Th1 and Th2 cytokine profiles in uncomplicated measles infection among a group of Ghanain children.

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DECLARATION

I do hereby declare that except for references to other investigators' work which I have duly acknowledged, this thesis is the result of my own original research and has not been presented elsewhere, either in whole or in part, for another degree.

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DEDICATION

This script is dedicated to the Glory of Almighty God for his Grace and Faithfulness to me. It is also dedicated to Georgina, Scinthia and Michael my family and my inspiration.

I also wish to dedicate this work to my mother Madam Elizabeth Esi Thompson and to the memory of my father, late Mr. Clemence K. Yeboah.

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ABBREVIATIONS

JICA	Japan International Cooperation Agency
EDTA	Ethylene diamine tetraacetic acid
ELISA -	Enyzme-linked Immunosorbent Assay
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
OD	Optical Density
pH	Negative logarithm base of hydrogen ion concentration
WHO	World Health Organization
IL	Interleukin
INF	Interferon
γ	Gamma
β	Beta
α	Alpha
pg	Picogram
ng	Nanogram
PCR	Polymerase Chain Reaction
RT	Reverse Transcriptase
DTH	Delayed Type Hyper-sensitivity
CD	Cluster of Differentiation
TNf	Tumour Necrosis Factor
mRNA -	Messenger Ribonucleic Acid
DNA	Deoxyribo Nucleic Acid
Th	Helper T cell
TGF-β	Transforming Growth factor Beta

ABSTRACT

Measles remains a major severe infectious disease causing deaths of many children in most countries including Ghana. The disorder is associated with prolonged immunosuppression resulting in complications and mortality in children. Although attempts have been made to identify the specific immune functions altered during measles, the exact immune changes have not been fully characterized. The pro and anti-inflammatory response balances (Th1/Th2) are important in determining the pathogenesis and course of viral infections in general.

CD4 mediated T cell responses to infectious agents may be dominated by type 1 or type 2 cells. Cell types are identified by the arrays of cytokine produced by each and have been clearly defined for mouse T cell clones. Similar distinctive patterns of cytokine production have been described for human T cell clones. Type 1 CD4 T cells secrete IL-2, IFN-γ and preferentially induce macrophage activation and delayed type hypersensitivity. Type 2 CD4 T cells produce IL-4, IL-5, IL-6 and IL-10, provide help for B cell responses and decrease macrophage activation. These subtypes of CD4 T cells are cross-regulatory. IFN-γ inhibits proliferation of type 2 CD4 cells whereas IL-4 and IL-10 inhibit cytokine production by type 1 CD4 cells either directly or through their effects on macrophages.

In this study, the profiles of Th1 (IFN-γ, TNF-α) and Th2 (IL-4, IL-10, TGFβ) cytokine expression *in vivo* during uncomplicated measles among a group of Ghanaian children aged 12 years and below, mean age 6.2 ± 3.5 years were determined on days 0 (acute phase), 14 and 60 (convalescence stages). Results obtained were compared with age and sex-matched healthy controls. The results clearly showed high plasma levels of interleukin-4 (IL-4) a Th2 and an anti-inflammatory cytokine, early in the acute phase (day 0) of the disease, which declined

by day 14, and day 60 post infection. Accompanying IL-4 up-regulation, Transforming growth factor β-1 (TGF-β1) another anti-inflammatory cytokine also increased significantly and remained elevated in plasma samples of all patients throughout the study period (day 0, 14 and 60).

The data however showed low levels of tumour necrosis factor alpha (TNF-α), a pro-inflammatory cytokine in the plasma samples of the patients at the acute and convalescent phases of the illness, and there was no significant difference observed between measles patients and healthy controls.

Plasma levels of interleukin 12 (IL-12) and interleukin-2 (IL-2) were low in the study subjects on days 0, 14 and 60. IL-12 is critical for the orchestration of cellular immunity for the resolution of measles infection and in this study; IL-12 suppression was prolonged for 2 months after recovery from measles.

Plasma levels of the Th1 cytokine, interferon gamma (INF-γ) increased significantly ($p<0.03$) at the acute phase when compared with levels for the healthy controls. However, this increase was not maintained on days 14 and 60. Statistically significant reduction ($p<0.05$) in IL-10 levels was seen in days 14 and 60 samples of measles patients when compared with levels in plasma samples of healthy control group.

Taken together, these data provide evidence of down-regulation of pro-inflammatory cytokine, TNF-α, and up-regulation of IL-4 an anti-inflammatory cytokine during measles infection. The mechanism by which type 2 CD4 T cells are preferentially activated during measles infection is not clear. Suggested contributing factors include genetic characteristics of the host, antigenic make-up of the pathogen, antigen dose and characteristics of the antigen presenting cell and its ability to signal T cells. The ability of measles virus to replicate in macrophages and to increase macrophage

production of interleukin-1, could play a role in the preferential activation of type 1 CD4 cells that occurs during measles. This trend constitutes suppression of the immune system resulting in increased susceptibility of children to intracellular organisms and this could help to explain the many opportunistic infections associated with acute measles in Ghanaian children.

1.0 INTRODUCTION

Measles is a severe viral infection that leads to significant morbidity and mortality among children worldwide despite the availability of effective vaccines. In Ghana despite the over 80% vaccine coverage rates reported in the country (Torigoe *et al* 1986) sporadic outbreaks of measles still occur each year. The World Health Organization (WHO) estimates that between 1-2 million children die annually of measles and that the annual mortality due to the human immunodeficiency virus (HIV) pandemic has only recently surpassed deaths due to measles (WHO report 1998). The disease is associated with severe complications and secondary infections are attributed to generalize immunosuppression involving T cell disturbances. The nature of this mechanism continues to be investigated but has not been completely characterized. The central regulatory cells of the immune system are T cells whose responses can be categorized as Th1 (mostly cellular) or Th2 (mostly humoral). Antigen stimuli induce T cells to differentiate into at least two subsets of effector groups-types 1 and 2 . An intricate immune network is centred in Th1 and Th2 cells that are distinguished by their distinct cytokine production patterns that are pivotal in determining the nature of subsequent immune responses. (Abbass *et al* 1997 and Romagnani 1997). Th1 cells produce high levels of interleukin-2, interferon-gamma and Tumour necrosis factor while Th2 cells produce high levels of interleukin-4, interleukin-5 and interleukin-6. Th0 or uncommitted cells produce both types 1 and 2 cytokines. Th1 responses are important for controlling viral infections and other intra cellular agents. Also Th1 responses promote phagocytic cell-mediated immune responses by producing Th1 cytokines (IFN-γ, TNF-α, IL-2), leading to production of Immunoglobulin G antibodies that enhance opsonization (Abbass, *et al* 1997 and Romagnani, S., 1997). Th2 responses promote eosinophil-mediated inflammatory

responses and counter regulate Th1 responses by production of Th2 cytokines (IL-4, 10, TGF-β) and IgG / IgE antibodies (Abbass, *et al* 1997 and Romagnani, S., 1997).

The role of Th1 and Th2 cytokines (pro-inflammatory and anti-inflammatory) in the pathogenesis of measles virus infection is still unclear. Ohga *et al.* (1992), have shown high levels of interferon gamma (IFN-γ) in plasma of Japanese children during the acute phase of measles. As yet no work has been done on cytokine profile determination in Ghanaian children with measles virus infection. Previous studies have revealed a selective reduction of memory T suppressor cell frequency as well as significant suppression of the anti-CD3 monoclonal antibody induced expression of interleukin-2 receptor alpha chain during the course of measles in Ghanaian chidlren (Addae *et al* 1998). However, the functional characteristics of the cells in terms of cytokine secretion were not determined. This present study should therefore provide some clues to elucidate the role of cytokines in the pathogenesis of the secondary immunosuppression status of patients during the primary infection of uncomplicated measles in Ghanaian children.

The objective of the present study was therefore to examine profiles of plasma Th1 and Th2 cytokines and to contribute to a better understanding of the pathogenesis of measles virus infection among Ghanaian children. The study would be restricted to uncomplicated measles infection to eliminate any other contributory factor or illness to the pathology or immunosuppression.

1.1 STUDY HYPOTHESIS

Acute measles causes down-regulation of pro-inflammatory Th1 cytokine response (IFNγ and TNFα) and up-regulation of anti-inflammatory Th2 cytokine response (TGF/β, IL-4, IL-6 and IL-5), which contributes to increased susceptibility to other infectious agents and measles pathology.

1.2 GENERAL OBJECTIVES

To determine Th1/Th2 Tcell responses in acute uncomplicated measles infection in Ghanaian children.

1.3 SPECIFIC AIMS

1. To measure plasma levels of Th1 (IFNγ and TNFα) and Th2 (IL-4, IL-10, TGF-β) cytokine profiles in children with uncomplicated measles at acute stage and two other points post recovery.
2. To characterize pro-inflammatory and anti-inflammatory cytokines during measles.
3. To compare levels of these cytokines in measles with those of healthy age and sex-matched controls.

2.0 LITERATURE REVIEW AND BACKGROUND

2.1 THE DISEASE

Measles is a common and most often severe infectious disease of childhood that causes deaths of many children and infants in many countries of the world, (Aaby *et al* 1990). Measles has been recognized as an entity quite distinct from any other communicable disease for more than 1000 years and epidemics probably occurred about 5000 years ago in early centres of civilization. Many of the basic principles of measles infection were elucidated by the studies of Panum, a young Danish physician who was sent to Faroe Islands in 1846 to help during a large measles epidemic. Through his observation, he deduced the highly contagious nature of the illness, the fourteen-day incubation period and the life-long immunity present in older persons. The disorder has been recognized as a highly contagious illness in non-immune persons and which is spread from one infected ill person to the new host by the respiratory route (Black *et al* 1989).

John Hall who documented the epidemic disease in Boston in the fall of 1657 described the first account of measles in America. Another epidemic in Colonial America was reported in 1687 (30 years later) and over the next 150 years, the epidemic interval in Boston gradually decreased from 30 years to about 3 years. Epidemics during the seventeenth to the eighteenth centuries involved persons of all ages, including neonates. The reduction in epidemic interval coincided with a reduction in measles age incidence which were attributed to increased importation of wild measles virus due to increase in numbers of faster ships crossing the Atlantic and the equivalent increase in population density in North America both by immigrants and local citizens.

The incidence of clinical measles has been reduced from 315 cases per 100,000 population in the modern United States to less than 2 per 100,000 since 1981 mainly due to immunization and less crowding.

However, measles is still a major health problem because of its worldwide prevalence and its changing epidemiological pattern in the U.S. and in those countries where vaccine use has been widespread. In the developing world, measles virus infection continues to be a leading cause of significant morbidity and mortality among children. The World Health Organization (WHO) estimates that measles results in the death of about 1.5 million each year and that 3.5% of the infected children in developing countries either die from the acute disease or the long term adverse effects after the infection (Sutter *et al* 1991, Beckford *et al* 1985, Neal , 1993 and Aaby *et al* 1990). The mortality rate in much of Africa, Central and South America and Asia has been placed around 10% (O'Donovan *et al* 1971, Moraes *et al* 1962, Ristori *et al* 1962 and Taneja *et al* 1962).

In Kenya 25 to 30% of children contract measles before their first birthday and about 60% become infected before their 2nd birthday while virtually all children might have contracted measles by 4 years of age (Hayden, 1974). In Kenyan children, mortality peaked in the 17 to 20 month age group and the median age for hospital admission was 14 months. In Ghana measles was estimated to account for about 10% of all deaths in children below five years of age but with increased vaccine coverage rates estimated to be around 83.6% in the capital, Accra, mortality had dropped significantly below 10% although measles continues to be a staggering problem in Ghana involving older children.

2.1.1 TYPICAL ILLNESS

Acute clinical measles is characterized by viral multiplication in many organs of the body, then rapid subsidence of the infection by the 17th day. The infection is associated with both serum and secretory antibody responses and specific cell-mediated responses that coincide with clinical recovery.

The incubation period of measles is about 10 days (8-12). Although extensive virologic and immunologic events are occurring during this period, outward signs of illness are virtually absent. However, some patients have mild transient respiratory symptoms as well as fever (Goodall, 1925 and Partington *et al* 1959).

2.1.2 PRODROMAL PERIOD

The prodome of measles lasts about three days on the average. Initial symptoms are respiratory and suggest the possibility of a cold except for the fact that fever is a very early sign. Stokes (1954) reported slight temperature elevation occurring and then subsiding for a day or two before the appearance of typical symptoms. The onset of clinical natural measles is characterized by general malaise, fever, cough, conjunctivitis and coryza, which could worsen over a 2 to 4 day period. An urticarial or macular rash usually occurs with the initial onset of fever but this could disappear before the onset of the typical exanthem. During the prodomal period, body temperature increases gradually to a value 39.5°C ± 1.1°C over a 4-day period. The nasal symptoms are similar to those of the common cold or acute nasopharyngitis and sneezing, rhinitis, wheezing as well as congestion are common symptoms. Conjunctivitis is associated with considerable lacrimation with corneal and conjunctival lesions (Azizi *et al* 1965).

The cough associated with the infection worsens throughout the period and could be very troublesome with laryngeal and tracheal involvement. On about the 10^{th} day of the illness, Koplik spots appear. Originally described by Koplik, the spot appears on a bright-red mucosal surface as bluish white speck, though other workers could not confirm the blue tinge associated with the white speck. Koplik spots have been found on the buccal mucosa opposite the lower molars but usually quickly spread to involve most of the buccal and lower bubial mucosa.

The lesions initially are about 1mm in size but occasionally seem to coalesce into larger lesions. Initially there are only a few lesions, but within 12 hours, the number is usually uncountable. Of equal importance in diagnosis is the appearance of the background mucosal surface which is always bright red and granular.

During the prodromal period, erythematous maculopapular lesions also are observed occasionally on the palate. At the end of the prodomal period, the posterior pharyngeal wall usually is erythematous and some patients may complain of sore throat.

2.1.3 EXANTHEM PERIOD

Exanthem period represents the time characterized by the eruption of fever and in measles infection this appears on about the fourteenth day after exposure, which coincides with the peak of the respiratory symptoms and the body temperature is usually around 39.5^{o} C. The Koplik spots formation have peaked and over the next 3 days they disappear leaving the red, sandpapery mucosal background which remain for a further day or two.

The measles exanthem first appears behind the ears and in the forehead at the hairline and then continues to spread, by the third day sequentially to involve face, neck trunk,

upper extremities, buttocks and lower extremities. During this period fever usually peaks on or about the second/third day of the rash and then falls by lysis over a 24 hr period and fever that persists after the third day or fourth day of exathem usually is an indication of a complication. Conjunctivitis and nasal symptoms usually subside at about the time of defervescence and continued nasal discharge suggests bacterial secondary infection. Pharyngitis is common during the exanthem period as in cervical lymph node enlargement. Generalized lymphadenopathy with suboccipital and postauricular involvement is not an uncommon finding as well as splenomegaly.

Young children occasionally have diarrhoea, vomiting, laryngitis and croup, sometimes very troublesome abdominal pain. The exanthem begins to clear on the third to the fourth day, and the patterns of clearance follows the centrifugal course of progression from head, face, neck, trunk, upper extremities, buttocks, lower extremities down to the face in that order lasting between 6 to 7 days.

2..2 AETIOLOGY OF MEASLES

Measles virus is large with helical capsid and an RNA genome and is included in the genus *Morbillivirus*, which are members of the family *Paramyxoviridae*. Measles virus differs from the other members of the *paramyxoviridae* in that it does not possess neuraminidase activity and it does not adsorb to neuraminic acid-containing cellular receptors, (Morgan *et al* 1977, Norrby *et al* 1962, Peries *et al* 1962 and Waterson 1965). Measles virus haemagglutinates whereas the other members of its genus do not. Measles virus is roughly spherical measuring close to 100-250nm in diameter, (Bellini *et al* 1994, Fenner 1976, Melnick1976 and Nakai *et al* 1969). It is pleormorphic or has the ability to assume various distinct forms from a single organism. The virion is composed of an outer lipoprotein envelope and an internal helical nucleocapsid. The virion contains six structural proteins, three of which are complexed with RNA while three are related to the virus envelope. The outer virion or viral envelope is 10-20nm thick with short surface projections (peplomers) and contains three virus-coded proteins F, H and M, (Choppin *et al* 1981, Morgan *et al* 1977 and Norrby *et al* 1974). The F protein is a dumb-bell shaped peplomer that causes membrane fusion of virus and host cell and enables penetration into host cell of the virus. The H protein is the haemagglutinin and is a conical peplomer. The M protein or matrix is non-glycosylated and is associated with the inner lipid bilayer of the envelope. It plays an important role in virus maturation.

The nucleocapsid (N) protein is a coiled rod, with a diameter of 18nm and a length of 1μm that contains the viral genomic RNA (Norrby *et al* 1972, 1974, and 1975, Waters *et al*., 1972, 1974). The N protein has a molecular weight of about 60KDA. About 5 percent of the nucleocapsid consists of RNA (Hall 1973,1974, Udem *et al* 1984 and

Waters *et al* 1972). The other internal proteins of the virus are the large (L) and phosphate (P) proteins, which are parts of the transcription process complex (Norrby1975). The virus genome has a molecular weight of 45 x10 daltons with a linear single strand of RNA that contains about 15,900 nucleotides.

