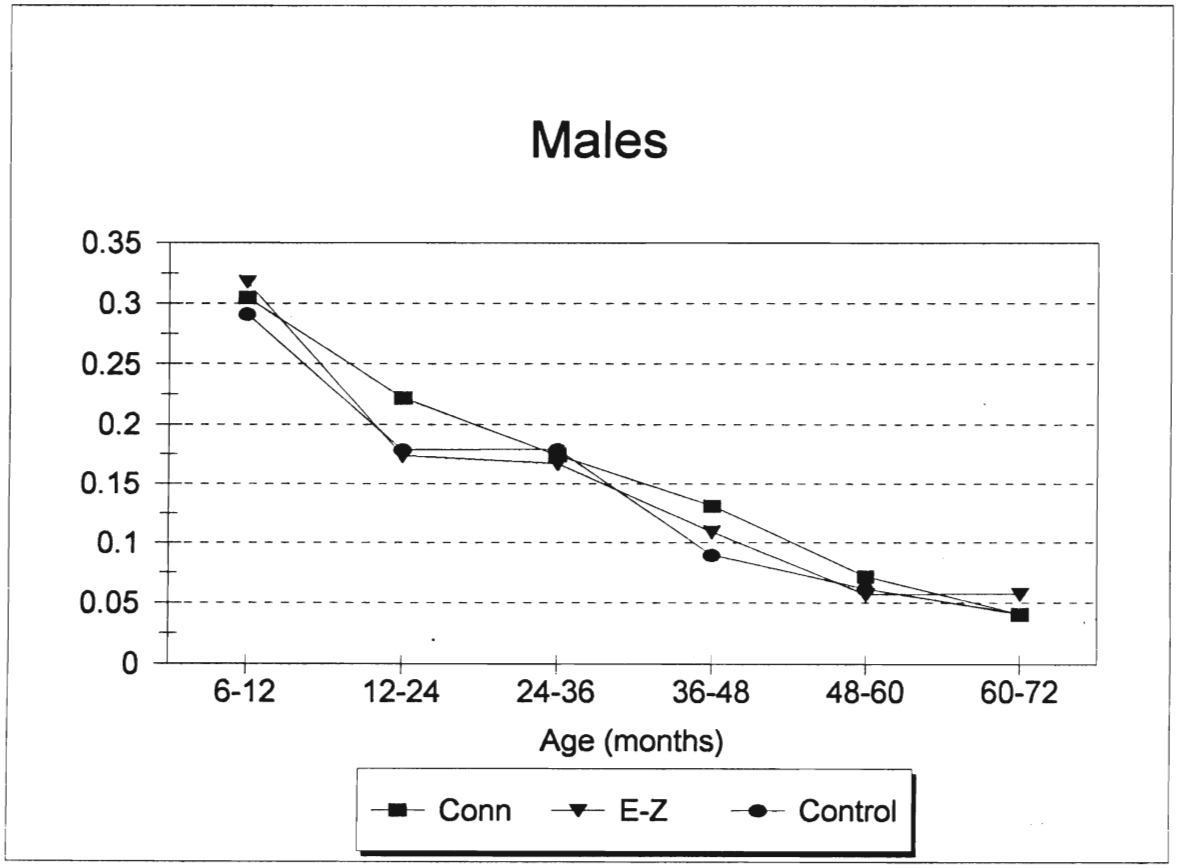


Figure 4 - Proportion of Visits with Illness (Males)



4.3 SPECIFIC AIM #2:

Mechanisms of Measles-Induced Immunopathogenesis

My work with samples from the HT vaccine trials yielded important results which were reassurring for the families of the children involved in these studies, but frustrating from the scientific point of view. Similarities between the "excess" mortality that occured following HT vaccination (Aaby P et al 1993b, Aaby P et al 1993c, Holt EA et al 1993) and that observed after asymptomatic exposures to wild-type virus (Aaby P et al 1990a, Aaby P et al 1993a) certainly suggested that mechanistic studies using samples obtained from HT vaccine trials might give insight into the immunopathogenesis of both natural infection and HT vaccination. Our inability to document immunologic differences between HT and LT vaccine recipients 4 - 5 years after vaccine exposure (Manuscript II and III), prompted us to look for more subtle markers of vaccine-induced immunologic disruption. The slow accumulation of deaths after HT vaccination, the absence of gross impact on morbidity (Manuscript IV) and the influence of sex on the risk of death all raised the possibility of vaccine-induced autoimmune processes as an underlying mechanism. In the first part of this section (Manuscript V), we demonstrate that HT recipients have small, but significant increases in reactivity to a range of self antigens. (Although this investigation is still ongoing, the results are presented here in a draft of the final report).

Among the many mechanisms by which acute infection with measles virus could plausibly induce long-term disturbance of immune cell function, apoptosis of mature immune effector cells was a particularly intriguing possibility. Both natural disease, and vaccine-type infections are known to abrogate established antigen-specific responses (Griffin DE & Bellini WJ 1996). In the second part of this section (Manuscript VI) the development of an *in vitro* infection model to demonstrate, for the first time, massive

measles virus-induced apoptosis of all PBMC subsets is described. We also explored the role of viral strain in mediating apoptosis, as well as the impact of vitamin A on measles virus-induced programmed cell death (Appendix I).

4.3.1 MANUSCRIPT V

Autoantigen Reactivity in Measles High Titer Vaccine Recipients

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Running Head: Autoantigen reactivity after measles vaccination
Key Words: Autoantibodies, autoantigen, myelin basic protein, rheumatoid factor, measles, measles vaccine, autoreactivity

Abstract

Background: The excess mortality associated with the use of high titer (HT) measles vaccines remains poorly understood. Although minor differences in $CD4^+$ T cell numbers and CD4/CD8 ratios between HT and standard titer recipients were detected 3 years after vaccination, such differences had disappeared by 4 - 5 years after vaccination. We investigated autoantigen reactivity as a possible marker for immunologic disruption at the time of or period after HT measles vaccination.

Methods: We measured the reactivity to 3 autoantigens in 405 Haitian children re-contacted 4 - 5 years after enrollment in a study of HT measles vaccines. These children had received one of five vaccines: Edmonston Zagreb high or medium titer at ~ 8 months (EZ-HT; $10^{5.6}$plaque forming units (pfu) or EZ-MT; $10^{4.4}$ pfu), or Schwarz high (SCH-HT; $10^{5.4}$ pfu) or medium titer (SCH-MT; and $10^{4.6}$ pfu), or standard titer Schwarz vaccine at ~ 12 months of age ($10^{3.0\text{-}3.7}$ pfu). Reactivity against Hela cell lysate (Hela) antigen, myelin basic protein (MBP) and IgG Fc (rheumatoid factor; RFc) were measured by ELISA (results expressed as log OD units). In the absence of a standard anti-sera, 130 sera from healthy young Canadians (1 - 16 years old) were tested for Hela or MBP reactivity to establish the range of normal values. Similarly, sera from 70 Canadian children at various times after acute measles virus infection was also tested.

Results: Low levels of autoreactivity directed against Hela (.54 ± .01 log OD) and MBP (.37 ± .01 log OD) were detectable in the large majority of healthy young Canadian children (mean age; 1.5 years old). Reactivity was highest in the youngest children and declined with age (.19 ± .02 log OD and .10 ± .02 log OD; age 10 - 16 years old, $p < 0.001$ for Hela and MBP respectively). Autoreactivity in the 5 - 6 year old Haitian children who had been enrolled in the trial of HT measles vaccines was similar to that observed in age-matched Canadian children overall (.30 ± .14 vs. .25 ± .02 and .21 ± .19 vs. .15 ±.01 for Hela and MBP respectively). When stratified by vaccine group, children who had received either medium or high titer vaccines at ~ 8 months of age had

significantly higher levels of autoreactivity against the 3 self-antigens tested compared with those who had received the standard formulation at ~ 12 months of age (Hela log OD: .31 ± .01 vs. .26 ± .02 $p < .02$, MBP log OD: .22 ± .02 vs. .15 ± .01 $p < .0005$, and RFc log OD: .041 ± .001 vs. .034 ± .002 $p < .005$). There were no differences attributable to vaccine strain or sex.

Conclusion: Haitian children vaccinated with formulations containing higher than normal titers of vaccine strain virus (EZ or SCH) at an earlier than normal age have elevated levels of antibodies directed against several self-antigens 4 - 5 years after vaccination. While the significance of this observation remains to be determined, these findings demonstrate for the first time the capacity of a vaccine to exert a sustained influence on autoreactivity.

Introduction

High Titer (HT) vaccines were developed in the late 1980's with the hope of inducing seroconversion in the presence of maternal antibodies and reduce measles associated morbidity and mortality in children < 9 months of age (Halsey NA 1993). The successful use of these vaccines in children as young as 4 months of age rapidly led the Expanded Program on Immunization to recommend their use in areas with high infant morbidity and mortality due to measles (EPI: Wkly Epi Rec 1990). In the early 1990's, enthusiasm for these vaccines was rapidly tempered when Aaby et al reported increased mortality in children who had received HT vaccines in Guinea Bissau (Aaby P 1991 personal communication, referenced in Halsey NA 1993). This alarming discovery triggered a re-evaluation of the other HT measles vaccine studies, and unexplained "excess" mortality in HT recipients was confirmed in three of the four largest HT measles vaccine trials (Aaby P et al 1993a , Aaby P et al 1993c, Holt EA et al 1993). In 1992, the World Health Organization (WHO) recommended that the use of HT measles vaccines should be stopped until the excess mortality associated with these formulations could be explained (EPI: Wkly Epi Rec 1992).

Initial investigations of the excess mortality revealed no problems in randomization for the original trials and no differences in anthropometric measures (e.g. height, weight) or socioeconomic status between HT and standard titer vaccine recipients. However, Auwaerter et al demonstrated transient changes in Vβ T cell receptor usage, for several weeks after HT measles vaccination in South African children (Auwaerter PG et al 1996a). Furthermore, studies in Guinea-Bissau (Lisse IM et al 1994) and Peru (Leon ME et al 1993) conducted 2 - 3 years after vaccine exposure revealed subtle differences between HT and standard titer measles vaccine recipients (e.g. lowered DTH, altered CD4 T cell numbers and decreased CD4/CD8 ratios). Such differences were no longer detectable in several large immunologic follow-up studies carried out in Africa and Haiti 4 - 5 after HT vaccine exposure (Samb B et al 1995b, Aaby P et al 1996, Bertley FMN et al Manuscript II

and III) and none of the differences observed suggested that the HT recipients had been immunosuppressed by vaccination. Lack of a gross immunologic effect of HT vaccines is further suggested by the absence of detectable impact on measures of morbidity in a placebo-controlled, prospective study of HT vaccines in the Sudan involving ~ 40,000 visits over a 4 - 5 year period after vaccination (Libman M et al Manuscript IV). These observations prompted us to look for more subtle evidence of immune disruption that may have been induced at the time of HT measles vaccination.

One of the most striking aspects of the reported excess mortality after HT vaccination was the gender bias; girls who received these vaccines were almost twice as likely to die than their male peers. A similar (or greater) gender bias is also a characteristic of many forms of autoimmunity; females are as much as 10-fold more likely to suffer certain autoimmune conditions than males (Steinman L 1993). Although there was no evidence that autoimmune "disorders" contributed to the excess mortality in the HT recipients, we reasoned that enhanced autoreactivity induced by an early childhood event (e.g. HT vaccination) could persist or even increase for years following the event. Initial observations revealed no significant differences between HT and standard titer recipients in "clinical" autoantibodies typically measured for the diagnosis of autoimmune disease (e.g. ANA, dsDNA). As a result, we chose to evaluate the reactivity of HT vaccine recipients to several self-antigens (e.g. MBP, Hela cell lysate, human IgG Fc) 5 years after vaccine exposure.

Materials and Methods

Subjects: The original Haitian HT vaccine trial has been described in detail (Job JS et al 1991). Briefly, a total of 1972 Haitian children were block randomized to receive one of four vaccines at mean age 8.1 months (range 5 - 11 months): i) Edmonston-Zagreb high titer (EZ HT; $10^{5.6}$) ii) Schwarz high titer (SCH HT; $10^{5.4}$) iii) EZ medium titer (EZ MT; $10^{4.4}$) iv) SCH medium titer (SCH MT; $10^{4.6}$). A fifth group of 371 children received standard low titer Schwarz vaccine (SCH LT; ~$10^{3.0\text{-}3.7}$) at mean age 12.2 months (range 6 - 20 months). Plasma samples from 405 children participating in the immunologic follow-up study (Bertley FMN et al Manuscript II) were available to test for the presence of autoantibodies. Based on socio-economic parameters and anthropometric data collected both at the time of the original study and the immunologic follow-up, these children were representative of the original study population (Bertley FMN et al Manuscript II).

Sample Preparation: For all samples, a single heparinized tube of blood (5 mL) was obtained by venipuncture from each child. All samples were processed within 6 hours of collection. The blood was centrifuged (300 x g for 10 minutes) and plasma was aliquoted and frozen at -20°C. Plasma was heat inactivated at 56°C for 30 minutes before use.

Antigen Source: Myelin basic protein (MBP) was donated by T Owens, McGill University, QC and Hela cell lysate was donated by M Newkirk, McGill University, QC. Human IgG Fc (hFc) was purchased from Jackson Research Laboratories, Inc (West Grove, PA).