Measles virus is labile (Katz 1962, 1965 and Musser *et al* 1960) and can be inactivated rapidly by heat, ultraviolet light, lipid solvents e.g. ether and chloroform as well as extreme degrees of acid or alkali agents i.e. with Ph <5 and >10. Longevity is prolonged when protein is in the viral suspending medium and when the virus is lyophilized with a protein stabilizer. Protein specially protects against the adverse effects of heat and light. Protein-stabilized measles virus can be stored at -70^{oC} for 5 years or longer without significant loss of infectivity. At room temperature, there is a 60 percent loss in titer within 3 to 5 days while inactivation occurs within 30 minutes at 56^{oC} (Black, 1959a and Musser and Underwood, 1960).

Recent nucleoside sequence analyses have identified distinct lineages among wild-type measles virus isolates and some properties associated with the virus include haemagglutinin, complement fixing antigens, haemolytic activity and a giant-cell inducing factor, (Bussell,1966, Hall *et al* 1974, Katz *et al,*1969 and Norrby,1962). Human measles virus infection results in serum antibodies that are capable of neutralizing viral infectivity, fixing complement with viral antigen and inhibiting viral haemagglutination and haemolysis.

Measles virus can be propagated in many different primary and cell line tissue cultures, (Black *et a,l* 1959b, Katz *et al,* 1969 and Matsumoto, 1966). However, for isolation of virus from specimen obtained from patients, primary human and monkey kidney cultures have been most successful over the years.

In one study, an Epstein-Barr virus- transformed marmoset lymphocyte line (B95-B) was found superior to primary monkey kidney cell cultures for the isolation of virus from nasopharyngeal specimens (Kobune *et al,* 1990). In tissue culture, measles virus has true distinct cytopathogenic effects. With initial isolation, syncytial formation occurs, being the result of cell fusion in which the resulting giant cell may contain 10 -50 or even more nuclei. On stained preparation, both the nuclei and cytoplasm contain eosinophilic inclusions. The second form of cytopathogenic effect is characterized by the alteration of single cells into spindle shapes or stellate forms.

Generally, tissue culture-adapted measles viral strains are more likely to cause this cytopathogenic effect than in giant cell formation. In most cultures, both forms of cytopathogenic effects are evident and change in medium composition make one or the other types predominate.

Measles virus is highly contagious but isolation of the virus from clinical samples is difficult and inefficient when primary monkey kidney cells and vero cells are used (Ruckle *et al,* 1957). Ihara *et al (* 1992), using B 95a cells isolated measles virus from peripheral blood mononuclear cells of 14 children suffering from natural measles one day before and six days after appearance of rash in all the children and concluded that viraemia persists longer than previously reported. B95a cells are 1000 times more sensitive to measles virus than are vero cells.

2.3 TRANSMISSION OF MEASLES

Transmission of measles is thought to occur mainly by aerosolized droplets of respiratory secretions. The acquisition by the new host of the infection is via the nose and possibly by the conjunctivae. Infection can occur by small droplet nuclei, which stay suspended in the air for considerable periods or by direct hits of large droplets at close range (Remington *et al,* 1985). It also seems possible that spread involves close person to person contact in young children with large virus-containing droplets of nasal secretions being picked up on the hands of the future host and then applied to the nose. Humans are the natural hosts of measles virus but with human contact monkeys are also infected easily (Meyer *et al,* 1962), although practically speaking, there is no animal reservoir. Available evidence suggests that humans who are with the infection spread the disease. Asymptomatic contagious carriers are unknown and persons with acute asymptomatic infection probably are not contagious or are minimally so.

The prevalence of measles throughout history has been affected markedly by population density. Extensive investigations by Aaby et al (1984) during last twenty years suggest that overcrowding and intensive exposure are more important determination of measles mortality than is nutritional status (Aaby 1988) although other workers (O'Donovan *et al,* 1971) have indicated that severity of measles and mortality correlate in general with the severity of malnutrition.

Measles susceptibility does not vary by race although severity of the disease in certain populations appears to suggest differences based on race, this probably is the result of nutritional and other environmental factors. Deseda-Tous (1976) and associates were unable to demonstrate any differences in measles antibody by HLA or ABO blood group and types. No sex differences in measles incidence have been documented

although a suggestion of higher complication rates in males than females has been made. Tidstrom (1968) found out that acute laryngitis was more than twice as common and deaths were slightly more common in males than in females. In other studies, Otitis media, pneumonia and deaths were slightly more common in males than in females (Babbott *et al,* 1954).

Studies of measles in the 20th century have indicated that secondary cases in households are likely to be more severe than are primary cases (Aaby *et al,* 1984), however this finding has not been generally confirmed by other workers. In the prevaccine era, the age of patients at measles infection was related inversely to the number of siblings in the family (Babott *et al,* 1954, Black *et al,* 1989 and Wright, 1942). Generally, measles occurred at an earlier age in city dwellers and in lower socio-economic classes, compared with rural families and well-educated upper income group. In the present era of improved healthcare delivery and other medical modalities, the greatest factors in measles morbidity and mortality are age and nutritional status.

2.4 PATHOGENESIS AND PATHOLOGY

The cellular events and reactions as well as pathologic mechanisms occurring in the development of measles by the virus have been investigated by a number of workers (Gresser *et al*1960, Griffin *et al.*1990, Grist *et al.*1950, Kamahora *et al.*, 1965, Kempe *et al.*, 1965, Kimura *et al.*1975, Lightwood *et al.*1970, Nii S *et al.*1964, Ono *et al.*1970, Peebles *et al.*1967, Robbins *et al.*1962, Ruckle *et al.*1957, Sergiev *et al.*1960 and Yamanouchi *et al.*1970). The primary site of infection seems to be the respiratory epithelium of the nasopharynx, resulting in an initial infection that is comparatively minimal which however sets the cycle of early spread of the virus to regional lymphoid tissue and lymphatics resulting in virus multiplication and primary viremia at about the 3rd day after the contact. After initial viraemia, extensive multiplication of virus occurs in the reticuloendothelial system at both regional and distant sites. Virus multiplication also continues at the site of initial infection. Between the third and 5^{th} day after initial contact, multiplication of measles virus in respiratory epithelium at the site of initial infection and in the reticuloendothelial system regionally and at distant sites continue, creating an extensive secondary viraemia by the 7^{th} day, resulting in the establishment of generalized measles viral infection. The skin, conjunctivae and respiratory tracts are obvious sites of infection, but other organs may be involved as well from the eleventh day to the fourteenth day. The viral content of the blood, respiratory tract and other organs peaks and then rapidly diminishes over the ensuing 2-3 day period. By the 17^{th} day viraemia decreases, then ceases, and viral content in organs also rapidly diminishes.

During infection, measles virus replicates in endothelial cells, epithelial cells, monocytes and macrophages (Griffin *et al* 1994 and Moench *et al* 1988) the cell

receptor for measles virus infection being CD46, a membrane cofactor protein (Dorin *et al.*1993, Manchester *et al.*1994, and Naniche *et al,* 1993, Karp et al 1996).

In persons who are naturally immunologically compromised with defects of cell-mediated factors, measles virus is not cleared from the secondary infection sites and progressive, usually fatal illness occur (Aicardi *et al.*1977, Enders *et al.*1959, Mitus *et al* 1965 and Murphy *et al,* 1976).

Clearance of measles virus from the tissue begins with the onset of the rash and is associated with the appearance of virus-specific cytotoxic CD8+ Tcells

(Van Binnendisk *et al*, 1990) but the characteristic pathologic feature of measles is the widespread distribution of multinucleated giant cells which are the result of cell fussion (Black *et al.*1959a, Kamahora *et al*1965, Katz *et al.*1969, Lightwood *et al.*1970, Sergiev *et al.*1960, Yamanouchi *et al.*1970, Roberts *et al* 1958).

Two main types of giant cell occur in measles namely (a) The Wartin-Finkeldey cells which are found in the reticuloenthelial cells and (b) the epithelial giant cells which are found principally in the respiratory epithelium but also on other epithelial surfaces (Lightwood *et al*1970). Warthin-Finkeldey giant cells are found throughout the reticuloentheial system in adenoids, tonsils, peyer's patches, appendix, lymph nodes, spleen and thymus. They vary in sizes and can contain up to 100 or more nuclei. The cells contain both cytoplasmic and intracellular eosinophilic inclusions with the cytoplamic inclusion being more common than the intracellular lesions. During the course of the disease, epithelial giant cells regularly can be found on the respiratory surfaces and are most frequently shed off freely.

2.5 COMPLICATIONS OF MEASLES

Measles virus is a singular agent that causes a relatively distinct exanthematous disease characterized by fever cough coryza, conjunctivitis and erythematous maculopapular confluent rash and a pathognomonic exanthema. In modern times in populous areas, measles has been a disease of children.

Before the present vaccine era, measles was an inevitable disease of childhood that was recognized readily by parents and other lay persons as well as physicians. Although occasional confusion with the exanthematous diseases occurred,

(Chery, 1969), the epidemic character of measles usually resulted in accurate diagnosis of the simple typical, natural disease; but in addition to typical measles, there are many other clinical manifestations and complications that have a broad range of frequency. By definition, unusual manifestations are the direct result of primary infection whereas complications are the result of damage by a secondary infection with another microorganism. In many instances, it is difficult to determine whether a particular manifestation is just viral or involved with a second agent. Combinations of infection are common.

One common complication of measles is pneumonia, a leading cause of death. It may be a manifestation of primary viral infection or it may result from a superimposed bacterial infection. Pulmonary involvement in measles, due to the viral infection is the rule rather than the exception. Kohn and Koiransky, (1929) performed careful radiographic studies in about 130 children with measles and noted that 55% had pneumonia infiltration and 74% had adenopathy. In the majority of instances, the pneumonia was observed early in the course of the illness, which suggested primary viral involvement rather than secondary bacterial infection which could be the result

of common respiratory pathogens particularly Streptococcus pneumonia, Haemophilus influenza, Streptococcus pyogenes and Staphylococcus aureus.

Otitis media is the most common complication and is age related. In the immediate prevaccine in the United States, almost 15% of patients with measles developed otitis media. Now it is a lesser problem because of the change in age incidence of measles.

Laryngitis and mild laryngotracheitis are common manifestations of typical measles and measles-associated bacterial tracheitis is not uncommon, (Conley *et al* 1993). Secondary bacterial infection of cervical lymph nodes and secondary bacterial pharyngitis also is rather frequent complications of measles.

Myocarditis and pericarditis occassionally occur in measles (DegenJr. 1937 and Finkel, 1964). Nonspecific, transient eletrocardiographic abnormalities were noted in more than half of 71 children with measles in one study (Ross, 1952). Although cardiac involvement appears to be common in measles, clinical consequences of this involvement are rare.

Also involved in measles infection is encephalitis and various workers have reported different ratios per 100,000 measles cases, worldwide. In general between 20 and 40% of patients who recover from measles encephalitis, have manifestations of brain damage. Patients with encephalitis frequently have multiple findings; headache abnormalities of respiratory rate and rhythm, twitching and other involuntary movement and disorientation. Some investigators have recovered measles virus from the cerebrospinal fluid and brain of affected patients which indicates that the virus may be involved directly in the process, (Esolen *et al* 1995, Foreman *et al* 1967, McClean, 1966 and Meulen *et al* 1972). Measles has been associated with many other manifestations and complications such as the often severe, fatal form of measles

called black measles characterized by a confluent haemorrhagic skin eruption, (Krugman *et al* 1965). Patients with this form of complication had extensive bleeding from the mouth, nose and bowel but this is not common today.

Other manifestations include hepatitis, appendicitis, cervicitis, acute glomerulonephritis, corneal ulceration and gangrene of the extremities, (Frederique *et al* 1969, Heinemann *et al* 1992, Katz, 1962, Khatib *et al* 1993 and Lin C-Y *et al* 1983). Measles in pregnancy results in significant maternal and fetal morbidity and mortality, (Atmar *et al* 1992, Eberhart Philips, 1993 and Stein *et al* 1991). Jespersen *et al(* 1977), working in Greenland noted that 32% of women infected during the first trimester had spontaneous abortion and 9% of these pregnancies that continued to term resulted in stillbirths.

Congenital malformations occurred in eight of 300 live born infants. Pneumonia is a common maternal complication of measles during pregnancy. Subacute sclerosing panencephalitis (SSPE) is a rare degenerative central nervous system disease of children and adolescents due to a persistent measles virus infection. Dawson first described it in 1933. He proposed a viral etiologic agent because of the occurrence of inclusion bodies in the neurons of patients dying of the disease. In 1967, Conolly and colleagues reported the observation of measles viral antigen in the brain of a patient with SSPE. They also noted high measles HI and CF titers in the sera and spinal fluids of three afflicted patients. Cultures of brain cells from patients with SSPE resulted in syncytial formation and the presence of measles antigen (Baublis *et al* 1968). The risk of SSPE in children who previously had natural measles is between 0.6 and 2.2 in 100,000 infections (Modlin , 1977). However, the risk is greater in patients that acquire measles at an early age.

The mean incubation period from measles illness to onset of SSPE is 7 years. In contrast with natural measles, the risk of SSPE after measles immunization is about one per million. Symptoms are frequently bizarre and complex and include psychic difficulties, motor incoordination, and seizures of various types, visual impairment and difficulties with speech. Progression of the disease leads to stupor, dementia central blindness and finally decorticate rigidity.

The average duration of illness from onset to death is about 6 to 9 months but with increased and extensive use of measles vaccine the incidence of SSPE has decreased dramatically worldwide, (Modlin *et al* 1977). Other well-known complications of measles include rubella, malnutrition (kwashiorkor, marasmus) gastroenteritis malaria and of late human immunodeficiency virus syndrome (HIV).

2.5.1 ATYPICAL MEASLES

Atypical measles is a clearly defined clinical syndrome that occurs in some previously immunized persons after exposure to natural measles. The overwhelming majority of cases have occurred in persons who initially received inactivated (killed) measles viral vaccines but some cases have been noted in children who received only live measles vaccines, (Cherry *et al* 1969 and Linnermann *et al* 1972). The prodromal period is characterized by the sudden onset of high fever and usually headache. Abdominal pain and myalgia are also common complaints. Dry non-productive cough is noted in most patients and vomiting occurs in about a third of those afflicted. Koplik spots appear to be rare in atypical measles. Rash appears 2-3 days after onset, coryza is not a prominent finding, so also conjunctivitis. In addition to pulmonary involvement, there is adenopathy and pneumonia, which is lobular, and pleural effusion is very common. Marked hepatosplenomegaly, weakness, numbness, marked

hyperesthesia and paresthesia are common findings. Measles antibody studies in atypical measles are remarkably diagnostic.

By the tenth day of illness, the CF and HI titers which stood at less than 1:5, could siginficantly be elevated markedly to around 1:1280. In contrast, in typical natural illness of measles infection, at the tenth day the titer is rarely greater than 1:160. Up to date, measles virus has not been isolated from a patient with atypical measles but only a few adequately performed studies have been carried out. Epidemiologic data presently available suggest that patients with atypical measles are not contagious, but patients with the disorder frequently are diagnosed erroneously as having Rocky Mountain spotted fever, other septic condition, lymphoma or collagen vascular disease and their work-up is associated with extensive blood cultures, other diagnostic procedures and vigorous antibiotic therapy. Careful attention to a history of prior administration of killed measles vaccine should clarify the diagnosis and prevent the unnecessary trauma associated with extensive diagnostic and therapeutic procedures. In atypical measles, chest radiographs always should be obtained because the pneumonia of these patients frequently is much more extensive than the clinical findings would indicate.

2.5.2 MODIFIED MEASLES

Modified measles is an infection that occurs in the partially immune person. It is characterized by a generally mild illness that usually follows the regular sequence of events in measles. The prodromal period is shorter, cough, coryza and fever are minimal. Koplik spots are few and transient, they frequently do not occur at all. The exanthem follows the progression pattern of regular measles, but confluence of the lesions does not occur.

Modified measles occurs under a variety of circumstances, the most important of which was the result of intentional disease alteration by the administration of immune serum globulin to the exposed child. Naturally occurring modified measles occasionally is seen in infants younger than 9 months of age because of presence of transplacentally acquired maternal measles antibody. Modified measles occurs as an occasional manifestation of live vaccine failure. In these instances, patients have had modified illness but demonstrated a secondary measles serum antibody response, only IgG antibody (Cherry *et al* 1973, Cherry *et al* 1972 and Smith *et al* 1982).

2.6 CELL-MEDIATED RESPONSES

It was recognized about three decades ago that patients with defective cellular resistance factors frequently died of measles virus infection (Nahmias *et al* 1967).

Ten years later (two decades ago) techniques were developed that demonstrated measles-specific lymphocyte sensitization reactions (Ruckdeschel *et al* 1975, Gallagher *et al* 1981 and Grazieno *et al* 1975), in which lymphocytes from persons who had measles previously had blastogenic response when they were incubated with measles antigen. Labowskie *et al* (1974), utilizing an *invitro* lymphocyte-mediated cytotoxicity assay noted that lymphocytes from persons who previously had measles caused cell destruction in a tissue culture infected with measles virus. Other workers also demonstrated excessive measles-specific lymphocyte blastogenic responses in some persons previously immunized with killed measles vaccine.

Presently, it is known that Tcells are important both for providing help for B cells leading to a mature (IgG and IgA) and adequate antiviral antibody responses and as effector cells in clearance of virus-infected cells from tissues.

Both helper (CD4+) and suppressor (CD8+) cells participate in the cellular responses. Measles virus-specific CD8+ cytotoxic T lymphocytes and proliferating CD8+ T cells are detectable in blood at the time of the rash and soluble CD8+, a by-product of the interaction of activated CD8+ T cells with target cells is increased in plasma. During infection, CD8+ T cells eliminate virus-infected cells by major histocompatibility complex class I-restricted cytotoxic mechanisms (Griffin *et al* 1994). CD8+ T cells recognize viral antigens after peptides derived from newly synthesized viral proteins associate with Major Histocompatibility Complex (MHC) class 1 molecules and migrate to the surface. β2 microglobulin, a component of MHC class 1 is also increased in plasma for a prolonged period. Lymphocytes and monocytes are found within the rash and although the types of cells have not been identified it is presumed that CD8+ Tcells are an important component of the lymphocytic infiltrates found at the sites of viral replication and eliminate infected cells by MHC class I-restricted cytotoxic mechanisms.

CD4+ Tcells are also activated in response to measles virus infection (Kiepela et al 1987) and during the rash period they proliferate while soluble CD4+ becomes elevated for several weeks. However, the two parameters most often used to assess CD4- dependent T cell immunity, delayed type hypersensitivity skin test responses and in vitro proliferation to viral antigens are often not demonstrable or are only minimally reactive in measles (Nelson *et al*. 1966, Hirsch *et al* 1984 and Fulginiti *et al* 1969). CD4+ T cell clones can lyse infected cells expressing viral antigens in the context of MHC class II molecules (Jacobson *et al* 1989, Van Binnendijk *et al* 1989) but CD4+ T cells exert most of their influence through secretion of cytokines resulting in attraction, activation and deactivation of macrophages and also in proliferation and differentiation of B cells to produce antibodies and in proliferation

and differentiation of T cells. Antibodies are first detectable on appearance of the rash (Graves *et al* 1984 and Bech 1959).

2.7 HUMORAL RESPONSES

Antibody responses are induced to most viral proteins namely N, F, H and M proteins and these can be demonstrated after infection (Norrby *et al* 1981, Fulginiti *et al* 1965, Graves *et al* 1984, Kleiman *et al* 1981, Krugman *et al* 1965 and Neumann *et al* 1985). Measles virus-specific antibody is initially IgM and primarily IgG1 and IgG4 (Mathieson *et al* 1990 and Ehrnst 1978). Antibody to individual viral proteins can be measured by immunoprecipitation by Enzyme Immunosorbent Assays (EIA) using purified viral antigens and by bioassays. The most abundant and most rapidly produced antibody is to the nucleocapsid (N) protein and is the major antibody detected in the complement fixation (CF) test (Norrby *et al* 1972). Because of the abundance of anti-N antibody, its absence is the most accurate indicator of seronegativity. The matrix, M, protein elicits only small amounts of antibody, except in atypical measles (Graves *et al* 1984 and Machamer *et al* 1980). Antibody to the fusion (F) protein is measured by inhibition of the haemolysis of monkey erythrocytes by measles virus haemolysin inhibition antibodies or by immunoprecipitation. These antibodies may contribute to virus neutralization by preventing fusion of the viral membrane with the cell membrane. Antibody to this protein is not induced by inactivated measles vaccine (Panum 1939, Norrby *et al* 1975 and Annuziato *et al* 1982). Antibody to the haemaglutinin (H) protein is measured by inhibition of the agglutination of monkey erythrocytes by measles virus haemagglutination inhibition. Antibodies to the H protein are also the primary antibodies measured by neutralization of virus infectivity in tissue culture. Neutralizing antibody is thought to play a

prominent role in preventing reinfection and therefore HI or neutralization tests are most often used to evaluate vaccine responses and to assess susceptibility to measles.

A most significant evidence for the importance of antibody in providing protective immunity is protection of infants by maternal antibody for several months after birth (Black 1989) and protection of exposed individuals by human gamma globulin containing antibody to measles virus (Krugmann 1963).

In general there is a strong correlation between levels of neutralizing antibody and protection but exceptions raise the possibility that, other components of the immune response are also important for protection from reinfection or that the protection is associated with a particular class or specificity of antibody that may not be measured by the tests used. Antibody is also capable of lysing infected cells (Ehrnst 1975), of modulating viral antigen from the cell surface (Joseph *et al* 1975), and of suppressing intracellular synthesis of viral protein and RNA in vitro, (Schneider-Schaulies *et al* 1992, Barrett *et al* 1985 and Fujinami *et al* 1979). Therefore, antibody may play a role in controlling viral replication or establishing persistent infection in vivo. Infection in the presence of maternal antibody is a specific explanation for the association of subacute sclerosing panencephalitis with measles at an early age. In response to primary infection, neutralizing antibody probably clears cell-free virus from the blood and failure to produce a good antibody response correlates with poor outcome, (Coovadia *et al* 1978), although this may be just an indicator of a poor immune response in general. However, clearance of virus from tissue is thought to be dependent on cellular immune responses.

Several workers have investigated some aspects of non-specific but immunologically related responses during natural measles virus infection as well as vaccine measles infections. Anderson and colleagues (1976) demonstrated a temporary defect in neutrophil mobility during acute measles that resolved by the eleventh day after the most of rash, while Black *et al* 1963, described leucopenia in natural measles as well as after immunization. With immunization, both neutrophil and lymphocyte numbers are reduced and this reduction can last up to one week with its onset about seven days after vaccination. Coovadia *et al* (1977 & 1978), have also shown that the numbers of T, B null cells of the lymphocyte population are reduced although the ratio between helper and suppressor cytotoxic cell phenotypes have not changed (Joffe *et al* 1983, Arneborn *et al* 1983 and Alpet *et al* 1984). Interferon -alpha and interleukin-2 receptor and neopterin levels have been reported to be elevated (Griffin *et al* 1990) while thrombocytopenia occasionally has been associated with natural measles (Huderson *et al* 1956) and after immunization (Wilhelm *et al* 1967).

2.8 LABORATORY DIAGNOSIS OF MEASLES

Medical laboratory based studies or investigations rarely are indicated in acute uncomplicated typical measles because the diagnosis can be established on a clinical and epidemiological basis and results of studies rarely affect patient management. During the period of prodrome and rash, the total leukocyte count is low, resulting in reduction of absolute numbers of neutrophils and lymphocytes. Most specific diagnosis in measles infection can be made by serologic study performed most easily by measles complement fixation test or haemagglutinin inhibition antibody studies. If serum sample is obtained during the prodrome and another one is obtained 10 days later and an antibody titer is determined in the samples, a 4-fold increase in titre is usually demonstrated and is diagnostic enough.

Measles infection can be diagnosed specifically by viral isolation in an appropriate tissue culture system, by demonstration of measles antigen in exfoliated cells and tissues by FA technique or by the polymerase chain reaction, (PCR methods) (Matsumoto *et al* 1966). Enzyme-linked immunosorbent Assays (ELISA) have been employed in specific diagnosis of measles infection by measuring levels of IgM antibodies in single serum sample. Most measles cases can be diagnosed by demonstration of specific IgM antibody in an acute phase serum specimen although false routine IgM ELISA results could also occur (Jenkerson *et al* 1995). Because antibody responses are induced to most viral protein (Norrby *et al* 1981), several methods have been employed by different workers to measure measles virus-specific antibodies - IgM, IgG1 and IgG4 (Mathiesen *et al* 1990, Ehrnst 1978) in the laboratory diagnosis of measles infection e.g. Enzyme immunoassays using purified viral antigens and by bioassays.

The most abundant and most rapidly produced antibody is to the nucleocapsid (N) protein and is the major antibody detected in the complement fixation (CF) test (Norrby *et al* 1972).

There are other non-specific immunologically related responses which have been associated with measles virus infection. Griffin et al (1990) reported elevated plasma levels of neopterin while complement activation studies showed frequent pathologic activation of the complement system; a reduction of C1q,C4,C3 and C5 (Charlesworth *et al* 1976). C reactive protein has been shown to be elevated at onset of rash (Griffin *et al* 1983). Circulating immune complexes have been noted in blood samples in 25% of patients 7 to 13 days after onset of rash (Ziola *et al* 1983). Coovadia and colleagues noted slight but significant reductons in serum IgA levels and elevation of IgM values in acute measles. They found the IgG concentrations to be normal. Increased levels of serum or plasma IgE have been noted in two studies (Griffin *et al* 1985). Delayed hypersensitivity responses in skin are suppressed in both natural and vaccine measles virus infections, similarly *in vitro* lymphocyte blastogenic responses to common antigens are suppressed (Wilkins *et al* 1978).

2.9 METHODS OF ASSAYS

There are several methods or assays for laboratory diagnosis of measles. The choice of a particular assay method over the other could depend on several factors as:

a. Simplicity

b. Reproducibility

c. Sensitivity

d. Specificity

e. Cost

2.10 SEROLOGICAL DIAGNOSSIS OF MEASLES

The basis for these serological assays are the detection of antibodies in plasma or sera samples of individuals infected by measles virus or through previous immunization. Specific antiboies can be revealed using anti-human radio labeled, fluorescein or enzyme labelled antibodies in the diagnosis of measles infection. In all these assays the detection of measles virus specific IgG and/ or IgM antibodies is diagnostic of exposure to the virus.

The serological assays or methods are: Enzyme-linked immunosorbent assay (ELISA), Complement fixation (CF), Haemagglutination Inhibition (HAI), Indirect fluorescent antibody test (IFAT), Viral Neutralization Assay (VNA) and Radioimmunoassays (RIA).

So far, ELISA assay for measles has been reported (Kleiman *et al* 1981) to have well documented advantages over CF, HAI, IFAT and other RIAs. The method uses commercially available reagents and is read by eye without the necessity of expensive photometric equipment. The absolute measles ELISA titers exceeded those determined with the other techniques tested. The frequency of CF-confirmed seroconversions that were undetectable by ELISA was therefore low. Comparison of techniques during measles revealed that the HAI assay detected antibody earlier than the CF, ELISA and IFAT techniques. This may perhaps result from a greater sensitivity of the HAI assay to immunoglobuin M antibody in early specimens. Therefore in an HAI test of paired sera in which the sample was drawn late in the acute phase of illness, a four-fold or greater increase in titer might not be detected; ELISA, CF or IFAT could be more useful in this situation.

2.11 VIRAL ISOLATION TECHNIQUE (VIT)

Measles virus could be isolated from peripheral blood mononuclear cells (PBMC) after centrifugation using Ficoll-Hypague Density Gradient separation method, cell suspension made at 1x 10^{6}/ml in RPMI 1640 with 5% FBS and added to primary monkey kidney cells, vero cells or B95a cells and incubated at 37°C in humidified atmosphere. Viral isolates can be observed and identified by the characteristic syncytium formation of culture cells under an inverted microscope. B95a cells have been reported (Ihara *et al* 1992) to be 1000 times more sensitive to measles virus than vero and monkey kidney cells. The technique has been utilized to isolate measles virus in urine samples of patients (Ihara *et al* 1995).

2.12 POLYMERASE CHAIN REACTION METHOD (PCR)

Immunofluorescence assay (IFA) of viral antigens in nasal aspirates is largely used for the diagnosis of respiratory syncytial virus (RSV) infection because it is more rapid and sensitive than the virus isolation technique (VIT). The use of IFA and VIT can provide an increase in the population of positive results but a significant number of specimens remain negative inspite of clinical and epidemiological presumptions of RSV infection. Reverse transcriptase-PCR (RT-PCR) should be able to improve sensitivity of RSV detection. PCR is able to amplify viral genetic materials which is then identified in gels after electrophoresis.

2.13 VACCINES AND IMMUNIZATION

Measles virus causes a severe systemic illness that could lead to substantial morbidity and mortality and efforts to produce effective measles vaccine proceeded rapidly after the identification of the causative agent. The initial measles vaccines were an inactivated vaccine modeled on the previously successful vaccine for polio (Warren *et al* 1962) and a live attenuated vaccine (Enders *et al* 1960).

In the prevaccine era in the United States of America and in other concentrated populations of the world, measles epidemics regularly occurred. In the US, urban-centred measles epidemics occurred every 2 to 5 years with each epidemic lasting three to four months, (Babbotte *et al* 1954, Black *et al* 1989 Gunn 1938, Hedrich 1930 and Yorke *et al* 1973).

Fifty to hundred years ago the case fatality rates for measles were as high as 8-10% in the United Kingdom and in other industrialized countries but the severity of measles has decreased with industrial development as a result of less crowding; a shift to an older mean of infection, improved nutrition and improvements in the ability to care for children with measles-related complications. In developing countries today measles case fatality rates are 10 times higher than is seen in the United States. In these countries, high case fatality rates being reported are observed in children who acquired measles from a household contact as compared to children who acquired the infection from a community contact (Aaby *et al* 1986, Koster 1988, Garenne *et al* 1990). Other factors associated with high case fatality rates in developing countries include the young age at the time of measles infection and inadequate therapy for bacterial infection and other measles-related complications. (Hasley *et al.*, 1990). The attenuation of measles virus for use in measles vaccines has been one of the most important developments in the prevention of infectious diseases.

The widespread use of measles vaccine in the US has led to over a 99% reduction in the incidence of reported measles (Gindler *et al* 1992 and Peter 1992). Studies in several developing countries have shown that measles vaccination has resulted in about 80% reduction in mortality after 9 months of age as compared to unvaccinated children (Holt *et al* 1990 and Koenig *et al* 1990). In developing countries, measles vaccines are now administered to the majority of children before they reach their first birthday and WHO estimates that measles vaccination has resulted in the prevention of over 1 million deaths each year (Aaby *et al* 1989). The effectiveness of measles vaccines has been limited in part because of interference by passively acquired maternal antibodies that limit the effectiveness of measles vaccination in the first year of life (Albrecht *et al* 1977).

In developed countries, low-titre, live attenuated measles vaccines are safe, immunogenic and effective when given in the second year of life, however in the developing countries the profile of maternal antibodies differs somewhat (Black *et al* 1986 and Van Ginnenken *et al* 1984) and the usually recommended age for vaccination is 9 months. This strategy leaves open a window of high risk of death from measles below 9 months. The live Edmonston-Zagreb (EZ) vaccine, (Preblud *et al* 1988), produces a better immunological response than standard vaccines even when given as early as 4 months of age (Sabin *et al* 1983, Sabin *et al* 1984, Whittle *et al* 1984).

Comparative randomized vaccine trials involving Edmonton-Zagreb High Titer (EZ-HT) and Schwartz-High Titer (SW-HT) have been carried out to determine efficacy, safety and immunogencity of the vaccine in many parts of the world.

Results indicate that WHO expert committee recommended that the use of high titer measles vaccines should be discontinued because of excess mortality observed in recipients in Senegal, Guinea-Bissau and Haiti, countries with high base-line infant and childhood mortality rates. The high titer vaccines contained approximately 100-fold higher doses of live measles vaccines than the standard vaccines routinely administered and no matter the make of the vaccine, EZ, SW or AiKC, the standard vaccines prevent deaths of infants and children when administered at the appropriate period to the children concerned although now it appears there is a shift to an older mean age of infection in most developing countries including Ghana.

2.14 FUNCTIONAL CHARACTERIZATION OF T CELLS

The central regulatory cells of the immune system are the T cells, which mediate much of their function by expression and secretion of cytokines. Initally, non-committed or Th0 cells are activated as a result of IL-1, secreted by antigen presenting cells to give rise to Th1 and Th2 cells. Th1 cells produce interleukin-2 and interferon-γ, which activate NK cells and macrophages as well as promoting secretion of other cytokines especially Tumour necrosis factor-α. Th2 cells secrete interleukin-4, interleukin-5, interleukin-6 and interleukin-10, all of which activate B cells to secrete immunoglobulins. The two arms of this response also inhibit one another (IFN-γ inhibits Th2 response, while IL-4 inhibits Th1 response). The reasons for the selective activation of one response over the other are not well understood and also the exact cytokines which mediate protection or aggravation of measles have to be clearly defined. There have been accumulating evidence suggesting that T helper type 1 and 2 (Th1/Th2) cell responses participate in pathological and immunological processes via cytokine secretion.

Cytokines are a family of hormone-like signalling protein mediators of both natural (innate) and specific (acquired) immunity. They are synthesized in response to inflammatory or antigenic stimuli and act locally, in an autocrine or paracrine fashion by binding to high affinity receptors on target cells. The discovery of particular cytokines can often be traced to investigations of infectious diseases or of antigen-induced immune responses. Early studies of cytokines extending from about 1950 to 1970 largely involved the description of numerous protein factors produced by different cells that mediated particular functions in bioassays.