ELISA: Briefly, 96-well plates were coated overnight with 2 μg/mL of Hela lysate, MBP or hFc in carbonate-bicarbonate buffer (pH 9.6). For Hela and MBP antigens plates were blocked with 5% goat serum (Life Technologies, Grand Island, NY) plasma samples were diluted 1:200 in SuperBlocker (Pierce Chemical Co., Rockford, IL) and incubated

overnight at 4°C. The assay was completed with mouse monoclonal antibody directed against human IgG (ATCC #1757, Rockville, MD) followed by incubations with biotinylated goat anti-mouse IgG (Fab_2; Boehringer Mannheim, Laval, QC) for 1 hour at 37°C, HRP-conjugated streptavidin (Boehringer Mannheim) for 30 minutes at 37°C and ABTS (Boerhinger Mannheim) with 0.4 μL/mL of hydrogen peroxide (Fisher, Ottawa, ON) for 20 minutes at room temperature. For RFc, the blocking step was omitted and the serum was diluted 1:500 in SuperBlocker for a 2 hours incubation at room temperature (~23°C). The RFc assay was completed with biotinylated goat monoclonal anti-human IgM (Fab_2; Jackson ImmunoResearch, West Grove, PA) for 1 hour at 37°C, HRP-conjugated streptavidin for 30 minutes at 37°C and ABTS with 0.4 μL/mL of hydrogen peroxide for 20 minutes at room temperature. Optical density (OD) was determined by spectrophotometry (Titertek Multiskan® MCC/340, Flow Laboratories, Mississauga, ON) at 405 nm. These are not clinically standardized tests for autoreactivity and "gold-standard" reagents (e.g. standardized sera) were not available. As a result, the differences between children in this study are relative and we refer to the increased optical density in the ELISA as "reactivity" rather than autoantibody levels. Results are expressed as $OD_{sample} - OD_{blank}$.

Statistics. Samples were grouped into standard titer (LT), medium titer (MT) and high titer (HT) and Statview statistical software (Cary, NC) was used for analysis. OD values were logarithmically transformed to approximate a normal distribution using the formula $\log_{10}(1+X)$. Data are expressed as mean ± standard error of the mean (SEM). For all statistical tests, p values were obtained from two-tailed analyses; $p < 0.05$ was considered to be significant.

Results

Healthy Canadian Children. Since the tests we used were not routine clinical assays for autoantibody production, no "gold standard" reagents were available, and little was known about the levels of these "autoantibodies" in normal, healthy children. We therefore tested a range of plasma samples from healthy, Canadian children at various times after natural measles (n = 70) or routine MMR vaccination; pre-vaccination at 12 months (n = 100) and between 6 months and 16 years post-vaccination (n = 138). The levels of autoreactivity (log OD) detected for Hela and MBP assays were highest in Canadian children at 12 months of age (immediately prior to MMR vaccination; Hela .54 ± .01; MBP .37 ± .01) and fell progressively with age (at 10 - 16 years; Hela .19 ± .02 ; MBP .10 ± .02) (Figures 1a and 2a). Hela and MBP reactivity were elevated during the first 2 - 3 weeks compared with age-matched controls after natural measles-virus infection (Hela: .41 ± .06 vs. .30 ± .04, and MBP: .21 ± .04 vs. .14 ± .02) but did not persist at elevated levels in the small number of adolescents studied ~ 1 year after acute illness (Figures 1b and 2b). Indeed, increase in autoreactivity induced by acute infection appeared to be quite transient. No sex differences were observed when the various control groups were divided by gender. At the time of writing, no data were available for IgG Fc reactivity in control children.

Haitian Children Exposed to HT Vaccines. In the Haitian children (age range 5 - 6 years), the mean log OD for Hela was 0.30 ± 0.14 (range 0.02 - 0.653), MBP was 0.21 ± 0.19 (range 0.015 - 0.629) and RFc was 0.04 ± 0.017 (range 0.006 - 0.101). These values were very similar to those observed in the small number of healthy 6 - 8 year old children in Canadian studies (Figures 1a and 2a). Analysis of the Haitian data by vaccine strain and titer showed no differences attributable to strain, but marked differences were observed between lower titer recipients (standard SCH at 12 months) and higher titer recipients (medium and high titer EZ or SCH at 8 months). Compared with the standard

measles virus recipients, those who had received higher titer formulations earlier in life had higher OD values for Hela ($p < 0.04$), MBP ($p < .001$) and RFc ($p < 0.001$) antigens (Table 1 and Figure 3). Analysis of the Haitian data by gender revealed elevated levels of RFc reactivity in the female higher titer recipients only ($.043 \pm .01$ vs. $.038 \pm .02$ in higher titer boys; $p < 0.002$)

Discussion

There is growing consensus that autoimmune disease is driven by T cells that respond in an aberrant fashion to self antigens (Burlingame RW et al 1993, Radic MZ & Weigert M 1995). Autoantibodies can occasionally play a role in the immunopathogenesis of autoimmune processes but are often a reflection of the ongoing T cell attack on self (Naparstek Y 1993). Autoreactive T cells can be found in "normal" individuals at all ages in low numbers (Abbas AK et al 1997). Similarly, a wide spectrum of low affinity autoantibodies are readily detected in the serum of most individuals and levels generally increase with advancing age (Naparstek Y 1993). Using three self-antigen preparations (e.g. Hela, MBP and RFc) we have demonstrated a relatively transient increase in autoreactivity during and shortly after natural measles infection, and a marked difference in autoreactivity (elevated levels) between Haitian children who received higher titer measles formulations before the age of routine vaccination and those who received standard titer vaccines.

In the context of acute measles infection, enhanced autoreactivity has been described at least once before; fibrillar anti-cellular IgM autoantibodies were detected in ~ 70% of acute measles patients (Haire M 1972). Although these investigators did not look at total immunoglobulin levels (IgM or IgG), our preliminary data looking at autoreactive IgG suggests that the transient increases in autoreactivity seen after natural disease cannot be explained on the basis of elevated IgG levels alone (data not shown). Given the intense B cell mitotic activity (Ward BJ & Griffin DE 1993a) and polyclonal B cell activation known to accompany acute disease (Griffin DE & Bellini WJ 1996) it is certainly plausible that the control of pre-existing autoreactive clones might be transiently lost during measles. However, it is also possible that measles virus participates more directly in the induction of autoreactivity. The receptor for measles virus (CD46) is present on virtually all human nucleated cells (Dorig RE et al 1993) and as such, measles virus has a wide tissue tropism, including; endothelia, epithelia, several cell types in the central nervous system (CNS) and

immune cells (Griffin DE & Bellini WJ 1996). We and others have demonstrated the capacity of measles virus to cause apoptosis in a wide range of cells and cell lines (Bertley FMN et al Manuscript VI, Esolen L et al 1995 , Fugier-Vivier I et al 1997). While apoptosis is undoubtedly an adaptive mechanism in this setting, there is accumulating evidence that programmed cell death is also intimately linked to the development of autoimmunity (Rosen A & Casciola-Rosen L 1999). Pathogen-driven apoptosis may result in the competent presentation of otherwise cryptic self-antigens in a pro-immune context leading to aberrant autoimmune reactivity (Rosen A & Casciola-Rosen L 1999). In recent years, several investigators have linked viral infections with the induction of cell and or antibody-mediated autoimmune processes (Aichele P et al 1996, Naucler CS et al 1996, Hansen KE et al 1998). Similarly, there is a growing list of vaccines including measles, influenza, rabies and hepatitis B (Cohen AD & Shoenfeld Y 1996), that have been implicated in the induction of short-term autoimmune syndromes (e.g. post-infectious encephalomyelitis and Guillain-Barre syndrome) (Johnson RT et al 1984, Hurwitz ES et al 1981). Although a great deal of energy has been expended trying to link viral pathogens and various vaccines with the development of chronic autoimmune, inflammatory and idiopathic conditions such as multiple sclerosis, diabetes mellitus, systemic lupus erythematosus, inflammatory bowel disease and autism, there is no conclusive evidence for any such associations to date (Andjaparidze OG et al 1989, Cosby SL et al 1989, Wakefield AJ et al 1993, Singh VK et al 1998).

In the context of the high titer measles vaccine trials, there was no evidence of increased autoimmune "disease" in the HT vaccine recipients as a factor contributing directly to the excess mortality (Aaby P et al 1993b, Aaby et al 1993c, Holt EA et al 1993). Indeed, we initially screened a subset of those children for "classical" autoantibody production (e.g. ssDNA, ANA) and found no differences. However, the children in this study who received higher than standard titer vaccine formulations before the age of routine immunization clearly had increased reactivity to all of the research self-antigens used,

which could not be attributed to elevations in total IgG levels. A limited analysis strongly implicated vaccine titer rather than vaccine timing in the induction of the autoreactive state. However, the transience of the burst of autoreactivity seen in the Canadian cohort with natural disease (age range; 16 - 19) raises the possibility that age at exposure and possibly, viral strain may influence this response. It is important to emphasize that these findings do not indicate that the higher titer vaccine recipients were immunologically compromised in any way. Furthermore, in the absence of sera from earlier time points, we cannot determine the kinetics of the development of the autoreactivity observed at 4 - 5 years after HT vaccine exposure. As a result, we do not know the full implications of these findings for the HT vaccine recipients, and many questions are raised about the potential for autoreactivity induction in a variety of settings such as natural infection and standard titer vaccination in the presence of maternal antibodies. However, these findings extend the period during which immunologic differences between HT and standard titer vaccine recipients persist, to at least 4 - 5 years after vaccination.

Table 1: Autoantigen Reactivity by Vaccine Titer and Gender in Haitian Children*

	STANDARD TITER		MEDIUM TITER				HIGH TITER			
	Schwarz		Schwarz		Edmonston Zagreb		Schwarz		Edmonston Zagreb	
	female (*n = 26*)	male (*n = 34*)	female (*n = 42*)	male (*n = 49*)	female (*n = 41*)	male (*n = 49*)	female (*n = 39*)	male (*n = 39*)	female (*n = 41*)	male (*n = 45*)
Autoantigen										
Hela	0.246(.024)	0.275(.020)	0.323(.024)	0.296(.020)	0.319(.024)	0.296(.020)	0.302(.028)	0.313(.020)	0.287(.018)	0.305(.020)
MBP	0.147 (.019)	0.153(.015)	0.211(.024)	0.218(.019)	0.207(.022)	0.218(.020)	0.224(.024)	0.248(.022)	0.226(.024)	0.237(.023)
RFc	0.036(.003)	0.032(.002)	0.043(.003)	0.040(.003)	0.046(.003)	0.037(.002)	0.041(.003)	0.039(.003)	0.047(.003)	0.041(.003)

*** Autoantibodies to Hela cell lysate (Hela), myelin basic protein (MBP) and Rheumatoid Factor (RFc) by vaccine titer and gender. Results are expressed as the mean (± SEM) of (log) optical density.**

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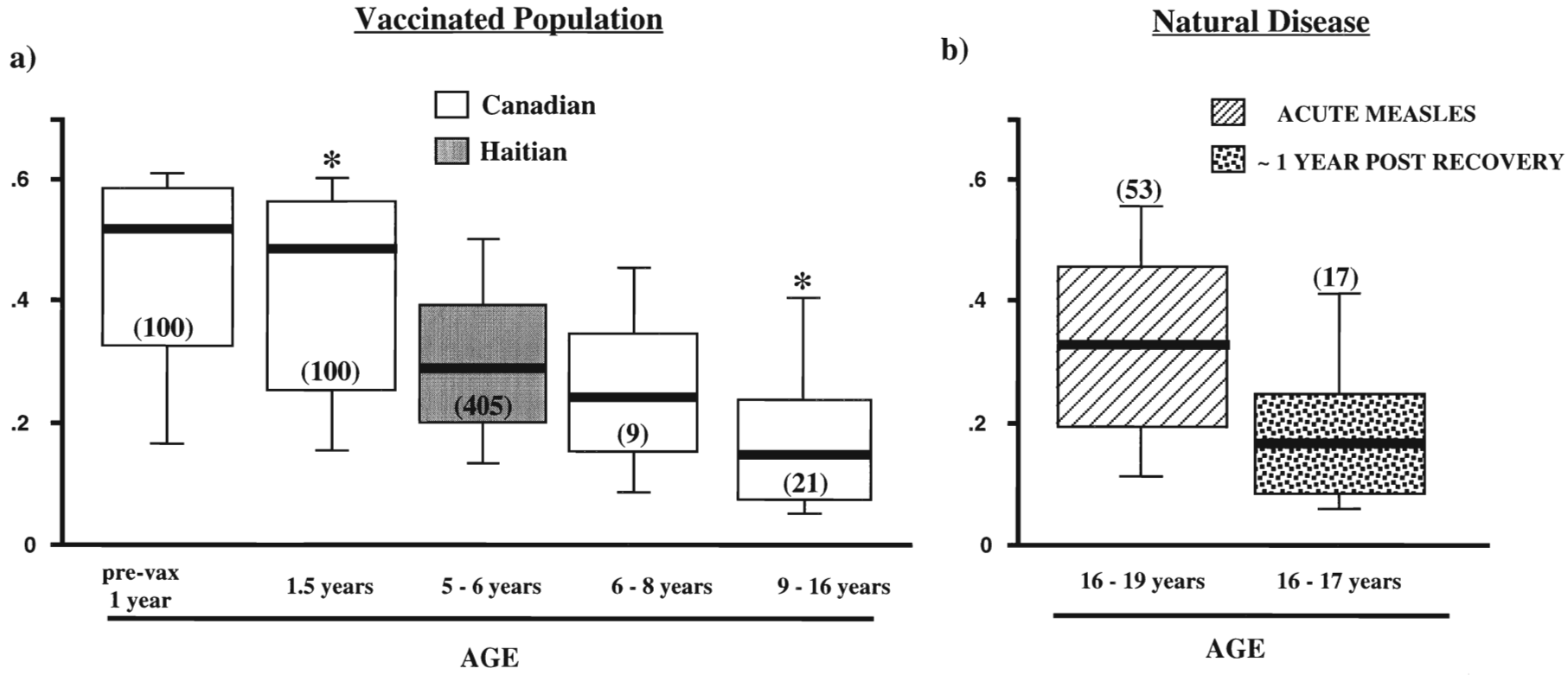
Figure 1 - Hela-Specific Reactivity in Control Subjects and Haitian Children



Autoreactivity to Hela cell lysate by ELISA in a) vaccine recipients by age or b) natural disease by time after wild-type exposure. Results are expressed as the log optical density represented by Box Plots with mean (▬) and range in percentiles (10% - 90% inclusive), (n), * p = 0.0001.