Following on these early discoveries were periods of partial purification and characterization of many individual cytokines as well as production of specific

neutralization antisera. The golden age of cytokine research began in the 1980s through 1990s, characterized by molecular cloning and expression of individual cytokine molecules as well as the production of completely specific often monoclonal antibodies resulting in many new previously unexpected properties of known cytokines. Because of these studies, there is now a wealth of information about the sources and biological activities of particular cytokines.

It is known that cytokines are produced during the effector phases of natural and specific immunity and serve to mediate and regulate immune and inflammatory responses.

Their secretion is a brief, self-limiting event i.e., they are not stored as preformed molecules and their synthesis is initiated by a new gene transcription. Many cytokines are produced by multiple-diverse cell types and can act upon many different cell types as well, a property referred to as pleiotropism.

Cytokine activities are often redundant, many functions being shared properties of several different cytokines. Some cytokines influence synthesis of other cytokines leading to cascades in which a second or third cytokine may mediate the biologic action of the first cytokine. The ability of one cytokine to enhance, support, or suppress the production of others may provide important positive and negative regulatory mechanisms for immune and inflammatory responses.

Most cellular responses to cytokines are slow, occurring over a period of hours and require new mRNA and protein synthesis while most cytokines initiate their action by binding to specific receptors on the surface of target cells. The relevant target cells may be the same cell that secrets the cytokine (autocrine action) a nearby cell (paracrine action) or like true hormones, a distant cell stimulated via cytokines

secreted into general circulation (endocrine action). For many target cells, cytokines act as regulators of cell division i.e. as growth factors. According to their principal actions, cytokines could be grouped into four major identifiable groups as follows:

a. Cytokines that mediate natural immunity e.g. Tumour Necrosis factor, Interleukin-1, and Interleukin-6. The predominant sources of this group of molecules are the mononuclear phagocytes.

b. The second group of cytokines is derived largely from antigen stimulated CD4+ T lymphocytes and serves to regulate the activation, growth and differentiation of B and T cell. This group includes interleukin-2, interleukin-4 and transforming growth factor-β, which inhibits lymphocyte responses.

c. The third group of cytokines produced by antigen-activated CD4+ and CD8+ T lymphocytes serves to activate inflammatory leucocytes and places them under T cell regulation. This group includes γ-interferon, the principal activator of mononuclear phagocytes, lymphotoxin an activator of neutrophils and interleukin-5 an activator of eosinophils.

d. The fourth group of cytokines called colony-stimulating factors consists of cytokines derived from both non-specific effector cells and T cells which stimulate the growth of bone marrow cells thereby providing a source of additional inflammatory leucocytes. Cytokines therefore serve many functions that are critical to host defence against pathogens and provide links between specific and natural immunity. Cytokines also regulate the magnitude and nature of immune responses by influencing the growth and differentiation of lymphocytes. Cytokines provide important amplification mechanisms that enable small numbers of lymphocytes specific for any one antigen to activate a variety of effector mechanisms to eliminate the antigen. Excessive production or actions of cytokines can lead to tissue injury and even death.

The administration of cytokines or their inhibitors is a potential approach for modifying biologic responses associated with disease.

The production of cytokines results in proliferation and differentiation of T cells. After initial stimulation by antigen, CD4+ T cells (TO cells) produce mainly interleukin-2, IL-2. After restimulation, two types of memory CD4 cells emerge. Type 1 cells i.e. those that produce primarily interferon γ (IFN-γ) IL-2 and tumour necrosis factors, TNF-β which is also known as lymphotoxin and type 2 cells, a group of cells that produce cytokines important for macrophage activation leading to delayed type hypersensitivity (DTH) responses (IFN-γ), lymphocyte proliferation (IL-2) and also (MHC class II-restricted cytotoxicity (TNF-β), lymphotoxin. Type 2 cells produce cytokines that are important for macrophage deactivation (IL-4 and IL-10) and B cells help (IL-4, IL-5 and IL-10), (Mosmann *et al* 1987 and Peltz 1991).

Measurement of cytokines released into plasma during measles shows an increase in IFN-γ, a product of natural killer cells (NK) as well as type 1 CD4+ and CD8+ cells before onset of the rash (Griffin *et al* 1990) and IL-2 which is a product of TO and type 1 CD4+ Tcells during the rash. As the rash subsides, plasma levels of IL-4 which is a product of type 2 CD4+ T cells increase and remain elevated for several weeks (Griffin *et al* 1993). This pattern of cytokine production suggests activation of CD8+ T cells (CD8 and IFN-γ) and of TO CD4+ cells during and before the rash followed by a more prolonged activation of type 2 CD4+ T cells after resolution of the rash. The lack of long term activation of type 1 CD4+ T cells (IL-2 and IFN-γ) is consistent with the observation that measles virus-specific delayed type hypersensitivity skin test responses do not develop (Nelson *et al* 1966 and Fulginiti *et al* 1969), and that measles virus-specific lymphoproliferative response are low (Hirsch *et al* 1984 and Greenstein *et al* 1983). The presence of long term activation of type 2 CD4+ T cells is

consistent with the polyclonal B cell activation and increased immunoglobulin E (IgE) that is present during acute phase of measles (Aicardi *et al* 1977, Griffin *et al* 1985) and with the predominance of virus-specific IgG 1 and IgG 3 (Mathieson *et al* 1990 and Ehrnst 1978).

It is therefore suggested that natural measles may result in activation of CD8+ T cells important for virus clearance and type 2 CD4+ T cells important optimal antibody production. However, much more experimental work is necessary to define the specific components of the cellular immune response necessary for virus clearance and for prevention of reinfection. Further investigation is also needed to identify the reason for preferential activation of certain T cells subsets and whether prolonged activation of type 2 T cells has an adverse influence on development of DTH and macrophage responses to new antigens.

2.15 ASSAY METHODS FOR CYTOKINES

Cytokine assays fall into six basic categories with readouts of: - proliferation, cytotoxicity, changes in intracellular or cell membrane, secreted proteins, cell motility and *in vivo* responses.

The choice of an assay system depends on the type of sample being studied and the purpose of measurement. Commercial producers of cytokines from recombinant DNA expression system are working with cytokines in isolation and for them any bioassay system is normally adequate to assess potency of the product. Potential problems arise when microbial expression systems also release other substances, eg endotoxin, which could give positive results in some bioassays. The optimal method for quantifying cytokine levels should involve using a specific biosassay in conjuction with two-site immunoassays for the cytokine and known inhibitors. Correlation of results between the two should inspire confidence in the value obtained. Usually the bioassay fails to detect material because of inhibitors that are not detected in the immunoassays.

Whether for research or clinical purposes, cytokine expression, synthesis, and secretion can be investigated by a variety of different techniques including:

a. Assay of secreted cytokine in supernantants or biological fluids.

b. Analysis of expression of cytokine specific mRNA by Northern blotting or reverse transcriptase polymerase chain reaction-RT-PCR.

c. Detection of cytokines in tissue samples by immunohistochemistry or in situ hybridization. The concentration of different cytokines present in biological fluids eg. Serum, plasma or urine and in culture supernatants can be determined based on their activity or on their immunogenicity or receptor-binding properties.

All assays require the use of standard preparation of recombinant cytokines of known activity and concentration. Cytokine assays should be calibrated against reference cytokine preparations. Reference standards are available from licensed commercial sources and agents.

2.15.1 BIOASSAYS

This method involves the use of "indicator cells", which either could be freshly isolated cells or established cell lines. Although bioassays are still a convenient way to identify or quantitate cytokines, it has become evident that most indicator cells are seldom cytokine specific and can usually respond to other cytokine in addition to the one they are intended to measure. Specificity may be a problem. Nevertheless when used in conjunction with appropriate neutralizing anti-cytokine monoclonal antibodies to either confirm the identity of the cytokine being measured or to neutralize potentially interfering cytokines, bioassays still offer relatively simple and sensitive means for cytokine assay.

ADVANTAGES

Bioassay methods measure only functional cytokines, which is a true reflection of biological activity. The methods are very sensitive, detection level of cytokines could be as low as $10pgml^{-1}$, however the methods could be relatively inexpensive.

DISADVANTAGES

In bioassays indicator cells are usually not cytokine specific-could be susceptible to potentiating or inhibitory effects of other cytokines; also respond to more than one cytokine. The cells could also be susceptible to inhibitors present in biological fluids

eg. Cytokine antagonists and soluble cytokine receptors. The assays do not differentiate between subtypes of cytokines e.g. IL-1α and IL-1β, TNF-α and TNF-β. Reproducibility may be compromised due to variability in indicator cells and also could take several days instead of hours to complete.

2.16 IMMUNO ASSAYS

These assays are based on the detection and quantitation of cytokines based on their reactivity with specific antibodies or binding to specific receptors. These types of assays are mostly preferable because of their speed and specificity, especially when monoclonal antibodies are used.

ADVANTAGES

The assay methods are highly specific, reproducible and fast and results could be available wintin 4 hours. They are not susceptible to inhibition and are less susceptible to interference by cytokine antagonists or soluble receptors.

DISADVANTAGES

Immunoassays may not differentiate between biologically active and inactive cytokines. Some immunoasays detect rather high levels of cytokines (50 pg ml^{-1}) making it difficult to measure lower levels of cytokines in samples.

3.0 SUBJECTS AND METHODS

SUBJECTS

Forty-four children diagnosed by physicians as having measles were recruited into the study. Criteria for inclusion in the study were based on the following: A characteristic febrile illness with body temperature > 38°C, a general maculopapular rash and at least one of the following: cough, coryza, conjunctivitis, koplic spot. Children with bronchopneumonia, septicaemia, persistent diarrhoea (lasting more than 2 weeks) or malaria were excluded from the study. A questionnaire was completed and informed consent of parent or guardian was sought before samples were taken. Venous blood was collected from patients on Day 0 (acute), day 14 and day 60 (convalescent).

Blood samples were collected at the La Polyclinic and 37 Military Hospital, Accra. These are locations of an earlier study on measles. In addition, blood samples from healthy, age and sex-matched children were obtained from the Madina Maternal and Child Health Centre and used as controls.

SAMPLE COLLECTION

Approximately 5mls of venous blood were taken into K3 EDTA vacutainer tubes. This was regarded as the first sample. Another sample was collected at two weeks after measles and a third sample was taken two months after measles and these represented the convalescent phase samples. For age matched healthy controls venous blood was collected once. All samples were delivered to the Immunology Unit of the NMIMR within 3 hours after collection.

Plasma was obtained from the anticoagulated blood by Ficoll-Hypague Density Gradient Separation, stored a -40°C, and used for the assays. Peripheral blood

mononuclear cells were obtained and stored in liquid nitrogen and used for cellular immunity studies.

BLOOD FILMS FOR MALARIA

Thick and thin blood smears were made for each sample taken and stained with a 1 in 10 dilution of Giemsa stain (Wako Pure Chemicals Ltd, Tokyo) and examined for malaria parasites, under a light microscope (Olympus, Tokyo, Japan).

REAGENTS & KITS

ELISA Kits were purchased to assay the following cytokines: TNFα, IFNγ, IL-2, IL-4, IL-10, IL-6 and TGF-β (R & D Systems Europe Ltd).

3.1 MEASLES ANTIBODY LEVELS - ENZYME LINKED IMMUNOASSAY

Measles antibody levels were measured using commercial ELISA kits for determination of IgG & IgM antibodies to measles virus (Denka Seiken Co. Ltd Japan).

PRINCIPLE

Purified measles antigen is attached to wells of microplates. Diluted test plasma samples are added to coated wells and incubated for 1 hour. During incubation, antibodies to measles present in plasma bind to antigen-coated well. After washing to remove unbound material, antibodies to human IgG (γ-chain specific) labelled with alkaline phosphatase (conjugate) are added. The conjugate binds to any IgG antibodies bound to measles antigen. The wells are washed to remove unbound conjugate and incubated with p-nitrophenyl phosphate, which is hydrolysed by alkaline phosphatase to form p-nitrophenol, a yellow-coloured end product with absorbance maximum at 405nm. The intensity of the absorbance at 405nm is proprotional to the amount of IgG antibodies to measles present in the sample.

MEASLES IgM

PRINCIPLE

Puried measles antigen is attached to wells of microplates. Test samples are first diluted with anti-human IgG antibody (pre-treatment reagent) and incubated. This immunologically inactivates any IgG antibodies present in the sample and eliminates interferences by measles IgG and IgM rheumatic factor. Pretreated samples are added to coated wells and incubated. During incubation IgM antibodies to measles present in sample will bind to antigen-coated wells. After washing to remove any unbound

material antibodies to human IgM (μ-chain specific) labelled with alkaline phospatase (conjugate) are added. The conjugate binds to any IgM antibodies bound to measles antigen. The wells are washed to remove unbound conjugate and incubated with p-nitrophenyl phosphate. The p-nitrophenyl phosphate is hydrolysed by alkaline phosphatase to form -p-nitrophenyl, a yellow-coloured end product with absorbance maximum at 405nm. The intensity of the absorbance at 405nm is proportional to the amount of IgM antibodies to measles present in sample.

MEASLES IgG

PRINCIPLE

Purified measles antigen is attached to wells of microplates. Diluted test samples are added to coated wells and incubated. During incubation, antibodes to measles present in serum will bind to antigen-coated well. After washing to remove unbound material, antibodies to human IgG (μ-chain specific) labeled with alkaline phosphatase (conjugate) are added. The conjugate binds to any IgG antibodies bound to measles antigen. The wells are washed to remove unbound conjugate and icubated with p-nitrophenyl phosphate. The p-nitrophenyl phosphate is hydrolyzed by alkaline phosphatase to form p-nitrophenyl, a yellow-colored end product with absorbance maximum at 405 nm. The intensity of the absorbance at 405 nm is proportional to the amount of IgG antibodies to measles present in sample.

3.2 VISUALIZATION OF VIRAL PARTICLES BY TRANSMISSION ELECTRON MICROSCOPIC (TEM) EXAMINATION OF MNCS

MNCS separated from patients whole blood were fixed in 2% Gluteraldehyde (GA) in sodium cacodylate buffer pH 7.3 (Taab Laboratories Berkshire UK) for 3 hours at $4^{o}C$ followed by a wash in cacodylate buffer alone and post-fixed in 1% O_s O_4 (Taab Laboratories, Berkshire UK) for 1 hour at $4^{0}C$. The cells were washed once more in the buffer and dehydrated by passage through graded series of ethanol (50%, 60%, 70%, 80%, 90%, 95% and 100%) for 10 minutes each.

The dehydrated MNCS were then placed in embedding medium Epon 812 (Taab Laboratories Berkshire, UK) and Propylene Oxide overnight during which time the cells were infiltrated with the medium. Infiltration was then followed with polymerization in Epon 812 in BEEM capsule at $60^{o}C$ for 48 hours. The embedded cells were trimmed with a glass knife and ultra-thin sections (75nm) were cut using Leica Ultracut R microtome and the Ultra thin sections were collected onto uncoated 200 mesh copper grids and stained with 2% uranyl acetate (Agar Scientific Ltd, Stanstead, Essex, UK), for 20 minutes after which the grids were washed in distilled water and double stained with lead citrate solution for 20 minutes, washed again with distilled water and air-dried on filter paper for 30 minutes and examined with the TEM, JEOL 1010, with acceleration voltage 80Kv and micrographs prepared.

3.3 CYTOKINE ASSAYS

Cytokine levels were measured using commercial quantitative enzyme ELISA kits (Quantikine™ R+D Systems, Inc. Minn USA)

Plasma samples were diluted one in 40 and added to pre-coated microtiter plates. Standards were included. Cytokines present were bound by the immobilized antibody. After washing away any unbound proteins, an enzyme-linked polyclonal antibody was added to the wells to "sandwich" the immobilized antibody during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of antibody found initially. The color development was stopped and the intensity of the color measured. A curve was prepared plotting the optical density versus the concentration of antibody in the standard wells. By comparing the optical density of the samples to this standard curve the concentration of the cytokine in the unknown plasma samples were determined.

PRINCIPLE OF THE ASSAY

Immunoassays employ the quantitative "sandwich" enyzme technique. A monoclonal antibody specific for the cytokine is coated onto the micro titer plate provided in the kit. Standards as well as sample are pipetted into the wells and the immobilized antibody binds any cytokine present. After washing away any unbound proteins, an enyzme linked polyclonal antibody specific for the cytokine is added to the wells to sandwich the cytokine immobilized during the first incubation. Following a wash to remove any unbound antibody-enyzme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of cytokine bound in the initial step. The colour developement is stopped and the intensity of the colour measured. A

curve is prepared, plotting the optical density against the cocncentration of cytokine in the standard wells. By comparing the OD of the samples to this standard curve, the concentration of the cytokine in the unknown is then determined.

In this study commercial kits obtained from R+D System, Europe Ltd, are utilized in the immunoassays. ELISA is one of the most versatile and widely available techniques with sensitivity between 100 pg ml^{-1} and 1 ng ml^{-1}. Incubation period is short (2 hours at room temperature) and the method is reliable and specific.

DATA ANALYSIS

Statistical Analysis were performed by the Sigma stats 2.0 software and data shown as means ± 95% confidence intervals (95% CI). Student t-test was used to compare groups while correlation (r^2) was calculated to estimate differences of quantitative variables. A 2-tailed P value <0.05 was considered significant.

4.0 RESULTS

4.1 DEMOGRAPHIC CHARACTERISTICS OF GHANAIAN CHILDREN WITH MEASLES

A total of 44 children all below 12 years, mean age 6.2 ± 3.5 were recruited into the study after informed consent was obtained from parents / guardians. Of the total, 59.1% were males and the male: female ratio was 1.14:1. No significant age differences were noted. Seventy-six percent 76% (20/26) males were followed up while 50% (9/18) females dropped out by day 14 (Table 1a). There were an improvement in the female group on day 60 when 66% (12/18) females were followed up. Table 1b shows measles vaccination record by age of measles cases. Only 4 (9.1%) were under 1 year. The number of children aged 6-12 years whose immunization profiles were unknown was 15 (34.1%). Measles vaccination status checks revealed that some of the children, 12 (27.3%) had previously received measles vaccine. Four cases had not received any vaccination. All the patients examined had no complications and all of them recovered completely with no mortality. The study was approved by the Government Ethics Committee of the Ministry of Health, Ghana.

4.1.1 CONTROLS

Thirty-nine children, mean age 6.3 ± 3.5 years examined by doctors to have no illness in the previous six to eight weeks served as controls. Their blood samples were taken once only.

4.2 MALARIA PARASITE TEST

Thick and thin blood films from measles cases and healthy controls and immunized were stained with a 1 in 10 dilution of stock Giemsa stain (Wako Pure Chemicals Ltd, Tokyo Japan) using a buffer pH 7.2, and examined for malaria parasites. Results obtained showed that none of the subjects recruited into this study had malaria infection at the time of recruitment.

4.3 MEASLES IgG AND IgM ANTIBODIES AND SYMPTOMS OF PATIENTS

SYMPTOMS: The most common symptom associated with the outbreak was skin rash. Rash occurred in all patients diagnosed with measles whether or not IgM was found alone or with IgG antibodies in the plasma of the patients. Fever was present in 83% (5/6) of patients who had only IgM in their plasma and completely absent in 10 patients who had only IgG antibodies in their plasma and present in 23% (5/22) in whose plasma both IgM and IgG antibodies were detected Table 4.1.

Koplik spot was found among patients in whose plasma samples both IgG and IgM antibodies were detected (64% 70%) and in 33% of patients in whose plasma samples, IgG antibodies were absent.

ANTIBODIES: Of the thirty-eight children tested for measles IgG and IgM by ELISA, IgM were detected in 73.7% (28/38) in the acute phase. IgG were detected in 84.2% (32/38) patients while six cases had only IgM and 10 patients had only IgG. Twenty-two cases had both IgM and IgG.

Table 4.2shows comparison of clinical symptoms with positive IgG and IgM titres in the acute phase of measles infection.

Table 4.3 shows combination of clinical symptoms of patients in the acute phase of measles infection. The most common combination of major symptoms of patients with measles who have been vaccinated was rash, koplik spot, cough, coryza and conjunctivitis. Only 2 out of the 32 cases (6.3%) had all the typical symptoms of measles.

4.4 VISUALIZATION OF VIRAL PARTICLES BY TRANSMISSION ELECTRON MICROSCOPE (TEM)

Viral particles were clearly visible at the acute phase of the illness, (Fig. 4.1) indicative of presence of measles viruses during the acute phase of the disease.

During the convalescent stage, 2 months after infection, the viral particles were no longer visible (Fig. 4.2) confirming absence of measles viruses. Taken together these results show that the subjects recruited into the study had measles virus infection and not any other infectious diseases. All patients recovered completely with no mortality.

4.5 CYTOKINE LEVELS IN PLASMA OF MEASLES PATIENTS AND HEALTHY CONTROLS

4.5.1 PLASMA INTERLEUKIN-4 (IL-4) LEVELS IN MEASLES INFECTION

IL-4 levels were measured in plasma of children diagnosed with measles and results obtained compared with healthy controls, Table 4.4. Plasma samples obtained from measles children 14 days and 60 days (convalescent period) were also assayed and results compared with those of the acute phase of the illness. IL-4 concentration of almost 1000 pg/ml was obtained for children during the acute phase of the illness while values <10 pg/ml were obtained on days 14 and 60 under investigation (Fig 4.3). In these plasma samples IL-4 concentration was very high compared with controls ($p<0.03$) but the high values were not maintained 14 or 60 days post infection.

Minimum detection levels in plasma is <10.0 pg/ml^{-1}

4.5.2 INTERFERON-γ (IFN-γ) LEVELS IN PLASMA OF MEASLES PATIENTS

IFN-γ levels were determined in plasma samples of children at the acute stage and compared with values at day 14 and day 60 (convalescent phases) and with healthy control group. Results obtained gave about 45 pg/ml IFN-γ in plasma samples of patients at the acute stage. This declined to around 15 pg/ml at day 14 days and then down to <10 pg/ml at day 60. (Fig:4.4). The level at convalescent stage was about the

same as obtained in the healthy controls. IFN-γ production was quite low in these samples and down-regulation occurred as the illness resolved.

Mininum detection dose is <8 pg/ml^{-1}

4.5.3 INTERLEUKIN-12 (IL-12) LEVELS IN MEASLES INFECTION

Plasma levels of IL-12 were determined in children diagnosed with measles and values obtained compared with recovery and convalescent phases of the illness. Value of 1.15 pg/ml was obtained at the acute phase and again a significant drop ($p<0.03$) to around 0.9 pg/ml was measured at 60 days (Table 4.4). In the healthy controls, a value of 0.57 pg/ml-1 was obtained (Fig: 4.5). IL-12 responses of these children were significantly low. Measles specific IL-12 production was not up regulated, although values in patient categories were mildly higher than values in healthy controls.

Minimum detection levels in plasma is <5.0 pg/ml^{-1}

4.5.4 INTERLEUKIN-10 (IL-10) LEVELS IN MEASLES INFECTION

IL-10 produced during measles was measured and compared with recovery and convalescent phases of the illness. (Fig: 4.6). Value of <7 pg/ml was obtained in the healthy controls. IL-10 values in the acute measles cases were mildly less than the controls (6.5 pg/ml^{-1}) but this dropped to <3.7 pg/ml-1 by day 60 (Table 4.4). There was an obvious non-significant response to natural measles virus and values obtained post infection declined with time.

Minimum detection levels in plasma is <3.9 pg/ml^{-1}

4.5.5 INTERLEUKIN-2 (IL-2) LEVELS IN MEASLES INFECTION

IL-2 production (Table 4.4) was low in plasma samples at all stages of the illness. The highest amount of 8.8 pg/ml was measured 14 days after measles virus infection. (Fig: 4.7). In the healthy controls, values around 6.9 pg/ml^{-1} were measured, significantly lower than the patients ($p=0.05$) yet overall, IL-2 response in measles patients was not high.

Minimum detection levels in plasma is <7.0 pg/ml^{-1}

4.5.6 TUMUOR-NECROSIS FACTOR (TNF-α) LEVELS IN MEASLES INFECTION

TNF-α concentration in plasma samples of measles patients studied were 9.7 pg/ml at the acute phase and 7.2 pg/ml^{-1}at the convalescent phase of illness. Levels in the recovery phases (d. 14, d.60) were the same (Fig: 4.8). TNF-α values were not elevated. They showed continuous reduction post infection period when compared with values of the control group.

Minimum detection levels in plasma is <4.4 pg/ml^{-1}

4.5.7 TRANSFORMING GROWTH FACTOR-BETA I (TGFβI) LEVELS IN MEALSES PATIENTS

TGFβI level in plasma of all study subjects were remarkably high (Fig:4.9). In the healthy controls, 854 pg/ml-1 of TGFβI levels were obtained while in the acute phase samples (day 0), patients had 548 pg/ml-1, increasing siginificantly ($p<0.05$) to 633 pg/ml^{-1}. Values dropped to around 381 pg/ml^{-1}, during the convalescent phase (Table 4.4) of the illness. TGFβ has been known to antagonize many responses of

lymphocytes and to measure almost 400pg/ml^{-1} of TGFβ in the plasma samples on day 60 post infection, a sign of readiness to shut off immune responses when challenged *in vivo*.

Minimum detection levels in plasma is <7.00 pg/ml^{-1}

REGRESSION AND 95% CI OF CYTOKINES

Furthermore ratios of plasma IL-10, IL-4, IL-2: TNF-α were determined (data not shown) and multiple regression analysis were performed (Fig 4.11). Correlation between IL-10 and TNF-α determined in day 0 (acute phase) samples was statistically significant ($p=0.028$, $r^2=0.016$) and highly significant in the day 60 (convalescent phase) samples ($p<0.001$, $r^2=0.110$). Correlation between IL-4 and TNF-α in day 0 and correlation between IL-4 and TNF-α in day 0 (acute phase) samples was significant ($p=0.006$, $r^2= 0.171$) Fig. 4.12. Correlation between IL-2 and TNF-α was statistically insignificant in both day 0 and day 60 samples ($p=0.979$, $p=0.162$ respectively). Ratios of IL-10, IL-4, IL-2 vrs. TNF-α were supportive of above findings.

TABLE 4.1a DEMOGRAPHIC CHARACTERISTICS OF PATIENTS AND HEALTHY CONTROLS

MEASLES PATIENTS

	Healthy Controls	Acute	14 days follow-up	60 days follow-up
Boys (n)	**26**	**26**	**20**	**21**
Age Mean (years)	6.6	5.8	6.5	6.0
Range	5.2 - 7.9	4.4 - 7.2	5.1 - 7.9	4.6 - 7.3
Girls (n)	**13**	**18**	**9**	**12**
Age (mean) years	5.8	5.4	7.1	6.3
Range	3.6 - 8.8	3.7 - 7.0	5.2 - 9.8	4.4 - 8.3
	39	**44**	**29**	**33**

TABLE 4.1b. MEASLES VACCINATION HISTORY BY AGE OF MEASLES CASES.

	Age				
Vaccination status	<1 Year	1-2 Years	3-5 Years	6-12 Years	Total
Vaccinated	0	0	0	4	4 (9.1%)
Not vaccinated	4	2	2	4	12 (27.3%)
Unknown	0	2	11	15	28 (63.6%)
Total	4 (9.1%)	4 (9.1%)	13 (29.5%)	23 (52.3%)	44

TABLE 4.2

MEASLES ANTIBODY AND CLINICAL SYMPTOMS IN ACUTE PHASE OF MEASLES INFECTION.

Symptoms Antibody	BT >37.5C	Rash	Koplik Spot	Cough	Coryza	Conjunctivitis
IgM + IgG- N=6	83% (5/6)	100% (6/6)	33% (2/6)	100% (6/6)	50% (3/6)	67% (4/6)
IgM - IgG+ N=10	0% (0/10)	100% (10/10)	70% (7/10)	70% (7/10)	70% (7/10)	70% (7/10)
IgM + IgG+ N=22	23% (5/22)	100% (22/22)	64% (14/22)	95% (21/22)	59% (13/22)	73% (16/22)

TABLE 4.3.

INCLUSION CRITERIA AND THEIR VARIOUS COMBINATIONS

Clinical symptoms	Number of patients
Rash + Koplik spot + Cough + Coryza + Conjunctivitis	7
Rash + Koplik spot + Cough + Conjunctivitis	5
Rash + Cough + Conjunctivitis	4
Rash + Koplik spot + Cough + Coryza	3
Fever + Rash + Koplik spot + Cough + Coryza + Conjunctivitis	2
Rash + Cough + Coryza + Conjunctivitis	2
Rash + Koplik spot + Cough	2
Rash + Cough + Coryza	2
Fever + Rash + Koplik spot +Cough + Coryza	1
Fever + Rash + Koplik spot + Cough + Conjunctivitis	1
Fever + Rash + Cough	1
Rash + Coryza + Conjunctivitis	1
Rash + Conjunctivitis	1

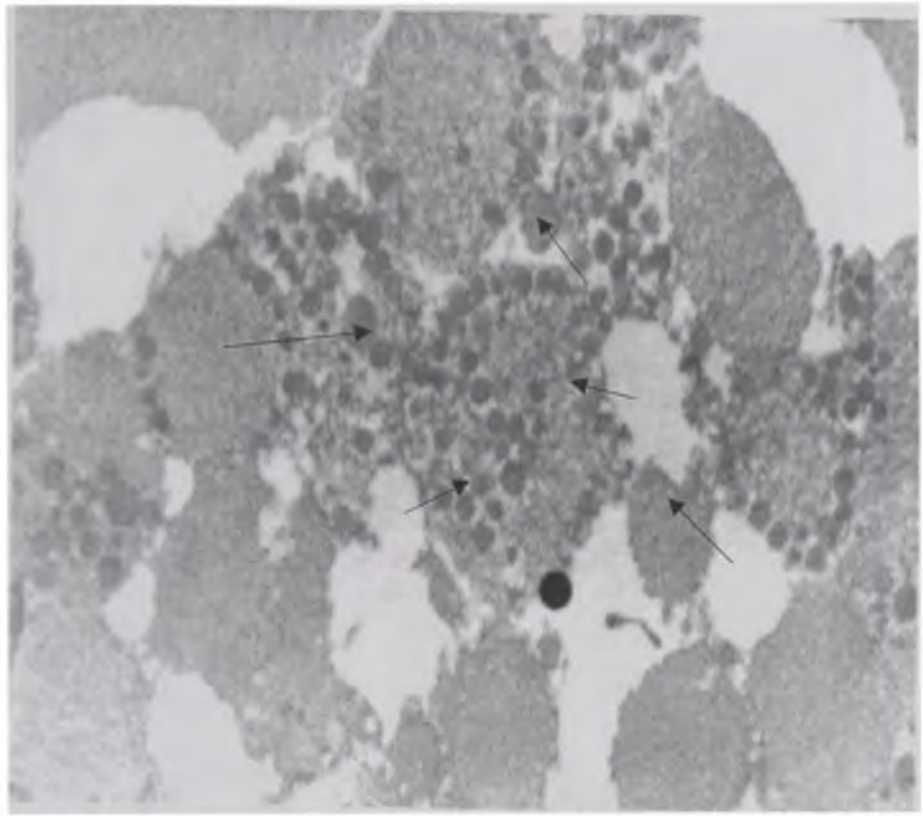
TABLE 4.4.

PRO- AND ANTI-INFLAMMATORY CYTOKINE LEVELS IN PLASMA OF HEALTHY CONTROL AND VACCINE-MODIFIED MEASLES (VMM) IN CHILDREN.

	Healthy control	Vmm acute	Vmm day 14	Vmm day 60
IL-12 (pg/ml)	0.57* (0.41 - 0.73)	1.15* (0.98 - 1.32)	1.1* (0.92 - 1.28)	0.91* (0.68 - 1.13)
INF-γ (pg/ml)	7.3* (3.75 - 10.85)	40.5* (13.49 - 67.51)	14.93 (0.01 - 29.82)	5.27 (3.79 - 6.76)
TNF-α (pg/ml)	8.19 (6.76 - 9.62)	9.7 (7.13 - 12.26)	7.23 (6.16 - 8.29)	7.23 (6.16 - 8.29)
IL-2 (pg/ml)	6.93* (5.97 - 7.88)	8.32 (7.29 - 9.35)	8.79* (7.54 - 10.44)	7.6 (6.58 - 8.62)
IL-4 (pg/ml)	3.85* (3.21 - 4.49)	984.51 (288.17 - 1680.86)	2.69* (2.09 - 3.28)	3.77* (2.4 - 4.4)
TGF-β1 (pg/ml)	854.1 (5563. - 1151.9)	548.8 (365.8 - 731.8)	633.4 (421 - 845.8)	381.4 (202.6 - 560.3)
IL-10 (pg/ml)	6.9* (5.32 8.48)	6.57 (5.48 7.66)	4.7* (1.69 - 7.7)	3.73* (1.05 - 6.42)
Mean age (yrs ± s.d)	6.3 ± 3.5	5.7 ± 3.4	5.7 ± 3.4	5.7 ± 3.4
Sample size	33	33	33	33

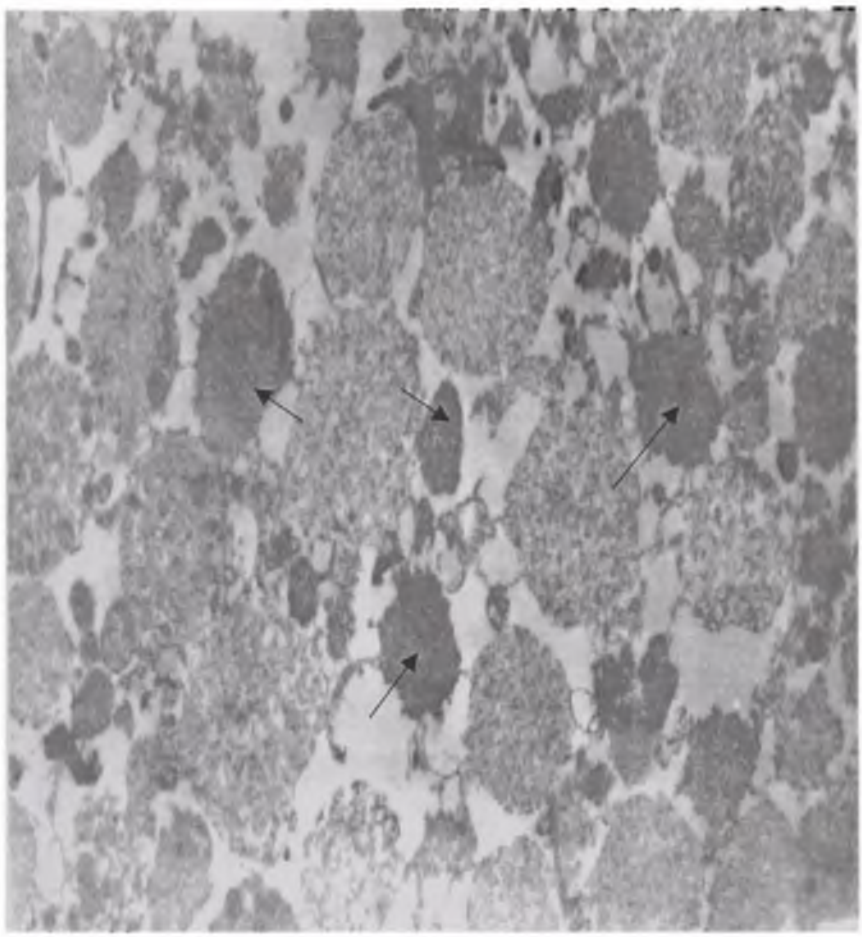
All plasma values are arithmetic means (95% Confidence Intervals).
*** Indicates significant difference at $p<0.05$.**

→ - **Pro-inflammatory cytokines**
◆ - **Anti-inflammatory cytokines**



X16 000

Fig. 4.1
Transmission Electron Microscope picture of mononuclear cells at acute phase of measles infection. Viral particles are clearly visible. (Arrows)
Magnification X16 000



X16 000

Fig. 4.2
Transmission Electron Microscope picture of mononuclear cells of convalescent stage of measles infection. Arrows show mononuclear cells. No viral particles are visible. Magnification X16 000.