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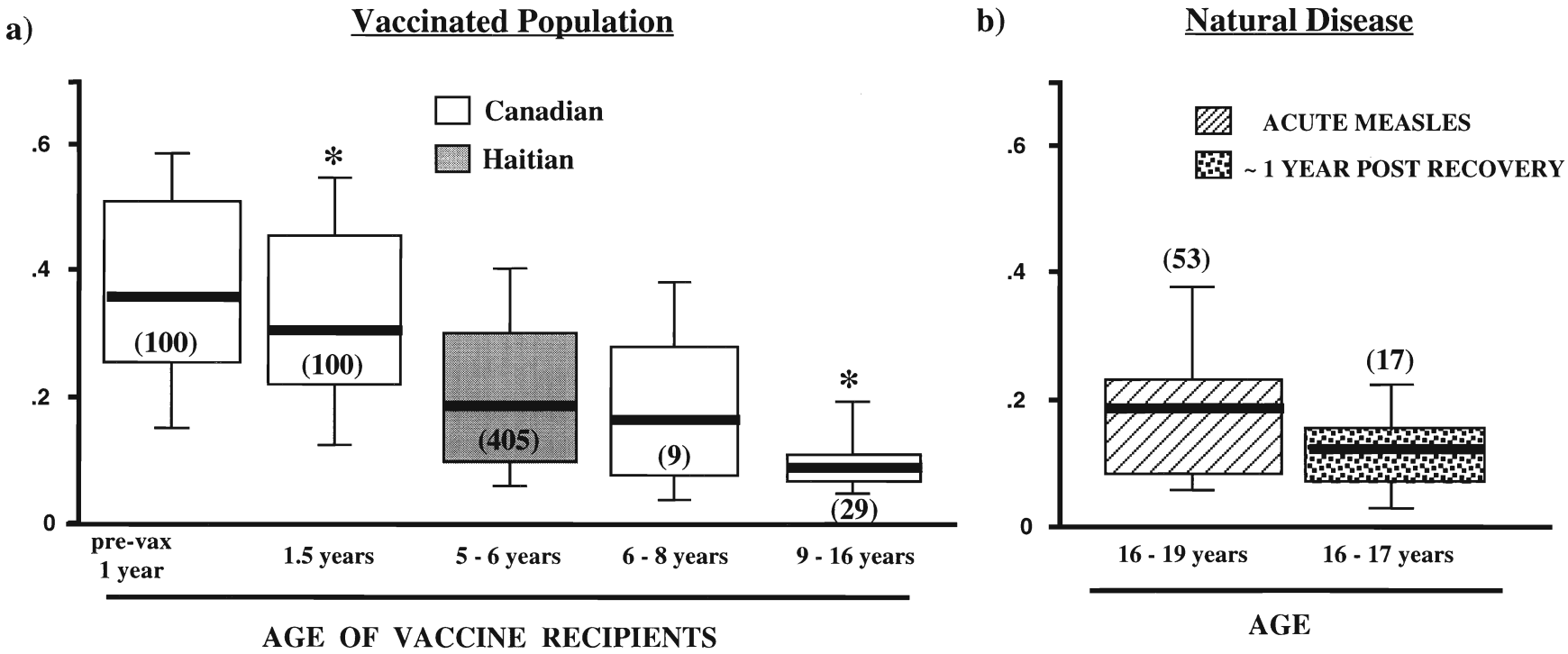
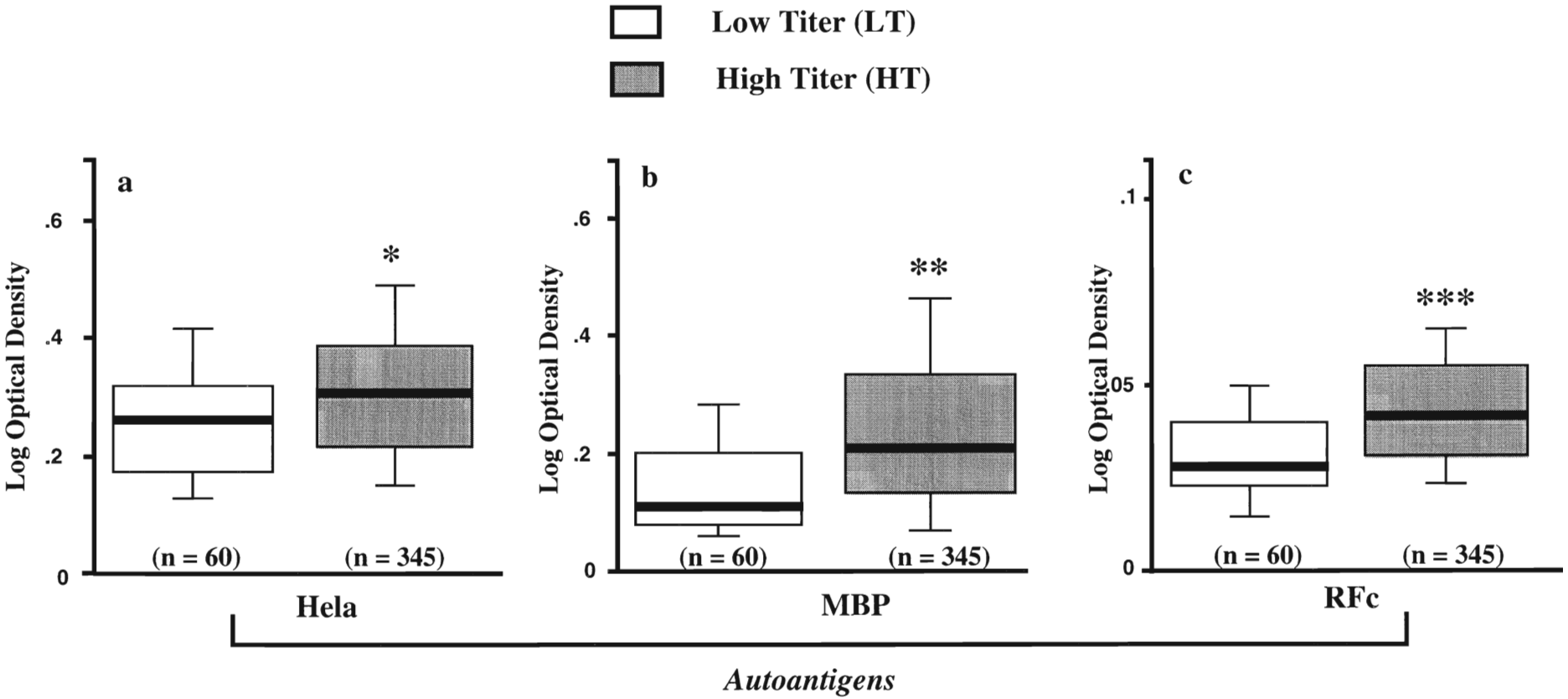
Figure 2 - Myelin Basic Protein-Specific Autoreactivity in Control Subjects and Haitian Children



Autoreactivity to MBP by ELISA in a) vaccine recipients by age or b) natural disease by time after wild-type exposure. Results are expressed as the log-optical density represented by Box Plots with mean (▬) and range in percentiles (10% - 90% inclusive), (n), * p = 0.0001.

Figure 3 - Reactivity Against Autoantigens in Standard Titer and Higher Titer Measles Vaccine Recipients in Haitian Trial



Autoreactivity in LT (Schwarz standard) and HT (Schwarz and Edmonston Zagreb high titer) measles vaccine recipients to; *a.* Hela cell lysate, *b.* myelin basic protein and *c.* Rheumatoid Factor (RFc) by ELISA. Results are expressed as the log-optical density represented by Box Plots with mean (▬) and range in percentiles (10% - 90% inclusive); * p = 0.04, ** p = 0.0001, ***p = 0.003.

4.3.2. MANUSCRIPT VI

Measles-induced Apoptosis in Human Peripheral Blood Mononuclear Cells

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Running Head: Measles-induced apoptosis
Key Words: apoptosis, PBMC, measles, measles vaccine, viral strain

Abstract

Background: Measles virus can productively infect a wide range of cells including macrophages, B and T lymphocytes. Natural disease is associated with profound lymphopenia, and established immune responses can be suppressed for weeks to months after infection. Measles-induced apoptosis has been reported in SSPE brain tissues, in SCID-hu thymocytes and in a variety of cell lines infected *in vitro*. Increased spontaneous apoptosis in peripheral blood mononuclear cells (PBMC) has been noted for weeks to months after natural measles virus infection. We examined the kinetics of apoptosis in PBMC subsets and the role of viral strain in promoting programmed cell death.

Methods: PBMC from healthy subjects were stimulated with PHA and infected with virus at a multiplicity of infection (m.o.i.) of 1.0. Virus-induced apoptosis in individual PBMC subsets was evaluated between 12 - 96 hours after infection using the TUNEL assay. The role of viral strain on apoptosis was evaluated using a panel of wild-type viruses (n = 8) and both partially (Edmonston B) and fully attenuated vaccine-strain viruses (n = 3).

Results: Background levels of apoptosis in uninfected PBMC were low (2% and 12% at 48 and 96 hours respectively). Apoptosis induced by wild-type virus (CHI-1) was first detectable at 24 hours after infection, was substantial at 72 hours (67 ± 6%; range 38 - 96%) and was massive by 96 hours (90 ± 11%; range 75 - 100%). Replicating virus was required since little increase in cell death was seen in the absence of mitogen or with inactivated virus. Macrophages were relatively resistant to measles virus-induced apoptosis compared to lymphocytes (e.g. 33 ± 3% vs. 61 ± 5%, respectively at 72 hours; $p < .01$) despite equal levels of infection. The apoptotic stimulus induced by measles virus appeared to be strain dependant as wild-type viruses were several-fold more potent than vaccine strains ($p < 0.003$; 72 hours).

Conclusion: These findings may help to explain both the lymphopenia and loss of effector cell function associated with natural measles. Differences in virus-induced

apoptosis between wild-type and vaccine-strain measles viruses may be a useful surrogate marker for virulence.

Introduction

Measles virus causes massive disruption of the human immune system. Although measles can occasionnally kill infected individuals outright (eg: giant cell encephalomyelitis, pneumonitis, SSPE) (Griffin DE & Bellini WJ 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 AP & Kaschula RO 1985, Griffin DE & Bellini WJ 1996). Measles-induced immune suppression is characterized by massive lymphopenia, decreased NK cell function, poor mitogen responsiveness, loss of antigen-specific lymphoproliferative responses and disappearance of DTH to recall antigens (Wesley A et al 1978, Griffin DE et al 1990b, Ward BJ et al 1991, Tamashiro VG et al 1987). The mechanisms that underlie these abnormalities have received considerable attention during the past decade. Factors that may contribute to measles-associated immune disruption include altered cytokine production patterns (Karp CL et al 1996, Bell AF et al 1997), soluble suppressors (Sun XM et al 1998), changes in co-stimulatory molecule expression and membrane receptor signalling (Ravanel K et al 1997), altered Vβ T cell receptor usage (Auwaerter PG et al 1996a), as well as induced stasis (Schnorr JJ et al 1997a, Nanaiche 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 measles virus infection (Wesley A et al 1978) and the transient loss of pre-existing immune responses such as DTH (Tamashiro VG et al 1987).

Measles-induced apoptosis has been documented in monocytes and other human and primate cell lines (Esolen LM et al 1995), follicular dendritic cells (FDC) (Fugier-vivier I et al 1997), thymocytes in a HuSCID mouse model (Auwaerter PG et al 1996b) and in brain tissues of SSPE patients, (Mcquaid S et al 1997). Furthermore, increased spontaneous PBMC apoptosis had been reported for weeks to months after natural diseases (Pignata C et al 1998). It is somewhat suprising therefore, that the only reported attempt to

demonstrate measles-induced apoptosis in isolated human PBMC showed little or no effect (Ito M et al 1997). We describe the development of an *in vitro* model for measles virus-induced apoptosis in PBMC and demonstrate marked differences between wild-type and vaccine-strain viruses in their capacity to initiate programmed cell death.

Methods

Measles Strains. A total of 12 virus strains were used in this study: 3 fully attenuated vaccine strains; Edmonston Enders, (gift from R Wittes, Connaught Laboratories LTD, Willowdale, ON), Edmonston-Zagreb and Schwarz (gifts from P Rota, Atlanta, GA), one partially attenuated vaccine strain; Edmonston B, (gift from M. Hilleman, Merck Vaccines), and 8 wild-type strains; Bilthoven (gift from A.Osterhaus, Rotterdam, Holland), Chicago-1, Massachussetts, Minnosota, Utah, Hawaii, Gambia, and Guam (gifts from W Billini and P Rota, Atlanta, GA). Viruses were plaque purified using African green monkey Vero cells, and re-seeded on Vero cell monolayers in MEM (Life Technologies, Grand Island, NY) + 5% heat inactivated FBS (Life Technologies) for 48 - 72 hours. Cell-free supernatents were harvested at 50% cytopathic effect (CPE) and at 90 - 95% CPE monolayers were freeze-thawed. Harvests were pooled, centrifuged at 500 x g for 10 minutes and supernatants were filtered (0.22 μm; Corning-Costar, Cambridge, MA). Viral titers were determined by plaque assay. Although the precise passage history was not available for all of the virus strains used, the vaccine strains and most of the wild-type strains grew well in Vero cells forming visible plaques in only 4 - 5 days. Guam and Utah wild-type viruses grew least well in Vero cells, but still formed detectable plaques within 6 - 7 days. Viral stocks were aliquoted and frozen at -70°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 measles virus proteins was detected as previously described (Ward BJ et al 1990). Briefly, immunoperoxidase staining was carried out on cultured PBMC with or without measles virus 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 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 BJ et al 1995). PBMC were washed twice in HBSS (Life Technologies), and 1 x 10^6 PBMC in MEM were infected with vaccine strain or wild-type virus at a m.o.i of 0.1 or 1.0. Virus particles (viable or inactivated) 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 phytohemaglutinin (PHA: 2.5 µg/mL, Sigma Fine Chemicals, Oakville, ON) and cultured in 1 mL of RPMI with 10 mM HEPES, 2mM glutamine and 50 µg/mL gentamicin (all Life Technologies) 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 from replicate cultures to quantify viral titers 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 (Life Technologies) and stained with FITC or PerCP-conjugated monoclonal antibodies to CD4, CD8, CD19, CD14 (Becton Dickinson, San Jose, CA) and CD95 (Dako, Toronto, ON) according to the manufacturer's instructions. Labelled cells were washed with ice cold PBS and resuspended in PBS containing 1% paraformaldehyde and .05% sodium azide (American Chemicals, Montreal, QC) and .05% sodium azide (American Chemicals). The TUNEL assay was used to detect cells undergoing apoptosis. Briefly, fixed 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 TUNEL reaction mixture: (TUNEL reaction buffer: 200 mM potassium cacodylate, 25 mM Tris-HCL, 1 mM $CoCl_2$; Pharmingen, Mississauga, ON) dUTP-Biotin (Boerhinger Mannheim, Toronto, ON) and terminal-deoxytransferase (TdT) (Pharmingen) for 1 hour at 37°C in the dark. PBMC were then washed and incubated in the dark with 5μL of PE-conjugated streptavidin (Dako) for 30 minutes at 37°C. After washing dUTP incorporation was measured by flow cytometry within 2 hours of staining and data was analysed using CellQuest software (Becton Dickinson). PBMC exposed to ultraviolet-radation (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 a percent of PBMC or PBMC subset undergoing apoptosis or expressing a given marker (e.g. CD95).