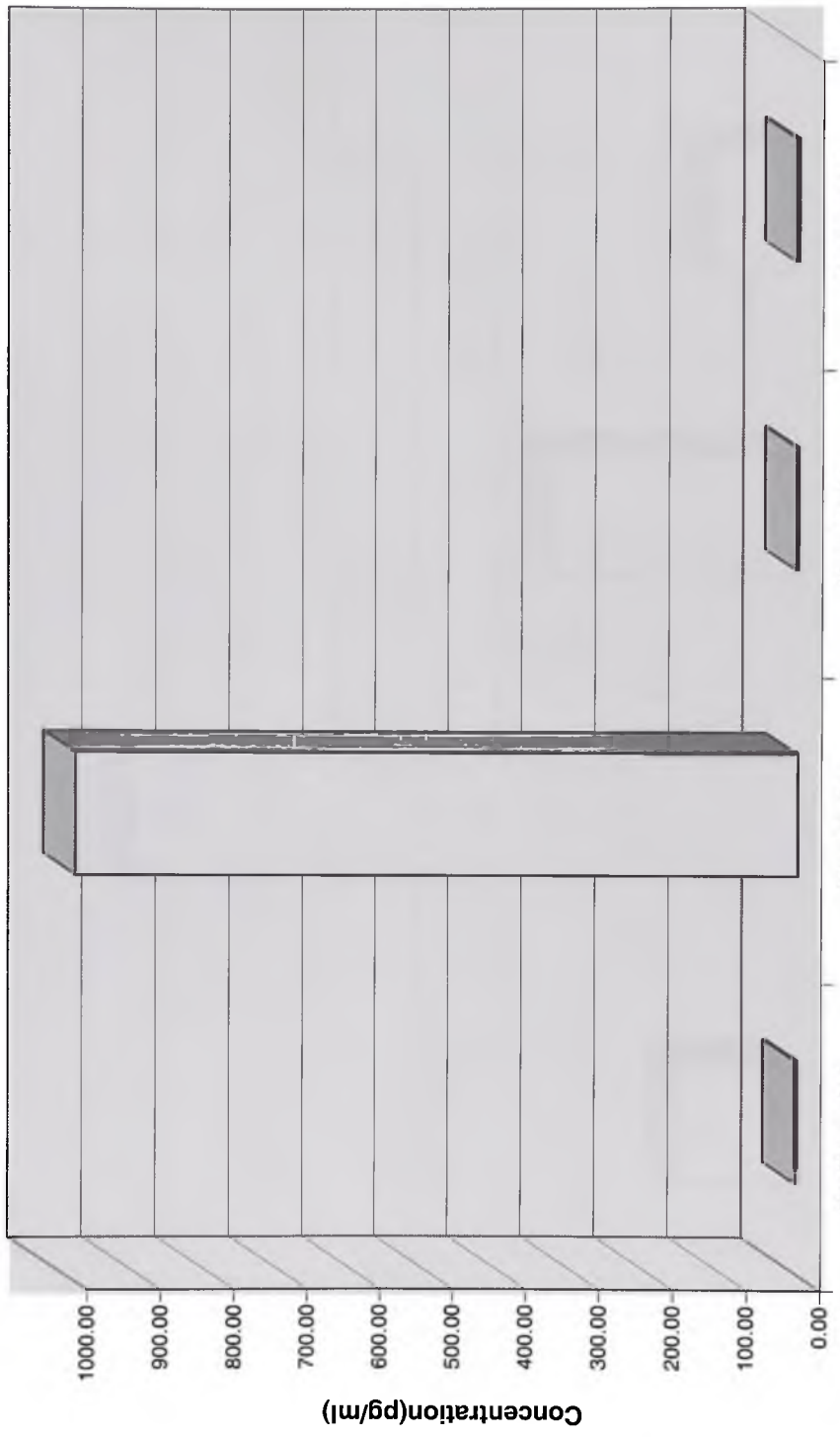
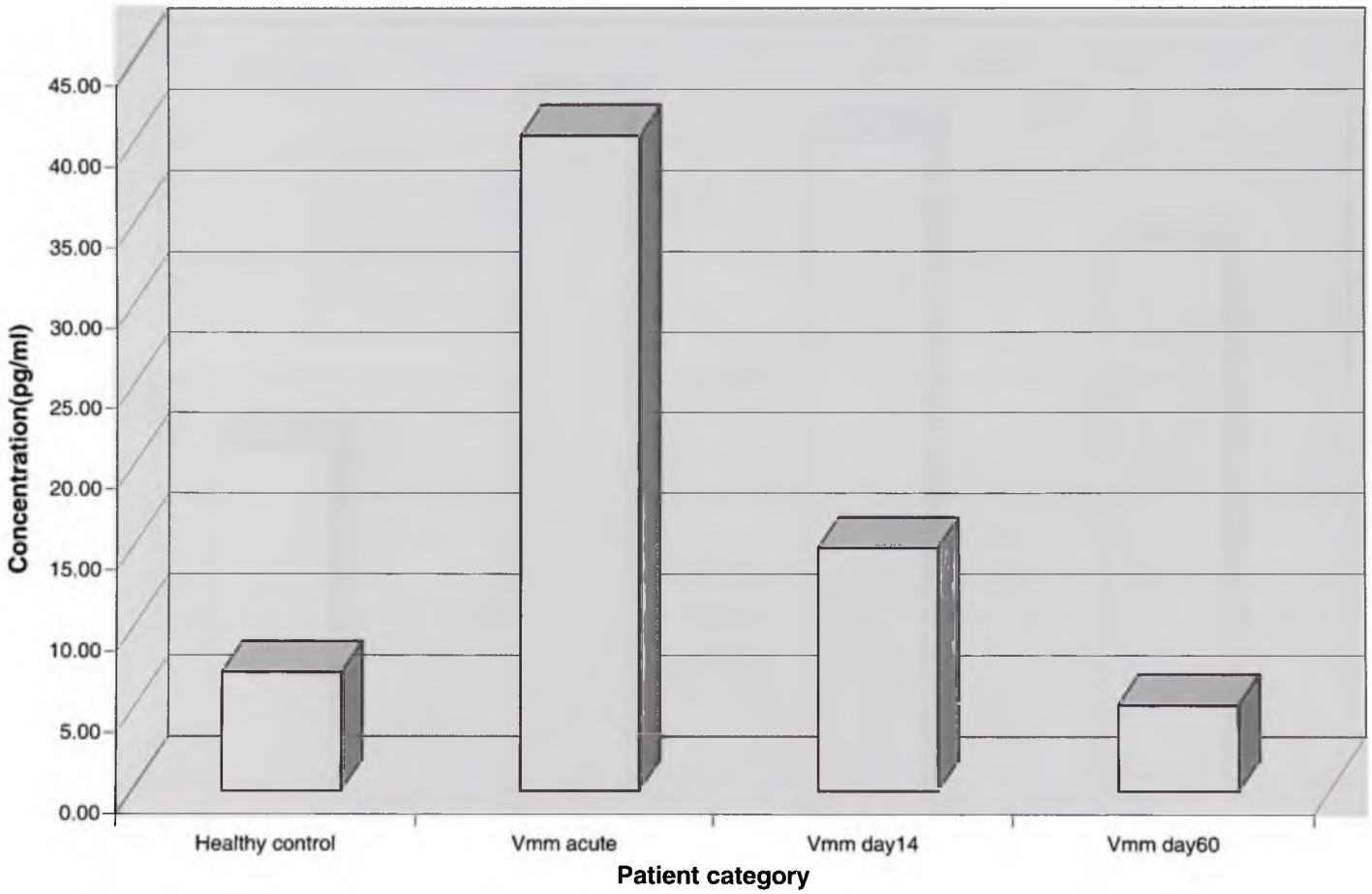


Fig 4.3: Levels of IL-4 plasma cytokine in vaccine modified measles(vmm)

Fig 4.4: Levels of INF-gamma cytokine in plasma in vaccine modified measles(vmm)



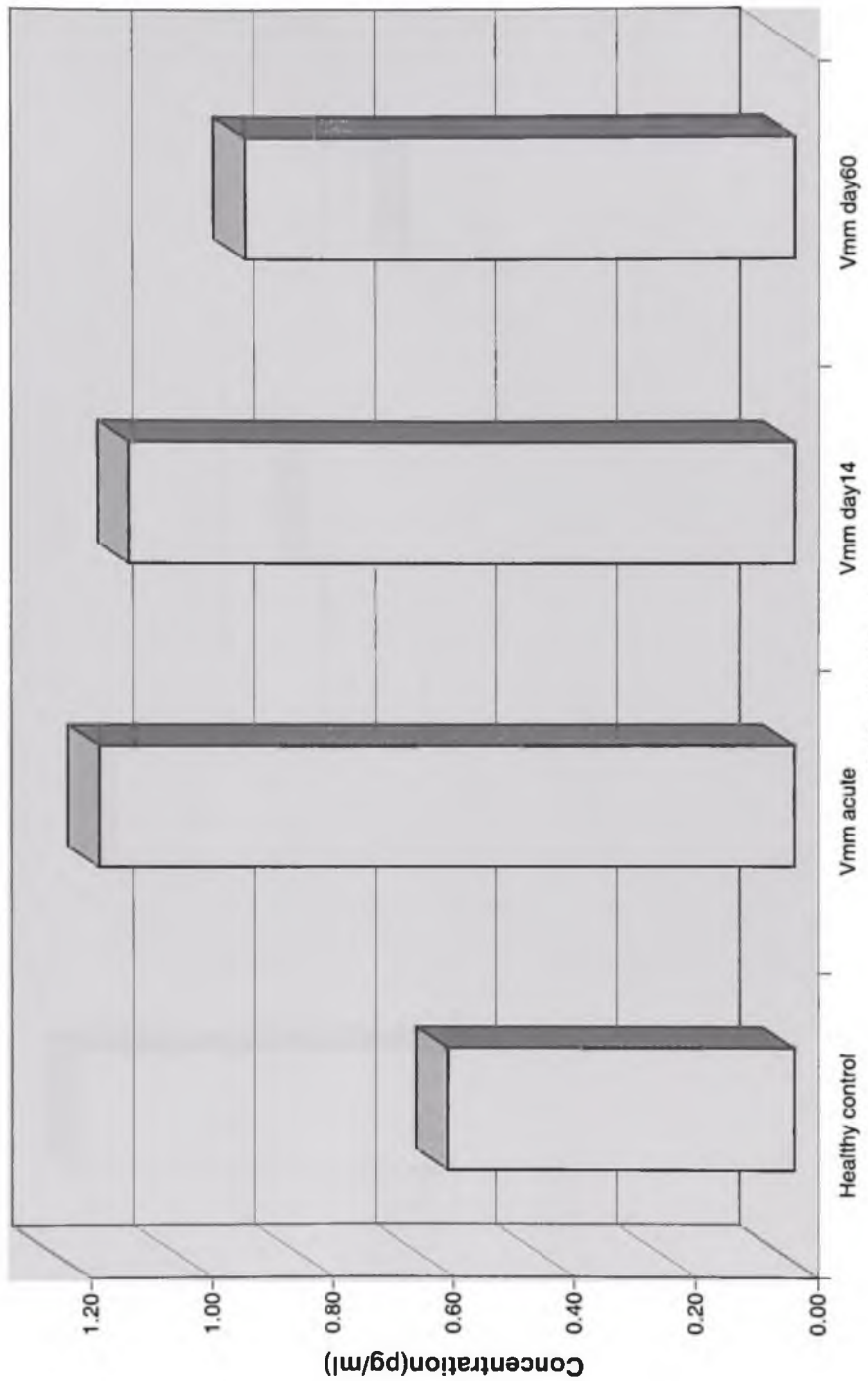
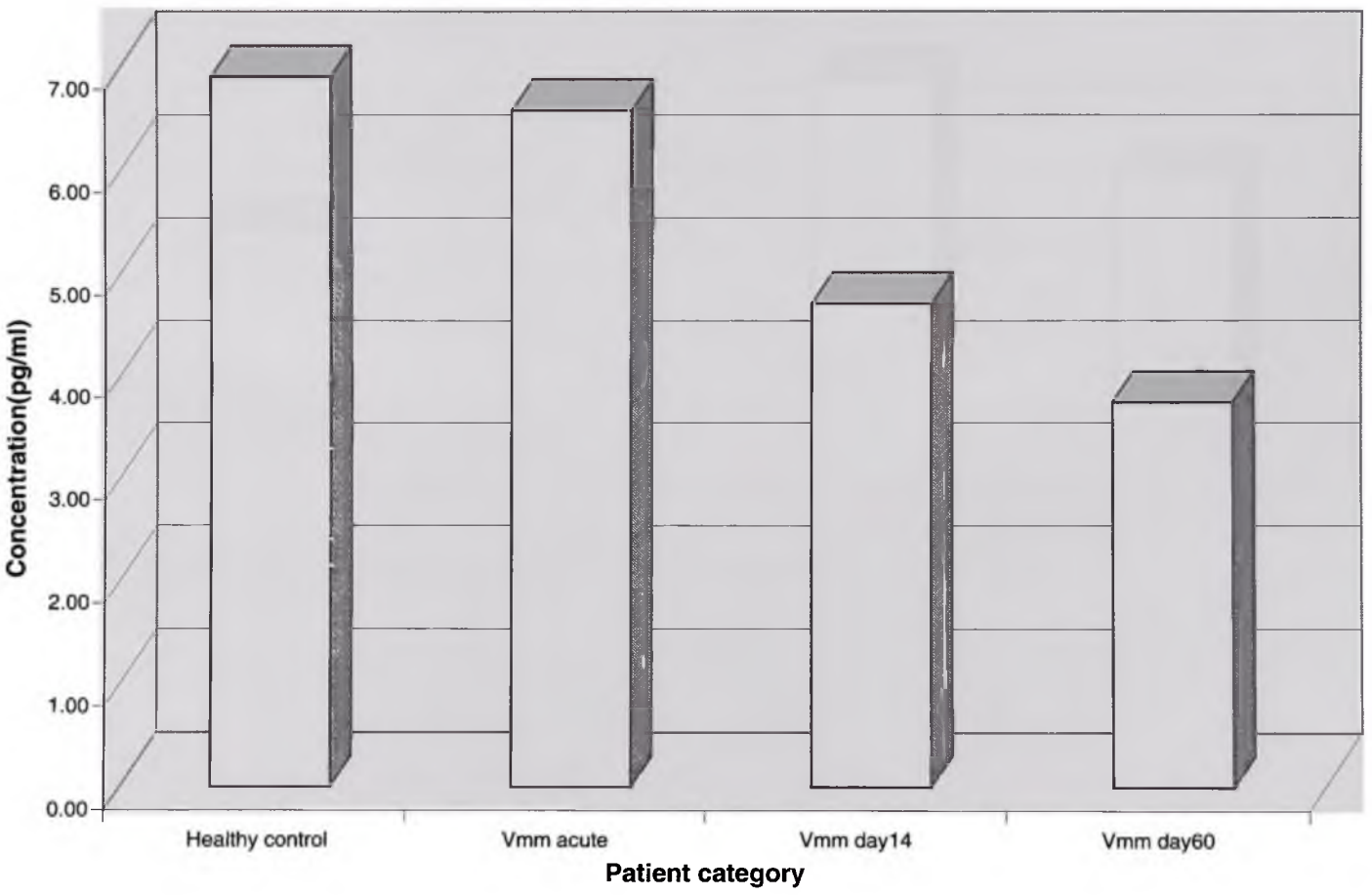


Fig 4.5: Levels of plasma IL-12 cytokine in vaccine modified measles(vmm)

Fig 4.6: Levels of IL-10 plasma concentration in vaccine modified measles(vmm)



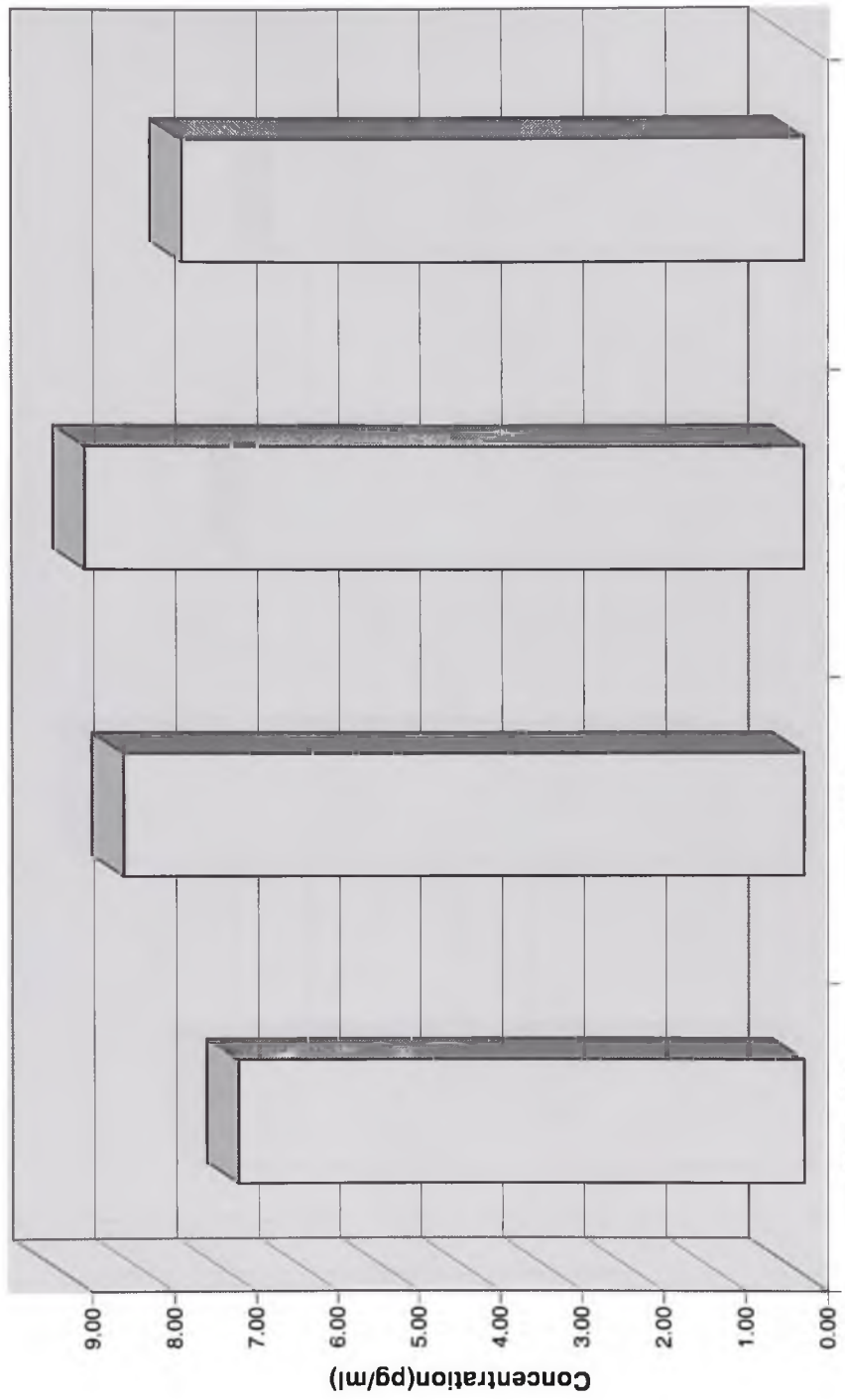


Fig 4.7: Levels of IL-2 plasma cytokine in vaccine modified measles(vmm)

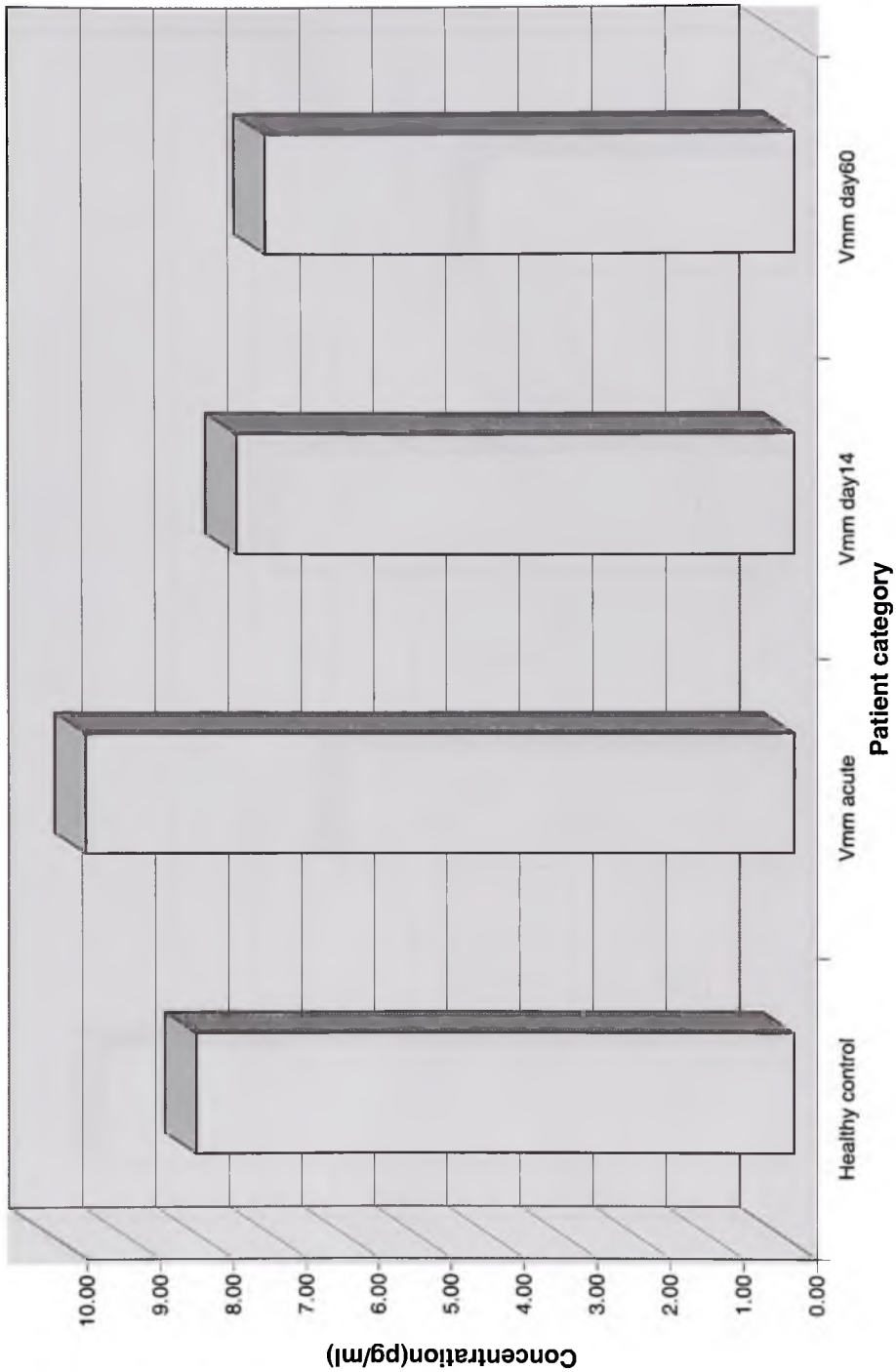


Fig 4.8: Levels of TNF-alpha plasma cytokine in vaccine modified measles(vmm)

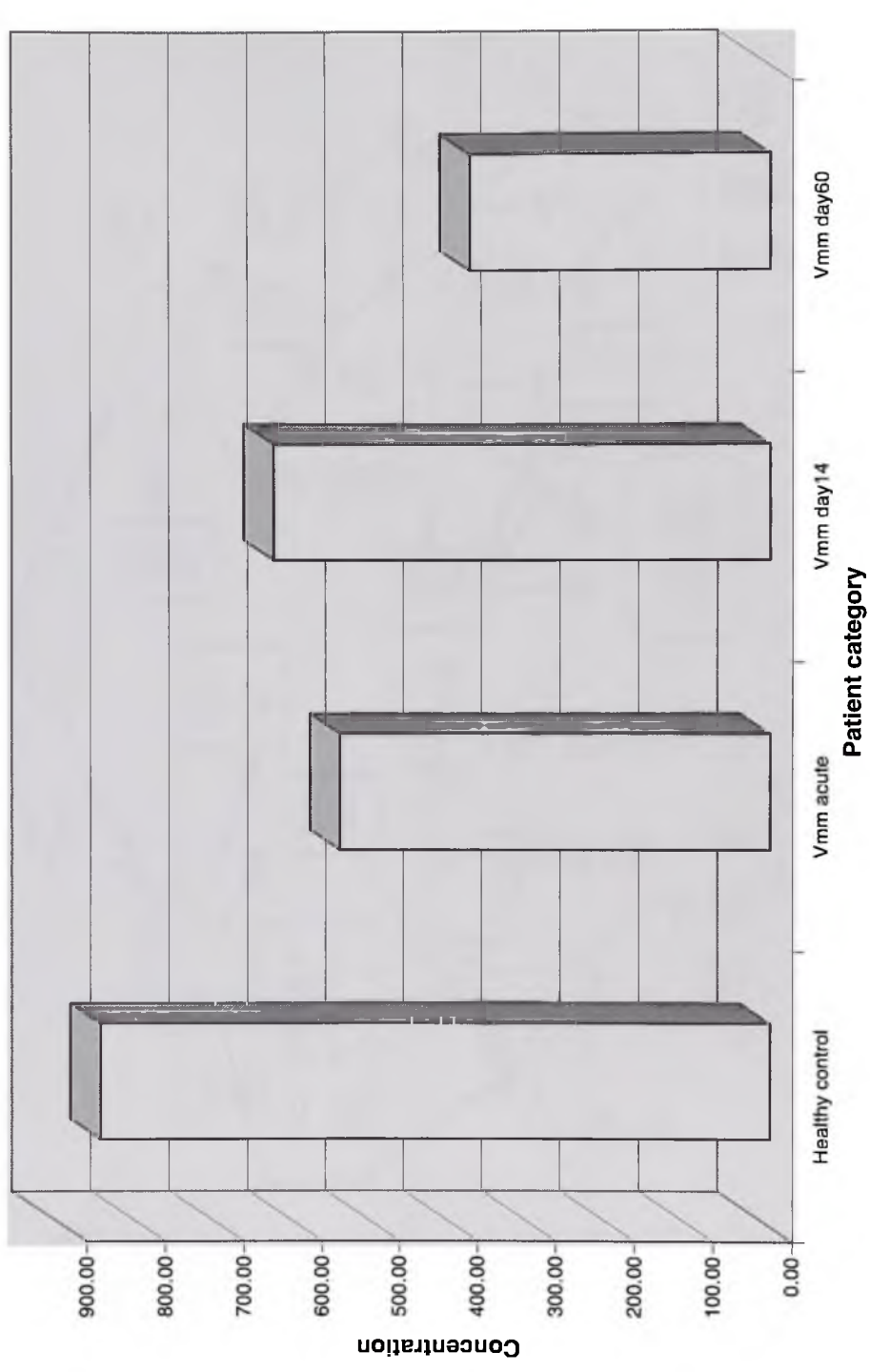


Fig 4.9: Levels of TGF-beta1 plasma cytokine in vaccine modified measles(vmm)

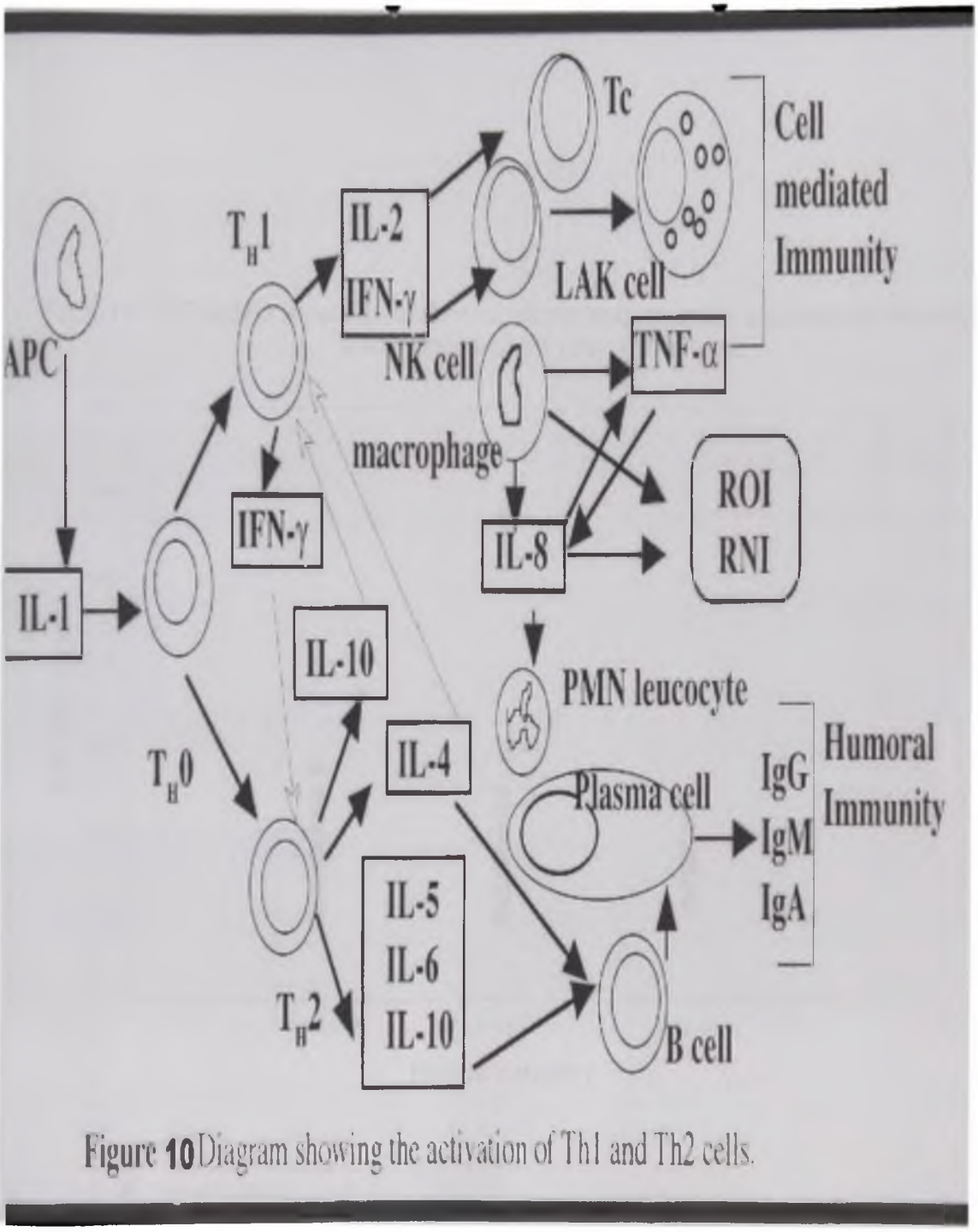


Fig. 4.10: Diagram showing the activation of Th1 and Th2 cells

Fig 4.11 TNF-alpha levels in healthy controls and measles patients at acute (day 0) and convalescent (day 60) phase.