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 serial 10-fold dilutions of culture supernatant for 90 minutes at 37°C in 5% CO_2 (duplicate wells, 100 μL/well diluted in HBSS). A 16% methylcellulose (Life Technologies) overlay was applied to each well (200 μL) and allowed to gel prior to incubation 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 (Life Technologies) and visible plaques in duplicate wells were counted. Results are expressed as plaque forming units (pfu/mL).

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%) measles virus infection. Very few cells (< 5%; range 0 - 5.1%) were found to express measles virus 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 measles proteins (97 ± 2%). Background apoptosis at 72 hours after mitogen stimulation was also low in mock-infected cultures (7.8 ± .9%; range 1 - 12%), but increased dramatically with measles virus infection (e.g. CHI-1 mean 54.5 ± 4%, range 39 - 96%, $p < 0.003$) (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). Although all PBMC subsets were susceptible to measles virus-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%; $p< 0.001$, $p < 0.001$, $p < 03$, 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, measles-induced apoptosis was first detectable at 24 hours (Figure 3a-e). Incorporation of dUTP increased progressively thereafter to peak between 72 and 96 hours after infecton. The vast majority of infected, PHA-stimulated PBMC were dead by 96 hours (Figure 3e) as determined by trypan blue exclusion assay (data not shown).

Surface expression of Fas. Expression of CD95 on CHI-1-infected cells rose from a baseline level of 22 - 38% at the time of infection, to ~ 100% by 48 - 72h (Figure 4). Although CD95 expression on mock-infected cells rose slightly with time after infection, levels were significantly less than expression on infected cells throughout the 4-day period of observation (Figure 4).

Effect of measles strain on apoptosis. The degree of measles-induced apoptosis varied considerably between the strains tested. For example, one of the wild-type strains (Hawaii) consistantly induced only modest amounts of apoptosis compared with the Guam ($67 \pm 4\%$) and Bilthoven strains ($81 \pm 3\%$; $p < 0.001$ and $p < 0.001$, respectively) (Figure 5). However, overall the wild-type strains induced ~ 2 - 3 fold more apoptosis in PBMC than the vaccine strains; ($15.5 \pm 2\%$ vs $40.5 \pm 6\%$ at 72 hours in culture; $p < 0.003$) (Figure 5). It is particularly interesting that the partially attenuated vaccine strain, Edmonston B, induced intermediate levels of apoptosis ($26 \pm 4\%$) (Figure 5). Although the extent of apoptosis varied somewhat with the origin of the PBMC used in any given experiment, the rank order of potency between virus strains was consistently observed in different samples within an experiment and between experiments using samples from the same individual performed on different days. Plaque morphology on Vero cell monolayers varied considerably between strains but viral output in PHA-stimulated PBMC cultures was generally higher for wild-type viruses (5 - 10 fold) than vaccine strains overall ($p < 0.09$) (Table 2).

Discussion

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

In the current study, we observed striking levels of apoptosis induced by many wild-type strains of measles virus. Although vaccine strain viruses were generally less able to initiate programmed cell death in PBMC, there was considerable variability. One of the vaccine strains (the partially attenuated Edmonston B) consistantly induced more apoptosis than two of the wild-type strains (Hawaii and Massachussetts). Although some of these differences 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 tissue culture growth and apoptosis induction in PBMC. In ongoing experiments we are trying to relate the growth characteristics of viral strains in different cell lines with their capacity to induce programmed cell death.

In addition to the variable capacity of viral strains to induce apoptosis, we also noted considerable variability in the susceptibility of PBMC isolated from different individuals to undergo measles virus-induced apoptosis. The differences between individuals were independant of viral strains (ie: the relative potency of the various strains was maintained) and appeared to be a stable characteristic of the individuals whose blood

cells were used over a period of 10 - 12 months. 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 factor". We do not yet know if the individual differences in viral-induced apoptosis reflect permissiveness of the host PBMC (ie: higher viral output/cell) or are adaptive (ie: 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 programmed cell death in PBMC help to explain the differences in measles virus-induced apoptosis reported in the literature (Ito M et al 1997, Schnorr JJ 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 (ie: clinical presentation, immunosuppression, complications) (Griffin DE & Bellini WJ 1996) and possibly the increased mortality associated with HT measles vaccines (Halsey NA 1993, Knudsen KM et al 1996).

The relative resistance to measles-virus induced apoptosis that we observed in monocytes suggests that these cells may act as viral resevoirs *in vivo*. In this context, it is interesting that Esolen et al. found measles virus nucleic acid only in the monocyte fraction of PBMC isolated from children with natural disease (Esolen LM et al 1993).

The mechanism of measles-induced apoptosis remains unknown. Unlike HIV, SIV, SIV, and HTLV-1 (Adamson D et al 1996, Yamada T et al 1994, Li CJ et al 1995), receptor cross-linking alone is not a sufficient stimulus to trigger the cell death cascade, as UV- and heat-inactivated measles virus were incapable of inducing apoptosis. In the case of measles virus, 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 JJ et al 1997). 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 report on spontaneous apoptosis following natural measles infection, its is unclear whether or not the cells

undergoing spontaneous apoptosis in this study were infected by measles virus (Pignata C et al 1998). Other surface molecules, such as TNF, TNFr, CD95L, and TRAIL as well as the family of cytoplamic proteins, (caspase 1-9), will need to be examined to fully understand the cascade of events leading to measles-induced apoptosis (Green D 1998).

The findings of the current study further emphasize the extraordinary breadth of the attack initiated by measles virus against the human immune system. It is very likely that measles-induced apoptosis of PBMC contributes significantly to the lymphopenia and loss of memory cell functions associated with this disease (Wesley A et al 1978, Starr SE 1967, Tamashiro VG 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 1997, Schnorr JJ et al 1997) and the clinical presentation of the disease remain to be determined. Finally, our observations using a modest number of strains raises the possibility that measles virus-induced apoptosis might be a relatively simple surrogate marker for virulence.

Table 1 - Apoptosis in PBMC Subsets

Cell type	*Percent Apoptosis*	
	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

PBMC were infected with measles virus (CHI-1, m.o.i. = 1.0). At 24 and 72 hours cells were harvested, labelled with monoclonal antibodies to T cells (CD4, CD8), B cells (CD19) or monocytes (CD14) and apoptosis was detected by TUNEL assay. Results are expressed as the percent of a given subset undergoing apoptosis, (* $p < 0.001$, †$p < 0.01$ vs. monocytes).

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Table 2 - Viral Output in Wild-Type and Vaccine Strain-Infected PBMC

	Virus Output (pfu/100 uL)			
	24 Hours	**48 Hours**	**72 Hours**	**96 Hours**
Virus Strain				
Connaught	**375**	**2624**	**3450**	**22000**
Bilthoven	**3400**	**15500**	**29500**	**140000**

PBMC were infected with wild-type (Bilthoven) or vaccine strain (Connaught) measles virus (m.o.i. = 1.0). Culture supernatants were collected at various time points after infection and viral output was determinde by plaque assay (expressed as plaque forming units (pfu)).

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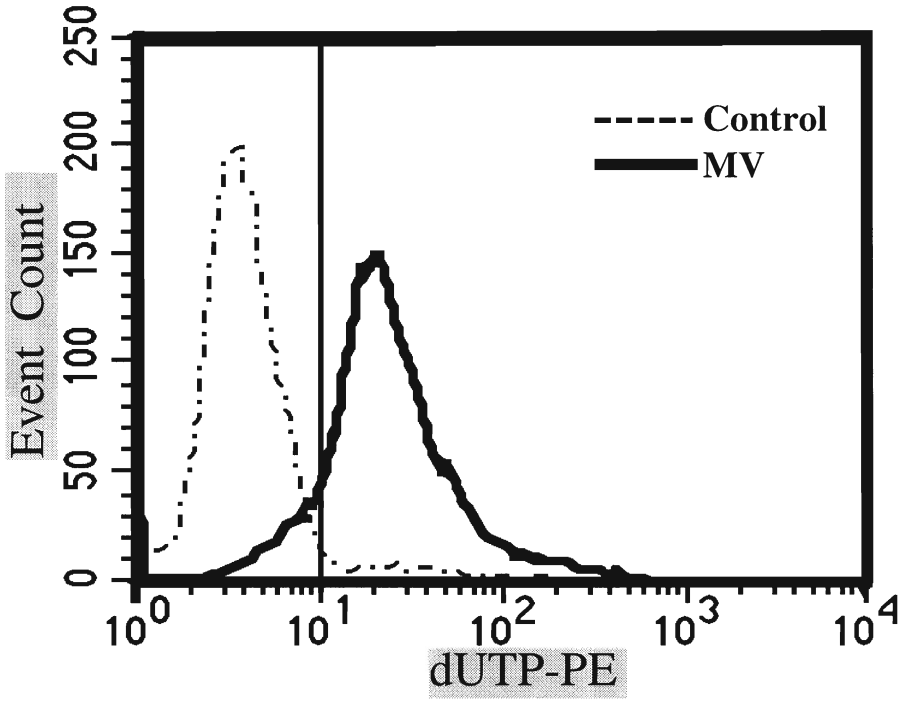
Figure 1 - Apoptosis in Measles-Infected PBMC



Histogram of representative data of PBMC apoptosis in TUNEL assay 72 hours after infection with CHI-1 (wild-type) measles virus (87.9%) or mock-infection (3.8%) .

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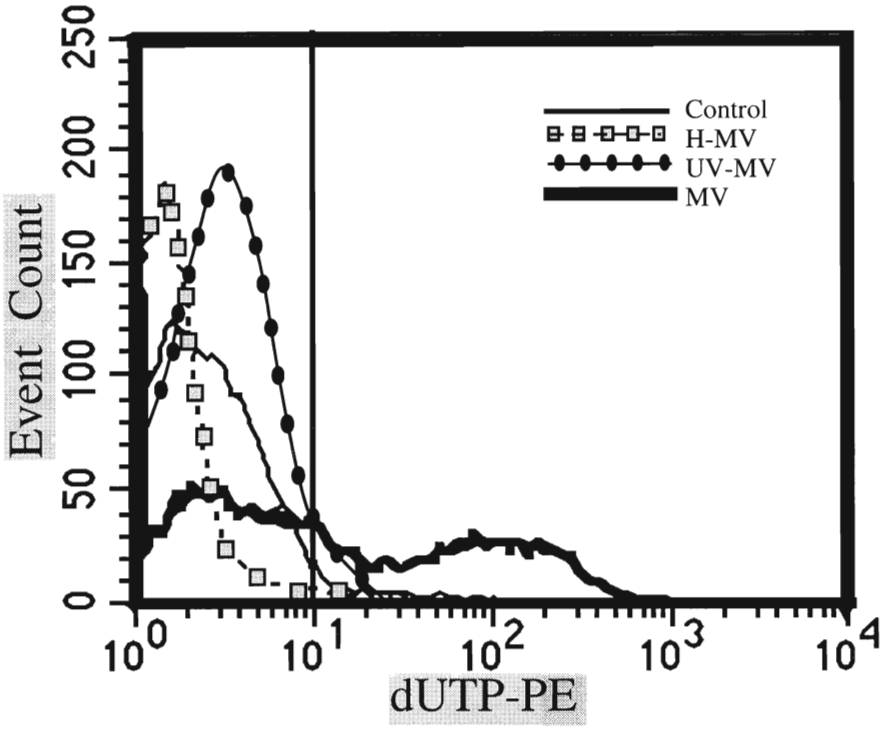
Figure 2 - Effect of Virus Inactivation on Measles-Induced Apoptosis



PBMC were infected (m.o.i. = 1.0) with CHI-1 virus inoculated by heat (H-MV) or UV-irradiation (UV-MV), or live CHI-1 virus (MV) and dUTP incorporation was measured. Percent apoptosis at 48 hours was 3.3%(Control), 1.2%(H-MV) , 6.6%(UV-MV) and 53.3%(MV).

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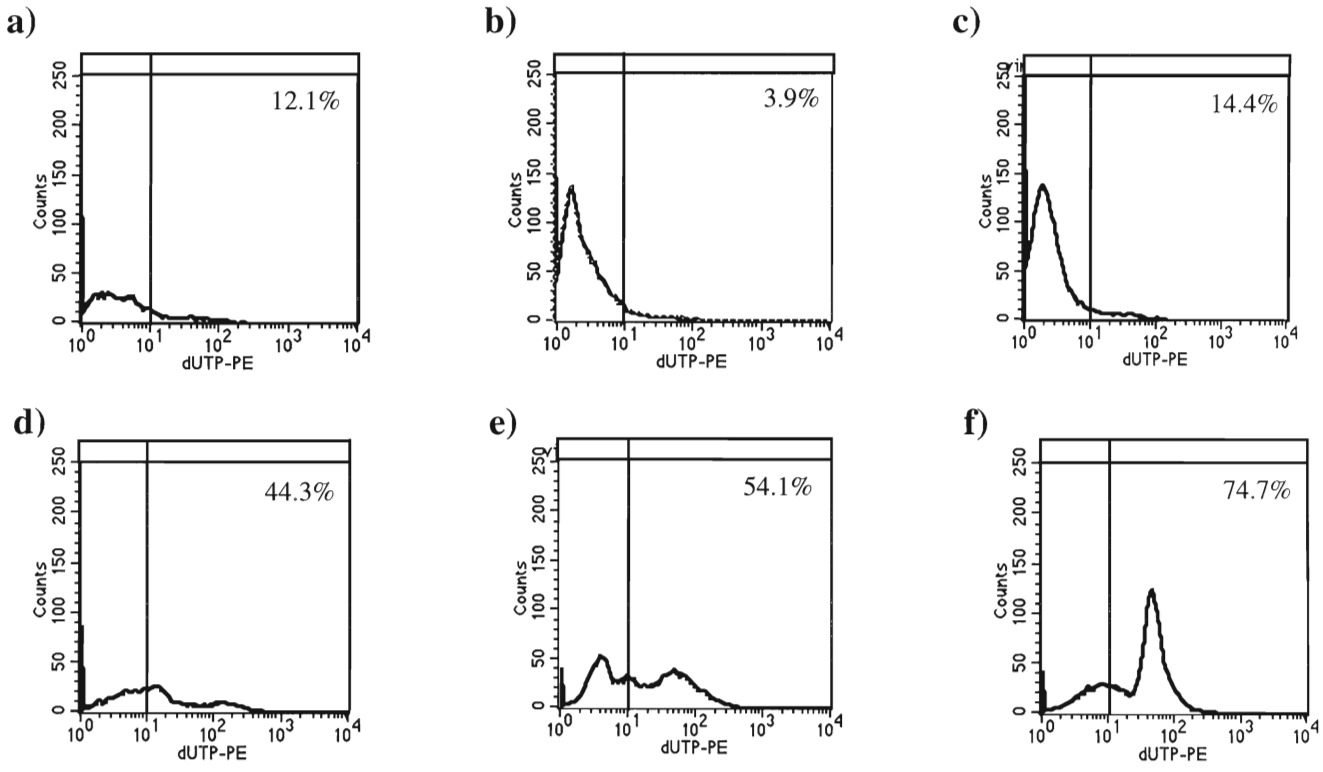
Figure 3 - Kinetics of Measles Virus-Induced Apoptosis



PBMC were infected with wild-type MV (CHI-1; m.o.i. = 1.0) and cultured for 5 days. Apoptosis was measured by TUNEL assay at 12, 24, 48 72 and 96 hours (b - f). (a) apoptosis in mock-infected PBMC at 96 hours.

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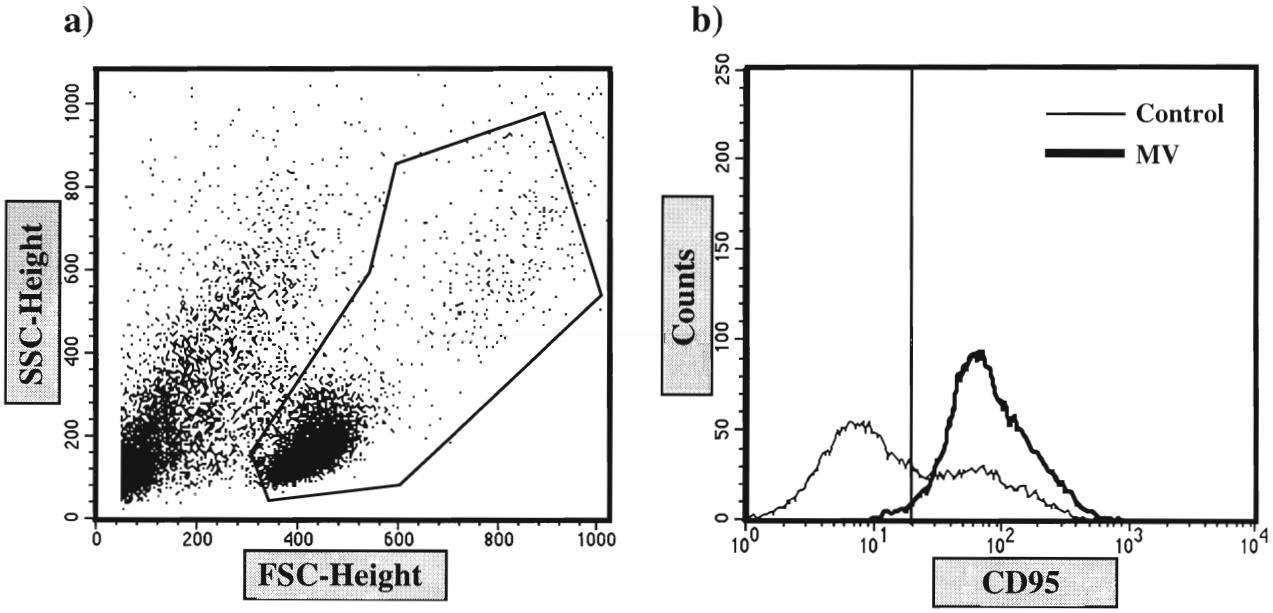
Figure 4 - CD95 Expression After Measles Virus-Infection



PBMC were infected with wild-type virus (CHI-1; m.o.i = 1.0) and CD95 expression was measured at 72. a) *Dot plot* indicating gated population b) *Histogram* indicating CD95 expression on measles infected PBMC (MV: 94.6%) vs. mock-infected cells (control: 34.5%).

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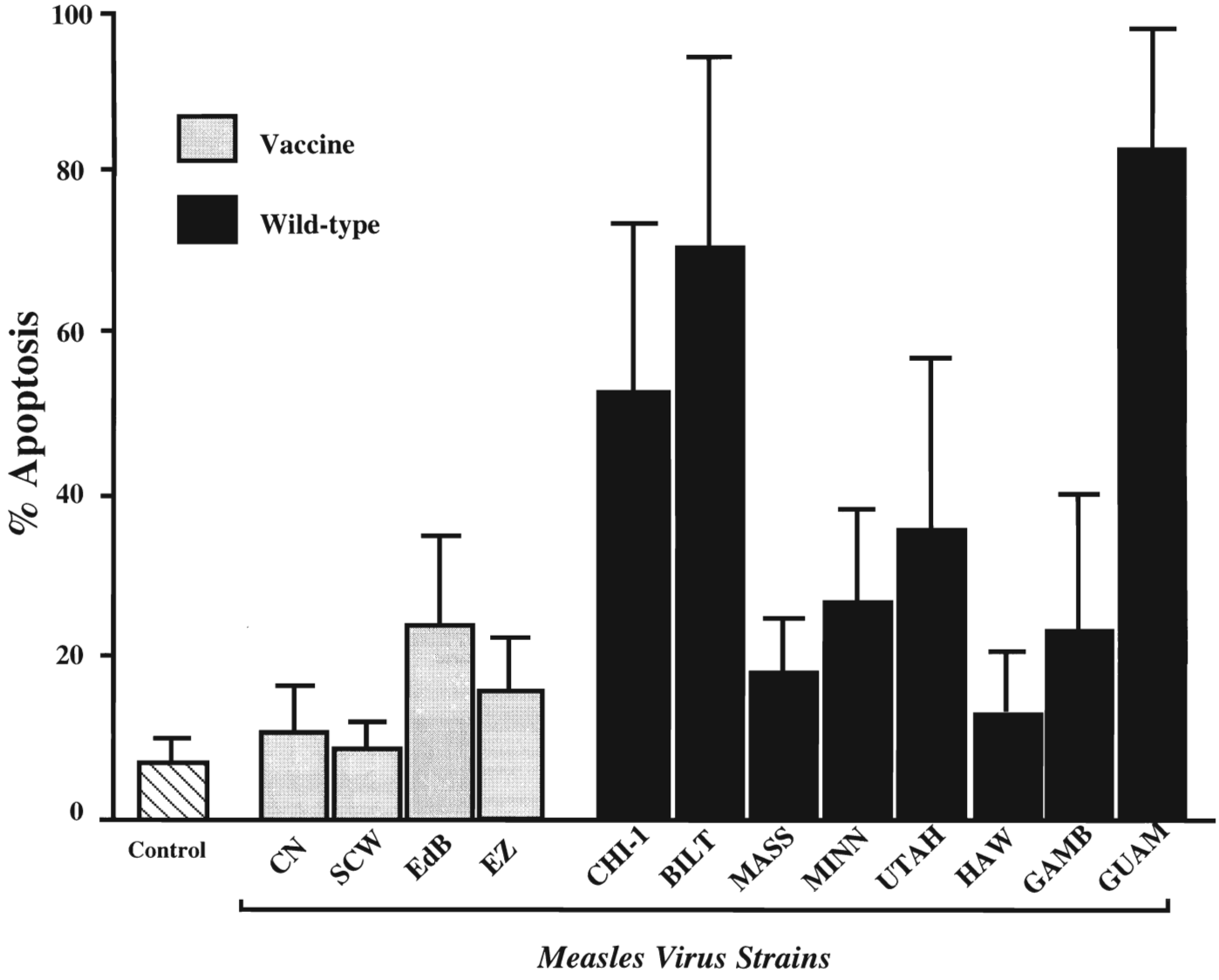
Figure 5 - Effect of Viral Strain on Measles-Induced Apoptosis



PBMC were infected with vaccine strain (CN; Connaught, SCW; Schwarz, EdB;Edmonston B, EZ; Edmonston Zagreb) or wild-type measles virus (CHI-1; Chicago- 1, BILT; Bilthoven, MASS; Massachussetts, MINN; Minnesota, Utah, HAW; Hawaii, GAMB; Gambia and Guam) at m.o.i. = 1.0. A control group of mock-infected PBMC was included. At 72 hours apoptosis was determined by TUNEL assay. Overall wild-type virus vs vaccine strain; $p < 0.003$.

APPENDIX I

4.3.2.1 Vitamin A Enhances Measles Virus-induced Apoptosis of PBMC

Introduction

The *in vitro* model described in Manuscript VI was also used to evaluate the impact of supplemental retinol on measles-induced apoptosis. Retinol (vitamin A) is an essential, fat-soluble vitamin that is tightly regulated and functions in the body as an exogenous hormone (Wolback 1925). Vitamin A plays a critical role in embryogenesis, the maintenance of ephithelial integrity and optimal immune function of the immune system (Sommer 1995, Deluca 1991). In the last 15 years vitamin A has been rediscovered as a major factor in the recovery from infectious diseases and prevention of infant mortality (Sommer 1983, Beaton 1993). The anti-infective nature of vitamin A is most impressive in the context of measles virus infection (Hussey 1990). For example, supplementation of hospitalized patients with serious measles with even modest doses of vitamin A can reduce measles-associated mortality by as much as 50% (Ellison 1932, Barclay 1987, EPI: Wkly Epi Rec 1987). The mechanism of benefit of vitamin A supplementation in measles is unknown.

One of the central interests of our laboratory is the investigation of possible mechanisms of vitamin A action. To date, we have found that retinoid treatment of measles virus-infected PBMC cultures results in a 30 - 40% reduction of virus output. Preliminary data suggests that the retinoid(s) can change viral transcription patterns and prevent measles virus-induced changes in the retinoid signal cascade. In a limited study, we evaluated the possible impact of retinoids on apoptosis in infected cultures.

Materials and Methods

Retinoids. Retinol (ROH) and retinol binding protein (RBP) (Sigma Fine Chemicals, Ottawa, ON) were solubilized in DMSO at 10^{-3}M, and stored at -70°C in opaque eppindorfs until use. Immediately prior to use, equimolars of ROH and RBP were mixed (1:1) to permit the evaluation of the more physiologic ROH/RBP heterodimer.

Measles Virus Infection. 1 X 10^6 human PBMC isolated from healthy adult volunteers were stimulated with PHA (2.5 μg/mL) and infected with CHI-1 measles virus at a m.o.i of 1.0 as in Manuscript VI. Infected cells were washed in cRPMI and cultured at 37°C and 5% CO_2 in cRPMI + PHA. Vitamin A (2μM of ROH/RBP) was added at 24 hour intervals, beginning at time 0 and until 72 hours after infection. Mock-infected vitamin A treated PBMC and infected PBMC without vitamin A served as experimental controls.

Detection of Apoptosis. The TUNEL assay was performed as in Manuscript VI.

Results

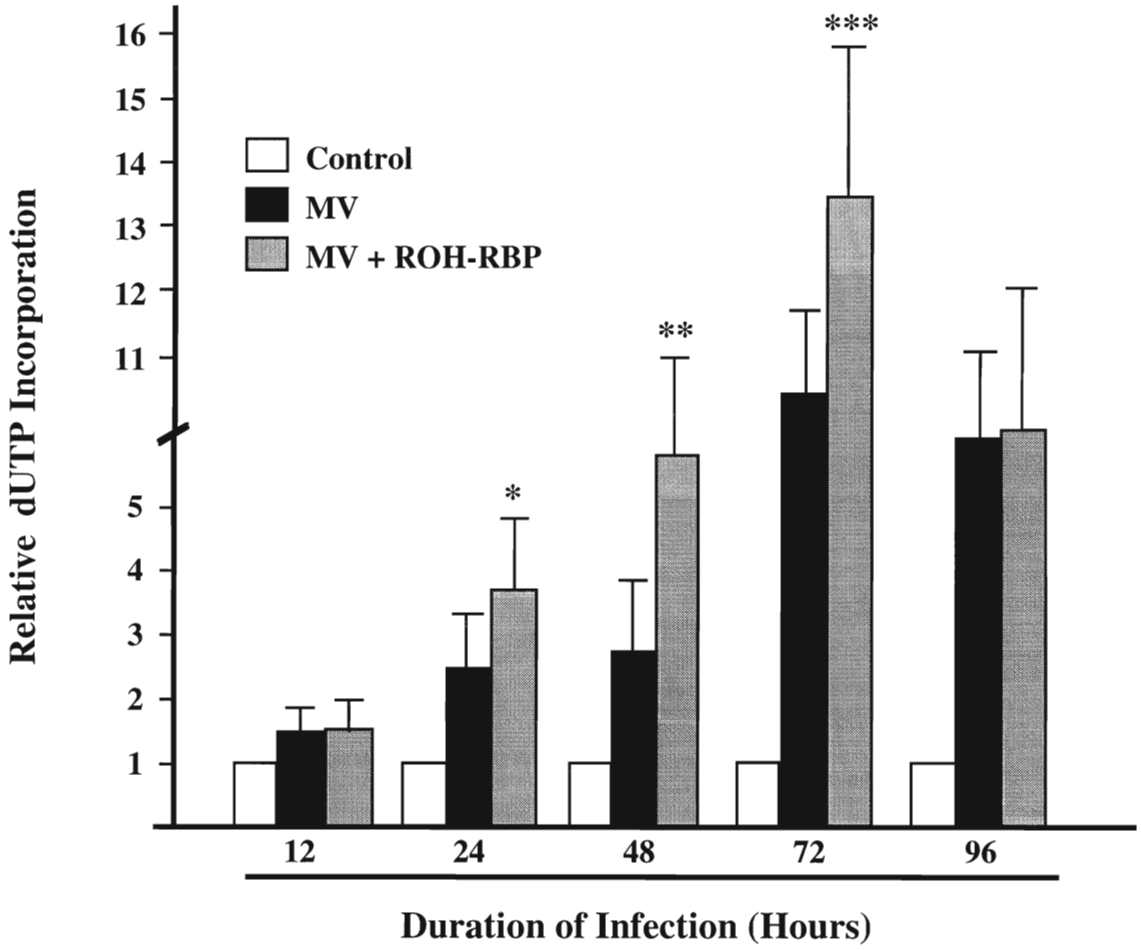
PBMC from adult volunteers were isolated and infected with measles virus. Apoptosis was measured at 24 hour intervals following infection. Vitamin A treatment of mock-infected PBMC had no effect on programmed cell death at any time. Mean levels of dUTP incorporation for vitamin A-treated and untreated PBMC at 72 hours were $11 \pm 2\%$ and $9 \pm 2\%$ respectively. In contrast, vitamin A treatment markedly altered the kinetics of apoptosis in the measles virus-infected cultures. Accelerated apoptosis was apparent as early as 24 hours but these differences did not reach significance. By 48 hours, infected PBMC exposed to vitamin A were entering programmed cell death at a much faster rate (mean $42 \pm 6\%$ versus $20 \pm 3\%$, $p < .02$) that persisted through to 72 hours (Figure 1; $p < .03$). At 96 hours following infection, virtually 100% of treated and untreated measles virus-infected PBMC were incorporating dUTP.

Discussion

Vitamin A treatment of infected PBMC accelerated the onset of measles virus-induced apoptosis and shortened the time required for 100% apoptosis of PBMC in culture. Together with the observation of decreased viral output in vitamin A-treated cultures, this finding of accelerated apoptosis provides the first biologically plausible mechanism of vitamin A benefit in measles.

The balance between measles virus and the human immune system is dynamic. In the vast majority of cases, the immune system eventually clears the infection, but even previously healthy individuals become seriously ill with measles for short periods of time. In North America, hospitalization rates in recent outbreaks have been as high as 50% for children < 1 year of age and 33% for adults. Even relatively modest changes in the balance between the virus racing to replicate and the human immune system's pressure to suppress it, may have a major impact on clinical presentation. Accelerated apoptosis in a major target tissue for measles virus may plausibly be such an influence. The mechanism by which retinoids enhance measles-induced apoptosis in our model is currently under investigation.

Figure 1 - Effect of Retinoids on Measles Virus-Induced Apoptosis in PBMC



PBMC were infected with wild-type measles virus (CHI-1; m.o.i. = 1.0) in the presence or absence of vitamin A (2uM ROH-RBP). Apoptosis was assessed by TUNEL assay at 24, 48, 72 and 96 hours and results are expressed as relative amounts of dUTP incorporation compared to mock-infected controls. * $p < .08$, ** $p < .02$, *** $p < .03$ vs. cultures with vitamin A.

APPENDIX II

4.4 SPECIFIC AIM #3:

Immune Priming by Early Measles Vaccination

The samples collected for the immunologic follow-up of the children in the HT measles vaccine trial in the Sudan allowed us to address the question of whether or not early exposure to measles vaccine in the presence of maternal antibodies might "prime" the immune system for a particular pattern of response upon re-exposure to measles antigens (via natural infection or booster vaccination). This question has considerable importance for the development and implementation of measles vaccination programs. Our results are described in Manuscript VII.

MANUSCRIPT VII

- Concise Communication -

Measles Vaccination in the Presence of Maternal Antibodies Primes for a Balanced Humoral and Cellular Response to Revaccination

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Running Head: Priming for lymphoproliferative response to measles antigen

Key Words: measles, measles vaccine, cellular immunity, immunologic priming, booster

Abstract

Background: The potential for initial antigen exposures to influence subsequent immune responses has only recently been recognized. Administration of measles vaccine in the presence of maternal antibodies is known to reduce the capacity of children to mount a durable antibody response to subsequent (booster) doses of vaccine. In the context of a high titer (HT) measles vaccine trial in the Sudan, we were able to assess whether or not such early exposure to measles virus antigens could "prime" the immune system for a balanced humoral and lymphoproliferative response to subsequent revaccination.

Methods: A trial of HT vaccines conducted in the Sudan included groups of children who received either Connaught HT measles vaccines (CN-HT: $10^{4.7}$pfu) or placebo (meningococcal A + C vaccine) at 5 months of age. At 9 months of age, all children (n = 340) received standard titer Schwarz vaccine (Placebo-SCH: $10^{3.9}$pfu). Neutralization antibody titers were measured before each of the vaccines (plaque reduction neutralization) and subsequently at ~ 5 years of age (syncytium inhibition assay). Lymphoproliferative responses to measles antigens were also evaluated at ~ 5 years.

Results: Twelve of the 54 children (22%) who received the CN-HT vaccine at 5 months of age had subprotective neutralizing antibody titers prior to revaccination at 9 months (mean log PRN: 1.6 ± .3 vs. 3.1 ± 2.3 in the remaining children; $p < 0.004$). Maternal antibodies at the time of vaccination in the 12 poor responders were high (2.6 ± 2.2 vs. 1.9 ± .9; $p < 0.0002$). All 12 of these children mounted excellent antibody responses following revaccination at 9 months of age and had neutralizing titers at 5 years of age that were slightly lower but not significantly different from those CN-SCH recipients who responded well to the first dose of vaccine (mean log SIA: 2. 3 ± 1.6 vs. 2.4 ± 1.7 respectively) and the Placebo-SCH group vaccinated only once at 9 months of age (SIA: 2.5 ± 1.9). In contrast, 7 of these 12 children (58.3%) had significant lymphoproliferative responses (SI ≥ 3) to measles virus antigens at 5 years of age (SI = 3.03 ± .51) compared

with only 14.3% in the remainder of the CN-SCH group (SI = 1.98 ± .32; $p = 0.07$) and 7.7% (SI = 1.44 ± .93; $p < 0.0003$) in the Placebo-SCH group.

Conclusion: Our data suggest that early measles vaccination in the presence of maternal antibodies primes for a balanced humoral and cellular immune response to revaccination.

Introduction

The role of antigen dose in determining the balance between antigen-specific humoral and cellular immune responses has recently gained considerable attention (Bretscher PA et al 1992, Hosken NA et al 1995, Singh RR et al 1996, Guery JC et al 1996, Partidos CD et al 1997). For example, low doses of *Leishmania Major* antigen (Lma) antigen appear to prime the murine immune system in a normally susceptible stain for a protective cellular response against subsequent leathal-dose challenge (Bretscher PA et al 1992). Increased γ-IFN production and cell mediated immunity have been reported in this murine Leishmania model following low-dose, "prime-boost" vaccination compared to single-time high dose immunization (Bretscher PA et al 1992). Although the mechanism of low dose immune deviation is not yet is not fully characterized, naive $CD4^+$ T cells are implicated with the generation of Th_1-like cytokines in response to intermediate doses (0.3-0.6μM) of antigen, while lower or higher concentrations of antigen induce a Th_2 response (Hosken NA et al 1995).

During vaccination, several parameters can effect the dose of antigen to which the immune system is ultimately exposed. In young children, the presence of antigen-specific maternal antibodies at the time of vaccination may be particularly important when live attenuated organisms are used (Redd SC et al 1999, Osterhaus A et al 1998, Siegrist CA et al 1998b). These vaccines typically contain inocula that are well below the threshold required to initiate an immune response if little or no replication occurs (eg: 10^3 pfu in most measles vaccines) (Redd SC et al 1999). As a result, even modest concentrations of measles-specific maternal antibodies can significantly influence the quantitiy of antigen available to the immune system, and perhaps the mechanisms by which this limited antigen is processed (Osterhaus A et al 1998, Siegrist CA et al 1998a,b). It has long been known that early measles vaccination in the presence of maternal antibodies, results in relatively poor antibody production and what appears to be a permanent state of hyporesponsiveness

(in terms of antibody production) to subsequent booster doses of vaccine (Redd SC et al 1999).

In recent years, the potential importance of cellular responses to measles virus vaccination has been recognized. Several studies in animal models and humans have demonstrated that some individuals with poor or undetectable antibody responses after vaccination have enhanced cell-mediated immunity (Ward BJ et al 1995, Osterhaus A et al 1998, Siegrist CA et al 1998a,c, Gans HA et al 1999).

In the context of a high titer measles vaccine trial in the Sudan, we were able to address the question of whether or not early measles virus vaccination in the presence of maternal antibodies influences the balance of humoral and cell-mediated immune responses to a subsequent dose of vaccine. This study included two groups of particular interest: (i) children exposed to measles vaccine at 5 months of age (in the presence of varying concentrations of maternal antibodies) and revaccinated at 9 months, and (ii) children vaccinated once at 9 months of age. At ~ 5 years of age, we were able to compare the pattern of immune response present in children who failed to produce protective titers of antibodies after the first dose of measles vaccine at 5 months, with that observed in children who responded well or received only one dose of vaccine.

Materials and Methods

Study Subjects: A trial of HT measles vaccine initiated in the Sudan in 1989 (Hoskins 1993) included groups of children that received either HT Connaught vaccine (CN-HT: $10^{4.7}$ pfu, Edmonston Enders, Connaught, Mississauga, ON) or "placebo" (meningococcal A + C, Smith Kline Beecham Pharma, Oakville, ON) at 5 months of age. Blood samples were obtained before the initial vaccination and immediately prior to revaccination with standard titer Schwarz vaccine (SCH: $10^{3.9}$ pfu) at 9 months of age. In the context of an immunologic follow-up study, we were able to collect a further blood sample at ~ 5 years of age from 54 children who had received 2 doses of measles vaccine (CN-SCH) and 59 children in the placebo group (Placebo-SCH) (Table 1).

Detection of humoral response: Measles-specific neutralizing antibody levels in the initial Sudanese trial (pre-vaccination and at 9 months of age) were measured by plaque reduction neutralization assay (PRN) by the Virology Laboratory of the London School of Hygiene and Tropical Medicine as previously described (Hoskins EW. In this assay, PRN titers ≥ 120 are thought to confer protection against symptomatic infection with measles virus. In the immunologic follow-up study (~ 4.5 years after vaccination), neutralizing antibody levels were determined by syncytium inhibition assay (SIA) as previously described (Ward BJ et al 1999, Manuscript I). Neutralizing antibody titers determined by SIA are highly correlated with PRN values and an SIA titer of 16 is roughly equivalent to a PRN value of 120 (Ward BJ et al 1999).

Plaque Reduction Neutralization assay (PRN). Briefly, serum or plasma specimens were heat-inactivated (56°C for 30 minutes) and serially diluted starting at 1:4 in Joklik's modified minimum essential media (SMEM; Bio-Whittaker, Walkersville, MD) in 96-well plates (120 μL/well). Samples were diluted either two-fold or four-fold depending upon the expected titer range: for example two-fold dilutions were used initially for pre-

vaccination samples and four-fold dilutions were used for samples obtained after vaccination. A standard inoculum of Edmonston vaccine-strain measles virus was added to each well (120 μL containing 50-70 plaque forming units (pfu)) and incubated at 37°C in 5% CO_2. After 100 minutes, the inoculum-plasma/serum mixtures were added to duplicate Vero cell monolayers at ~95% confluency in 24-well plates (100 μL/well) and incubated at 37°C in 5% CO_2. After 75 minutes, the inocula were removed and the monolayers were covered with an overlay of 1.6% methyl cellulose in Leibovitz's L-15 media (Life Technologies, Grand Island, NY; 500 μL/well). The plates were incubated for 4 days at 37°C in 5% CO_2 before the monolayers were stained with neutral red and the plaques were counted visually. The PRN values were determined using the Karber formula (Ballew HC 1986) to estimate the serum/plasma titer required to reduce the number of input pfu by 50%. PRN values < 8 are generally considered to be negative. Values ≥ 120 are thought to indicate protection from symptomatic disease (Chen RT et al 1989).

Syncytium Inhibition Assay. This assay was performed as previously described (Ward BJ et al 1999). Briefly, heat-inactivated plasma specimens were serially diluted (two or four-fold) starting at 1:4 in 96-well plates with Hanks buffered salt solution (HBSS; Life Technologies) containing 0.5% heat inactivated fetal bovine serum, 50 μg/mL gentamicin and 10 mM HEPES (Life Technologies; 100 μL/well). A challenge inoculum (125 pfu of either wild-type or vaccine-strain virus) was added to each well and incubated at 37°C in 5% CO_2. After 2 hours, 2 x 10^5 measles-susceptible B95.8 cells (ATCC #CRL-1612) diluted in acid RPMI 1640 with 50 μg/mL gentamicin and 30 mM HEPES and 20% fetal bovine serum (FBS) were added to each well (100 μL/well). Acid RPMI 1640 is prepared by adjusting RPMI 1640 (Life Technologies) to pH 6.8 with HCl. The plates were then incubated at 37°C in 5% CO_2 and giant cells (syncytia) were identified microscopically 72 hours later. SIA titers are reported as the highest dilution of plasma at which syncytium formation was completely inhibited.

Antigen Preparations. Measles antigen was prepared as previously described (Ward BJ et al 1995) from Vero cell monolayers infected with vaccine-strain CHI-1 wild-type virus (gift from W Bellini, Atlanta, GA) at a multiplicity of infection of 0.01. Culture supernatants were harvested at 90% cytopathic effect after one freeze-thaw cycle, centrifuged for 10 minutes at 500 x g and filtered (0.22 μM) (Falcon, Franklin, NJ). The filtrate was then centrifuged (20000 x g) for 2 hours at 4°C and the viral pellet was lightly sonicated and resuspended in PBS. Antigen prepared from mock-infected Vero cells processed identically were used as a negative control. Antigen concentration was determined by modified Bradford Assay (Biorad, Hercules, CA).

Lymphoproliferation Assay (LPA). Lymphoproliferation assays were performed as previously described (Ward BJ et al 1995). Briefly, measles and control antigens (10 μg/mL) were pre-coated onto 96-well plates in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C and washed twice with sterile Hanks buffered salt solution (HBSS; Life Technologies) immediately before use. Cryopreserved PBMC were quick-thawed, washed once in HBSS and resuspended at 1 x 10^6/mL in cRPMI (RPMI 1640, with 10 mM HEPES, 50 μg/mL gentamicin and 2μM glutamine; all Life Technologies) with 5%, heat-inactivated autologous plasma (56°C for 30 min). Cells were distributed at 2 x 10^5/well in triplicate wells for each antigen and incubated for 6 days at 37°C in 5% CO_2 before 1 μCi of ^{3}H-thymidine (ICN, Radiochemicals, Irvine, CA) was added to each well. Control and test antigens were coated onto the same plates to ensure similar culture conditions. Cellular DNA was harvested onto glass-fiber filters 24 hours later after one freeze-thaw cycle and incorporated ^{3}H-thymidine was estimated by beta-emission. Lymphoproliferative results are expressed as stimulation indices (SI = cpm of antigen-stimulated wells/cpm of control wells). A stimulation index (SI) ≥ 3 was considered to indicate a significant response.

Satistical Analysis. All results were analyzed using Statview® software (Cary, NC). Antibody data were log transformed to approximate a normal distribution. Student's t-test was used for all comparisons unless otherwise indicated in the text and results are expressed as mean ± standard error of the mean (SEM). All p values were generated using two-tailed comparisons.

Results

In the CN-SCH group, samples from all three time points were available for 54 children (serum at 5 and 9 months of age and plasma/PBMC at 5 years of age). In the Placebo-SCH group, samples from 59 children were available only at 5 years of age (plasma/PBMC). Despite the fact that maternal antibodies were detectable in the majority of the children in the CN-SCH group prior to the first dose of vaccine (mean log PRN: 2.7 ± 2.2) only 12/54 (22%) failed to generate levels of neutralizing antibodies believed to be protective (1.6 ± .3 vs. 3.1 ± 2.3 in the remaining 42 CN-SCH children; $p < 0.004$) (Figure 1). The pre-vaccination maternal antibody titers in the 12 children who failed to respond were very high compared with the remaining 42 CN-SCH children (2.7 ± 2.2 vs. 1.9 ± .9 respectively, $p < 0.0002$). Although pre-vaccination samples were not available for the children in the Placebo-SCH group, their maternal antibody status was presumed to be very low due to the ~ 28 day half life of these antibodies under the best of circumstances (Katz SL 1998).

At 5 years of age, all of the children in both groups had neutralizing antibody titers thought to be protective (log SIA > 1.2) (Figure 2). Overall, SIA titers for CN-SCH and Placebo-SCH groups were comparable (2.4 ± 1.5 and 2.5 ± 1.9, respectively). The highest titers were seen in the Placebo-SCH group (2.5 ± 1.9), the lowest were seen in the 12 CN-SCH children who had "failed" initial vaccination (2.3 ± 1.6) and the remaining 42 children in the CN-SCH group had intermediate titers (2.4 ± 1.7), but none of these differences reached significance. In contrast, lymphoproliferative responses to measles virus antigen were significantly higher in the 12 CN-SCH children who had "failed" initial vaccination (SI = 3.03 ± .48, $p < 0.0003$), intermediate in the remaining 42 CN-SCH children (SI = 1.98 ± .32) and lowest in children who had received only a single dose of vaccine at 9 months of age (SI = 1.44 ± .15) (Figure 3). Differences between the these same groups were also apparent when the proportions of children with SI ≥ 3 were compared: 58% in the 12 CN-SCH who "failed" primary immunization; 14% in the

remaining CN-SCH children ($p < .01$, Chi-Square) and 8% in the Placebo-SCH group ($p < .001$, Chi-Square) .

Although the 12 children in the CN-SCH group who failed to mount a strong antibody response to initial vaccination appear to have been primed to generate a lymphoproliferative response to the second dose of vaccine, there was no consistent relationship between maternal antibody status at the time of initial vaccination and the subsequent Ab titer at 5 years in the group as a whole (Spearman Rank correlation coefficient, $p = .258$). However, the maternal antibody status at the time of initial vaccination was strongly correlated with the lymphoproliferative responses at 5 years ($p = .01$).

Discussion

Measles remains one of the top 10 causes of infant mortality in the developing world (Cutts FT & Markowits LE 1994a). The mortality rates are highest in the youngest children and greater than 30% of measles-associated deaths occur in children under the age of "routine" immunization. Although measles vaccine failure is certainly multifactorial, neutralization of vaccine-strain virus by persisting maternal antibodies is widely acknowledged to be a cruicial cause (Redd SC et al 1999, Osterhaus A et al 1998, Siegrist CA et al 1998b). Differences in maternal antibody titers and the kinetics of their disappearance are major variables in designing vaccination strategies to minimize both primary vaccine failure and the period of susceptibility in young children (Redd SC et al 1999, Osterhaus A et al 1998, Siegrist CA et al 1998b).

Many studies have demonstrated that vaccination at an early age (in the presence of maternal antibodies) leads to what has been perceived as a persistent "defect" in the capacity to generate antibodies in response to subsequent booster immunizations with the same vaccine (Redd SC et al 1999). Antibody responses in these subjects tend to be weak and relatively transient (Redd SC et al 1999). Although no case of proven susceptibility to wild-type infection has ever been documented in these children to our knowledge, this apparent "defect" in antibody production has significantly dampened enthusiasm for strategies involving early vaccination in the presence of maternal antibodies (Redd SC et al 1999).

Despite an incomplete understanding of the correlations of immunity in measles, there is increasing evidence that cell-mediated rather than humoral responses are required for viral clearance and long-lasting protection against wild-type infection (Griffin DE & Bellini WJ 1996, Redd SC et al 1999). For example, children with isolated aggamma-globulenemia recover uneventfully from measles (Good 1956) while mortality rates in patients with T cell defects (eg: HIV, leukemia) can be as high as 50 - 100% (Griffin DE & Bellini WJ 1996, Halsey NA et al 1999). Although high titers of neutralizing antibodies

can protect against measles, both animal studies and epidemiologic observations in humans suggest that seronegativity cannot be equated with susceptibility in previously vaccinated subjects (Redd SC et al 1999). We have recently demonstrated that many young adults with weak or transient responses to measles booster immunizations have either pre-existing proliferative responses to measles virus antigen or mount strong lymphoproliferative responses following revaccination (Ward BJ et al 1995).

In the current study, we have essentially replicated this observation in developing world children who received a first dose of vaccine in the presence of elevated titers of maternal antibodies. Compared to children who received a single dose of vaccine at 9 months of age and children who responded "well" (ie: high antibody production) following primary vaccination at 5 months of age, children who initially failed primary immunization at 5 months of age had the greatest lymphoproliferative response to measles antigens ~ 5 years later (following booster vaccination at 9 months of age). These data strongly suggest that the first dose of vaccine had "primed" these children to mount a more balanced humoral and cellular response to the subsequent booster dose. The capacity to induce a balanced antibody and CMI response using prime-boost strategies has only recently been appreciated (Siegrist CA et al 1998a). Such strategies are under active investigation for a wide variety of microbial pathogens including Mycobacterium *tuberculosis*, malaria and leishmania that have frustrated vaccine development efforts to date (Bretscher PA et al 1992).

Several novel measles vaccines are currently under development, (e.g. DNA, ISCOM, and microparticle-based) that have been specifically designed to overcome the problem of vaccination in the presence of maternal antibodies (Hsu SC et al 1996, Partidos CD et al 1996 a,b Partidos CD et al 1997, Siegrist CA et al 1998a,c, Fennelly GJ et al 1999, Kovarik J et al 1999). Our data suggests that the capacity of the currently available vaccines to elicit balanced humoral and cellular responses in the presence of maternal antibodies using a "prime-boost" strategy should be evaluated more fully. Since the 12

children of greatest interest in the current study received a higher than standard titer ($10^{4.7}$ pfu) of a relatively unusual measles vaccine (Edmonston Enders; Connaught, Mississauga, ON), the role of vaccine titer and strain will need to be further evaluated. Such efforts are justified, however, since there is currently no acceptable strategy for protecting children in the developing world under the age of routine immunization (< 9 months of age). Two-dose vaccination stratgies have already had considerable impact in the developed world (Redd SC et al 1999, Durmaz R et al 1998, Davidkin I & Valle M 1998). A "prime-boost" strategy based on the currently available vaccines could be rapidly implemented in the developing world with enormous potential benefit for infant survival.

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Table 1 - Study Subjects

Vaccine Regimen		
6 months	**9 months**	**Samples available 5 years**
Connaught HighTiter ($10^{4.6}$ pfu)	**Schwarz Standard Titer ($10^{3.9}$ pfu)**	**n = 54 (CN-SCH)**
Placebo (meningococcal vaccine)	**Schwarz Standard Titer ($10^{3.9}$ pfu)**	**n = 59 (placebo-SCH)**

Children received either 2 doses of measles vaccine (Connaught HT at 6 months followed by Schwarz at 9 months or age) or 1 dose of Schwarz measles vaccine at 9 months of age.

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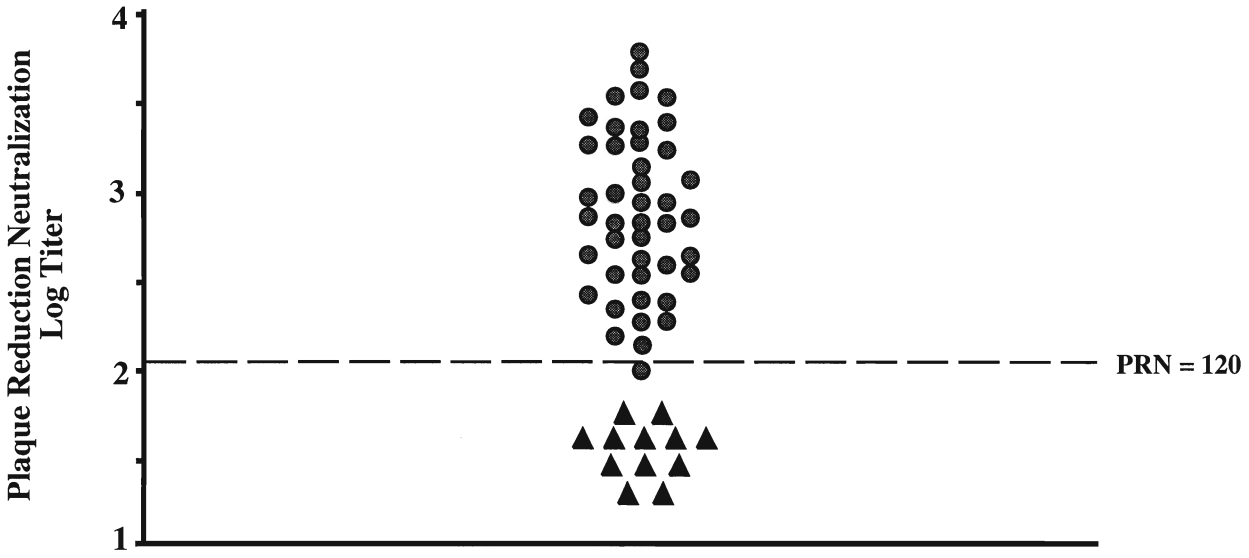
Figure 1 - Neutralizing Antibody Titers (PRN) in CN-SCH Group 3 Months After Primary Immunization



Plaque reduction neutralization (PRN) values at 3 months after HT-CN vaccination in the Connaught-Schwarz (CN-SCH) group Twelve of these children were identified as poor responders to primary immunization (▲ PRN < 80). PRN > 120 are thought to be indicate protection

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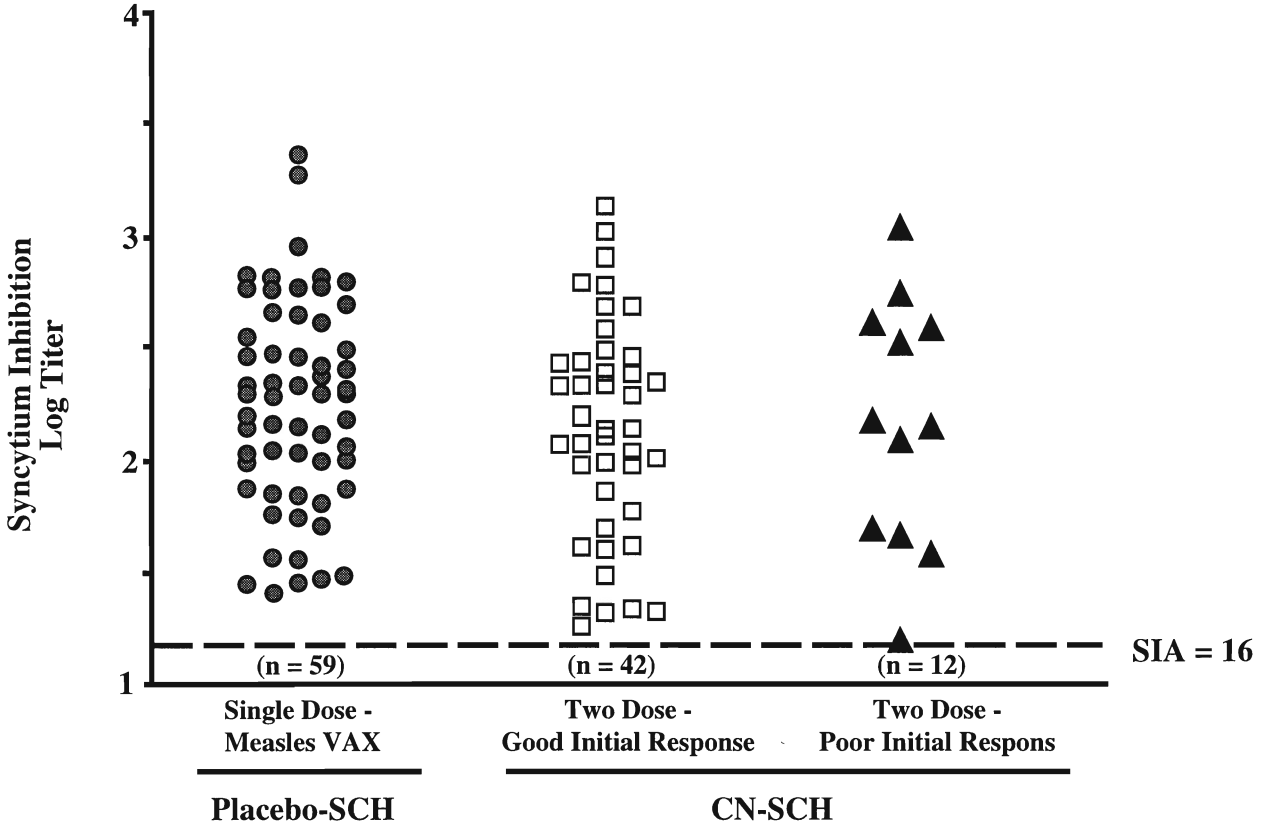
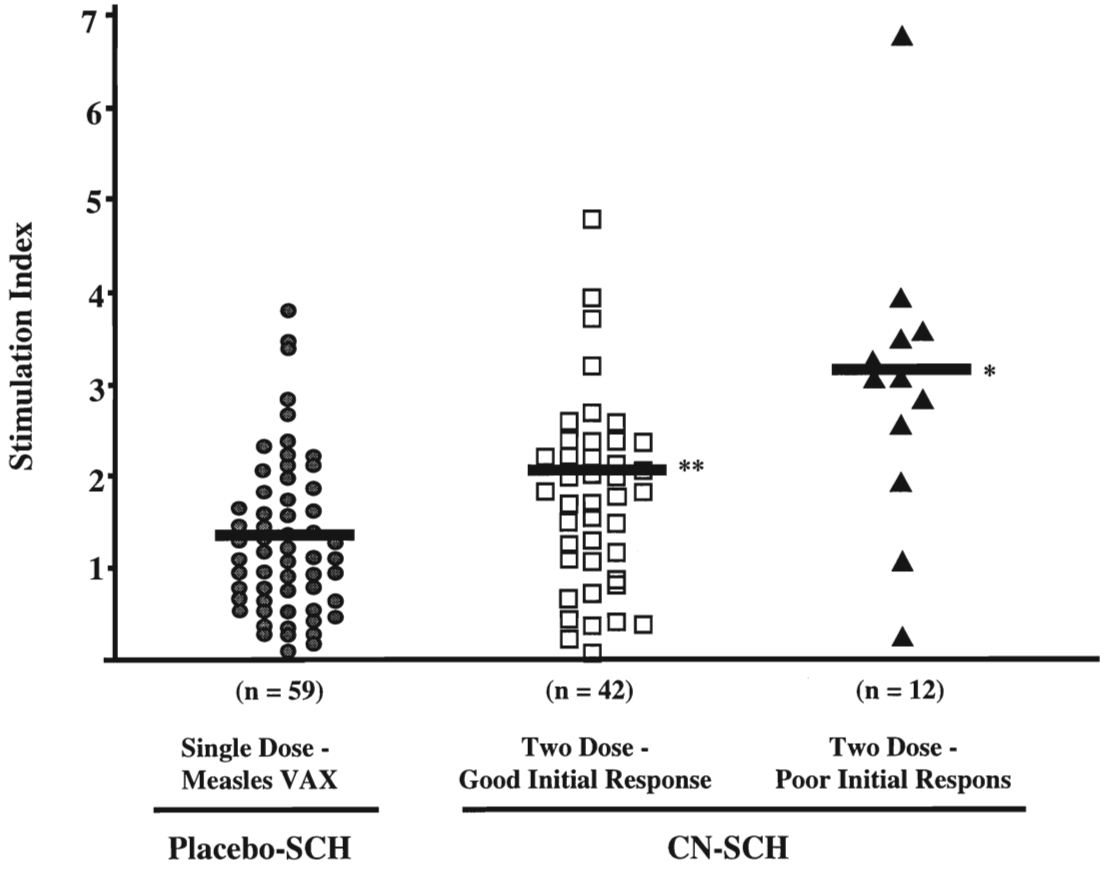
Figure 2 - Neutralizing Antibody Titers (SIA) at 5 Years of age



Syncytium inhibition titers (SIA) at 5 years of age in single dose measles vaccine recipients (Placebo-SCH,●) and 2-dose measles vaccine recipients (CN-SCH,□ : good responders after primary immunization and CN-SCH,▲ : vaccine failures after primary immunization). SIA titers ≥ 16 is thought to indicate protection from clinical infection.

Figure 3 - Measles Antigen-Specific Lymphoproliferation at 5 Years of Age



Lymphoproliferation to wild-type measles antigen (CHI-1) at 5 years of age in single dose measles vaccine recipients (Placebo-SCH,●) and 2-dose measles vaccine recipients (CN-SCH,□: good responders after primary immunization and CN-SCH,▲: vaccine failures after primary immunization). Results are expressed as mean SI (▬). SI = 3 corresponds to a significant lymphoproliferative response. * $p < 0.0003$, ** $p < 0.07$, vs. placebo group.

CHAPTER 5

DISCUSSION

Since the implementation of live attenuated measles virus vaccines in the 1960's, the incidence of measles and its associated morbidity and mortality have been dramatically reduced (Redd SC et al 1999). Despite the overall success of the measles vaccine campaign, however, greater than 30 million infections are reported in the developing world, with a mortality rate of ~ 1 million per year (WHO 1998). Although a combination of the socio-political, medical and economic problems contribute to measles endemicity in the developing world (Aaby P et al 1995a), measles outbreaks can also occur in wealthier countries with significantly better health care systems and excellent vaccine coverage (Boulianne et al 1991, Can Dis Wkly Rep 1993). In fact, in 1989 - 1990, a measles outbreak in the eastern United States resulted in ~ 40,000 cases with hospitalization rates of up to 33% in affected adults (CDC; MMWR 1991). Also, in 1995 - 1996, a Canadian outbreak of more than 2000 measles cases represented > 50% of all of the measles cases reported in the Americas in those two years (Redd SC et al 1999).

In the 1990's the continuous death toll in the developing world and the large outbreaks in developed nations sparked a resurgence of interest in measles virology, immunopathology and vaccine development. In particular, investigators focused on (i) the generation of effective vaccines for children younger than the standard age of routine vaccination (Job JS et al 1991, Markowitz LE et al 1990b, Berry S et al 1992, Tijani O et al 1989) (ii) increasing our understanding of secondary vaccine failure and the correlates of immunity (Ward BJ et al 1995, Pabst HF et al 1999b, Gans HA et al 1999) and (iii) elucidation of the mechanism of immunosuppression that is responsible for the bulk of the mortality associate with measles (Griffin DE & Bellini WJ 1996, Karp CL et al 1996, Schlender J et al 1996, Ravanel K et al 1997, Fugier-Vivier I et al 1997). This thesis work developed out of this renewed interest in measles and has significantly contributed to advancing our knowledge in some of these areas.

For example, our essentially "negative" findings in the Haitian and Sudanese HT measles vaccine trials were reassuring to all involved, especially for the families of the 20

million children who received a dose of the HT vaccine before these formulations were banned in 1992 (EPI: Wkly Epi Rep 1992). It is important to recall that, at the initiation of these follow-up studies, the impact on mortality by HT measles vaccines had only recently been recognized (Aaby P et al 1993b, Aaby P et al 1991, Holt EA et al 1993), and differences in immunologic parameters between LT and HT vaccine recipients had just been detected at 2 - 3 years after vaccine exposure (e.g. decreased CD4 T cell number CD4/CD8 ratios, DTH responses) (Leon ME et al 1993, Lisse IM et al 1994). At the time, it was uncertain whether or not the immunologic differences and "excess" mortality, were going to increase, stabilize or disappear. In this context, the absence of differences between HT and LT recipients that we documented in our studies in Haiti and the Sudan, along with the findings of other investigators (Aaby P et al 1996, Samb B et al 1995b) helped to close the door on this unfortunate episode in measles vaccine history.

Further reassurance regarding the safety of measles HT vaccine formulations was provided by our massive prospective study of post-vaccination morbidity in the Sudan (Libman M et al Manuscript IV). The original HT Sudan trial was unique in its inclusion of a prospective evaluation of HT vaccine benefit/harm and demonstrated conclusively that measles vaccines containing up to one logarithm more viral particles than standard titer preparations have no impact on any common measures of morbidity. It is very likely that this study is the only one of its kind that will ever be performed.

As reassuring as these data are, we were both gratified and apprehensive at our accumulation of evidence that HT measles vaccine exposure is associated with enhanced ELISA reactivity to several self-antigens for up to 4 - 6 years after exposure. While a great deal of work remains to be done to fully understand this enhanced auto-reactivity, the transient burst of reactivity to those same antigens in subjects with natural disease strongly suggest that the observation is worthy of further investigation. This is particularly true considering the association of some vaccines with autoimmune pathogenesis (Cohen AD &

Shoenfeld Y 1996, Tudela P et al 1992, Nieminen U et al 1993), and the growing consensus that apoptosis and autoreactivity are intimately linked (Rosen A & Casciola-Rosen L 1999).

Indeed, measles virus-induced apoptosis has been a topic of intense investigation (Esolen LM et al 1995, Schnorr JJ et al 1997a, Ito M 1997, Fugier-Vivier I et al 1997). Both wild-type and vaccine strain measles have broad tissue tropism and readily infect endothelial and epithelial tissues throughout the body as well as a wide range of immune cells (Griffin DE & Bellini WJ 1996). Although measles-induced apoptosis has previously been reported in a number of tissues and cell lines (Esolen LM et al 1995, Mcquaid S et al 1997, Fugier-Vivier I et al 1997), we were the first to convincingly demonstrate the capacity of this virus to induce massive programmed cell death in human PBMC and explore the role of virus strain in this process (Bertley FMN et al Manuscript VI). While interesting as a potential explanation for the profound lymphopenia and loss of memory cell effector function associated with measles virus-infection (Griffin DE & Bellini WJ 1996), the potential link between measles-induced apoptosis and both short-term and long-term autoreactivity is particularly intriguing. Although viral infections are often suspected in the induction of autoimmune processes (Aichele et al 1996, Naucler et al 1996, Hansen KE et al 1998), the hard evidence supporting such a role for measles virus in chronic autoimmune pathogenesis is relatively limited. Many investigations have tried to establish links between measles and a wide range of autoimmune, inflammatory or idiopathic conditions (e.g. multiple sclerosis, inflammatory bowel disease and autism) (Andjaparidze et al 1989, Cosby et al 1989, Wakefield AJ et al 1993, Singh et al 1998), however there is no convincing evidence for any of these associations to date. With further development, the potential links that we have established between viral strain, apoptosis and autoreactivity may finally give insight into this complex area of human disease.

At the current time, the measles vaccines used around the world are all based on so called "standard dose" ($10^{3\text{-}3.7}$ pfu) attenuated viruses. Unfortunately, these vaccines are

not uniformly successful in the presence of maternal antibodies (Redd SC et al 1999). The withdrawal of HT formulations from the market has therefore left the vaccine community with nothing to offer children under 9 months of age, some of whom are very susceptible to severe and often fatal disease. As such, a major priority of the WHO is the development of novel vaccines with the capacity to protect children despite the presence of maternal antibodies. Although candidate vaccines have been identified (e.g. recombinant vaccines, immune stimulating complexes, and DNA) (Hsu SC et al 1996, Partidos CD et al 1997, Siegrist CA 1998a,c, Kovarik J et al 1999), the typical delay before implementation of any new vaccine may be up to 5 - 10 years. In the meantime, children below the age of routine vaccination remain unprotected and thousands of these children will contract measles and die each year (Griffin DE & Bellini WJ 1996, Redd SC et al 1999). In our study, we have demonstrated that early vaccination with a medium titer vaccine (thought to be safe) induces seroconversion in most children and primes the remaining children for a balanced cellular and humoral response upon revaccination. These data strongly suggest that our hesitations regarding early measles vaccination may be ill-founded and that two-dose strategies using conventional vaccines should be tested immediately in young children in the developing world.

CHAPTER 6

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