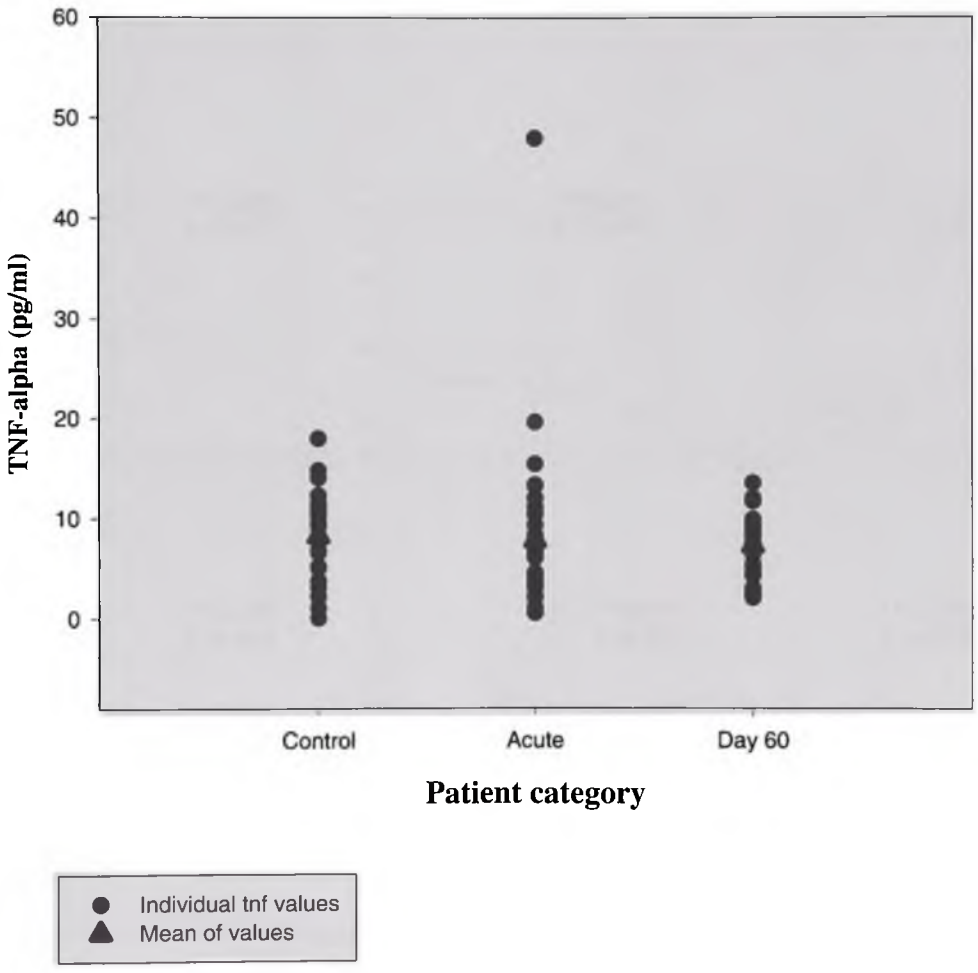
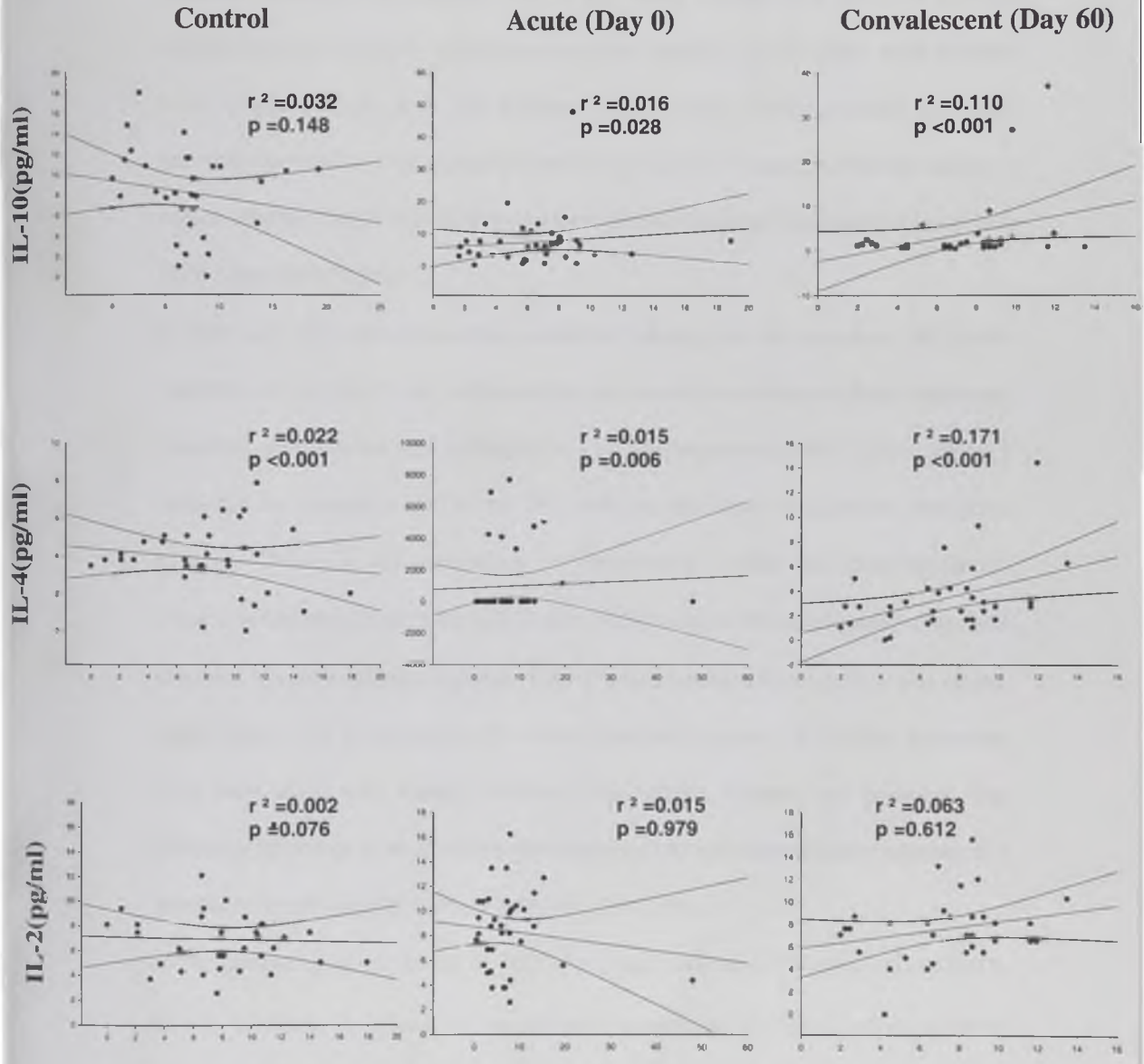


Fig 4.12 IL-10, IL-4 and IL-2 versus TNF-alpha in healthy controls and measles patients at acute and convalescent phase.



5.0 DISCUSSION

Antigenic stimulation of uncommitted T helper cells leads to activation of these TO cells along two arms of Th1 and Th2 cells that are committed to cell mediated and humoral immunity respectively (Fig 4.10). Both cellular and humoral immune mechanisms are involved in protection against measles. On the other hand children with specific defects in T cell function often develop severe measles with fatal parasitic, bacterial or viral complications (Li *et al* 2001). These observations support a critical role for T cells in both recovery from primary measles infection and protection from subsequent disease.

T cells and macrophages produce cytokines which play an important role in the regulation of immunity and inflammation and coordinate diverse cellular responses, including proliferation and differentiation of haematopoietic cells. CD4+ helper T cells can be classified as Th1 or Th2 cells on the basis of cytokine production patterns. These Th cell populations are functionally distinct and cross-regulatory. Type 1 cytokines include IFN-γ, IL-2 and TNF-β bias, a Th cell-dependent immune response toward a cellular response. Type 2 cytokines include IL-4, Il-10 and others, which favour the development of a strong humoral response. Cytokines alterations have been noted after human infection with viruses, bacteria and parasites. The cytokines responses to an invading microrganism can influence the development of a protective or pathological immune response in the host.

In the present study the levels of TH1 (IFNγ and IL-2) and TH2 (Th1, IL-4, IL-12, TGFβ) cytokines in plasma of patients with measles at the acute and convalscent stages were determined. This investigation was centered on patients with uncomplicated measles virus infection in comparison with a healthy control group whose clinical records show previous immunization with attenuated measles vaccines

and were without measles infection as at the time of the study. The results show that Th1/Th2 balance is affected by acute measles with a polarization towards Th2 responses in contrast with Th1. This trend has implications for controlling measles virus and other intracellular organisms including malaria. Upregulation of Th2 responses could contribute to the increased susceptibility of measles patients to other infections leading to complications and increased mortality. Th-2 (IL-4 and TGF-β) cyotkines may have an important regulatory and / or effector function during measles infection. There was a significant increase of IL-4 in the acute stage plasma samples and TGF-β (both Th2 cytokines) in plasma of patients during the recovery and convalescent phases. In contrast, there was a significant decrease in IFN-γ and IL-2 (Th1 cytokines) suggesting that Th2 cells were being preferentially activated during measles.

Acute measles virus infection induces a predominant Th2 immune response characterized by spontaneous release of IL-4 production of high levels of antibodies to measles virus and temporary suppression of cell-mediated immunity

(Li *et al* 2001). Furthermore, there is *in vitro* and *in vivo* evidence of a type 2 polarization in cytokine responses after measles (Atabani *et al* 2001). The present results confirm these findings.

Differentiated mouse $CD4^+$ T cells produce a restricted set of cytokines allowing their sub division into 2 discrete populations: TH1 characterized by secretion of IL-2,

IFN-γ and TH 2, which selectively produce IL-4, 5 and 10. (Mosmann *et al* 1987). A similar distinction also applies to human T cells (De Prete et al 1991).

The developement of antigen-specfic $CD4^+$cells into polarized Th1 and Th2 subsets is influenced by several factors, including antigen dose and the cytokine millieu during the initial phase of the immune responses (Abbas et al 1997). Among cytokines,

decisive roles are played by IL-4, 12 and 10, driving T cell responses towards the Th1 and Th2 phenotype, respectively (Paul *et al* 1994). In this stduy, Th1 cells failed to regulate IL-4 and TGF-β suggesting that Th 2 cells have a dominant negative effect on Th1 induced IL-10, 12, IFN-γ, TNF-α secretion. Conflicting results have been reported on the role of Th2 cells in the regulation of IFN-γ during acute measles infection. The results clearly show high levels of IL-4, which is a Th2 cytokine, at the acute phase of the illness. The fact that IL-4 is an anti-inflammatory cytokine and is produced in such high concentration during the acute phase of the illness is significant. Also quite high is the Th-2 cytokine TGF-β that is elevated at all phases (acute and convalscence) of study. The results however show low levels TNF-α and IFN-γ Th1cytokines in the plasma samples of children with uncomplicated measles. Taken together, the results therefore show an upregulation of Th2 cytokines in plasma samples of children diagnosed with acute uncomplicated measles. The findings suggest suppression of Th1 or cellular function while Th2 responses are upregulated. This could render children susceptible to any intracellular agents whose control or elimination would require Th1 responses. Incidentally, the study subjects were all measles patients without complications of any sort. It would be interesting to investigate measles patients with known or established complications in a comparative study.

It also appears that anti-inflammatory cytokines dominate appreciably leading to less inflammation, which is generally good for the well being of the patient, and this could be the reason for the absence of fever in the majority of measles patients in the present study.

Another potentially important finding of this work was the absence of malaria parasites in the blood of measles patients at the time of the investigation. Several lines

of evidence indicate that individuals that produce high levels of pro-inflammatory cytokines may be at higher risk of developing severe malaria and children who have suffered from cerebral malaria are more likely to produce higher levels of TNF-α and IL-1β.

Akanmori *et al* (2000) have shown significantly higher levels of TNF and TNF receptor 1 in cerebral malaria than in other clinical categories of *P.falciparum* malaria patients. In *P.falciparum* infection increase in levels of TNF exhibit antiparasitic effects (Rockette *et al* 1992) and a high TNF production capacity protects from severe malaria (Kremsner *et al* 1995). At the same time excessive TNF levels are associated with complications such as cerebral malaria or severe anaemia (Shaffer *et al* 1991. Ohga *et al.* (1992) have shown high levels of INF-γ in plasma of Japanese children during the acute phase of measles virus infection. The results presented herein indicate low level (s) of IFN-γ secretion. It is important to note that our study patients were vaccine modified measles cases and our findings may not be in conflict with Ohga et al. It is likely that measles specific secretion of IL-12 in modified measles may not be enough to induce high concentrations of IFN-γ to promote the maximal expansion of T cells that recognize measles antigens.

That Th 1 or pro-inflammatory cytokine levels in acute measles in Ghanaian subjects is genuinely down regulated is seen by the low levels of IL-12 which is critical for the induction of IFN-γ which is a major TH1 T cell cytokine that is involved in clonal expansion of antigen specific T cells (Marshall *et al* 1995, Hendrzak *et al* 1995, Schmitt *et al* 1994 and Trinchieri 1997). IL-12 provides a key link bewteen innate and acquired immunity. It is critical to the development of cell mediated immunity, since it is a potent inducer of IFN-γ from T and NK cells, required for the development of T helper (Th1) responses in most systems. It is also necessary for delayed-type

hypersensitivity responses and is an enhancer of NK cell cytotoxicity. Suppression of IL-12 production by measles virus provides a plausible underlying mechanism for many of the abnormalities in cell mediated immunity observed in measles. However, data on IL-12 and measles derive from *in vitro* models. The data presented potray an *in vivo* human condition.

Although the immunosuppression associated with measles is no doubt multifactorial, the specific defect in IL-12 production demonstrated here provides a unifying mechanism for many of the phenomena observed. Specifically, susceptibility to super infection, ablation of delayed type hypersensitivity responses, suppression of NK cell activity and polarization toward type 2 cytokine production are all consonant with a failure of IL-12 activity. Natural measles causes prolonged suppression of IL-12 (Atabani et al 2001). The accompanying compromise in IL-10 production may be relevant to type 2 cytokine polarization, as genetic deletion of IL-10 augments the type 2 polarization effected by IL-12 deprivation in murine models (Hoffman *et al* 1999). Defective IL-12 also is consistent with the development of successful measles virus specific antibody and cytolytic lymphocyte responses (CTL); CTL and overall antibody responses develop normally in the absence of IL-12 and genetic deletion of IL-12 is compatible with viral clearance. IL-12 suppression has been reported in children infected with *P.falciparum*, (Bont *et al* 2000 and Luty *et al* 2000). Chronic deficiencies of IL-12 are also seen with HIV infection (Chougnet *et al* 2000).

The mechanism underlying the *in vivo* IL-12 inhibition by measles is not clear and further studies need to be undertaken to address the mechanisms that underline the prolonged *in vivo* IL-12 suppression in measles infection.

Another important type 2, TH-2 cytokine whose level was significantly high is TGF-β which is synthesized by almost all cells in culture, although it is normally secreted in a latent form that must be activated by proteases. Both antigen-activated T cells and LPS-activated mononuclear phagocytes secrete biologically active TGF-β. The actions of TGF-β are highly pleiotropic: TGF-β inhibits the growth of many cell types and stimulates the growth of others.

Often TGF-β can either inhibit or stimulate growth of the same cell type. *In vivo* TGF-β causes the growth of new blood vessels, a " process called angiogenesis". As a cytokine, TGF-β antagonizes many responses of lymphocytes. It inhibits macrophage activation and acts on non-immune effector cells such as polymorphonuclear leucocytes and endothelial cells, again largely to counteract the effects of pro-inflammatory cytokines. TGF-β has thus been referred to as anti-cytokine and may be a signal for shutting off immune response. Signals that cause T cells to synthesize TGF-β may cause them to behave as suppressor cells. Therefore it is likely that in the studies reported herein, TGF-β decreased the activation of immune cells responsible for the secretion of IFN-γ and TNF-α (Th 1 cytokines) during measles virus infection

The contribution (s) of decreased IFN-γ, TNF-α and absence of malaria parasites cannot be discerned in our study. The reason (s) could be due to a combination of Th-1 type and Th2 type responses influenced by at least CD4, CD8 and / or γδ T cells. Functional responses to measles virus infection in humans have been demonstrated to be Th1 type and both Th1 and Th2 types.

Further sudies are needed to define the relationship between Th1 type and Th 2 types in protective measles infection. The upregulation of IL-4, a powerful anti-

inflammatory (Th2) cytokine in measles could render the patients susceptible to other parasitic, viral or bacterial infection. It suppresses eosinophils and inhibits production of IFN-γ by activated T cells. IL-4 inhibits the killing by macrophages of various parasites such as Leishmania and asexual erythrocytic forms of *P-falciparum*. Although IL-4 possesses potent anti tumour activity in humans, it can inhibit development of antigen-specific T cell mediated inflammatory response, induce activated B cells to produce more IL-6 and TNF leading to expansion of T cells and increase in synthesis of surface IgM.

TGF-β also a Th2 cytokine, described as anti-cytokine is immunosuppressive , suppresses T cells, NK cells and LAK cells. It down-regulates IgM and IgG production, inhibits IFN-γ, TNF-α, IL-1and IL-2. Cellular immune response is greatly affected.

5.1 CONCLUSION

The premise that acute measles causes down-regulation of Th1 (pro-inflammatory) cytokine response and up-regulates Th-2 (anti-inflammatory) cytokine response remains valid. Whether this situation moderates the pathogenesis of measles infection in Ghanaian children or not, is not immediately clear; however as no death (s) were recorded among the subjects under study it could be inferred that anti-inflammatory cytokine production in acute measles could be beneficial.

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7.0 APPENDIX

1 - REAGENTS

2 - QUESTIONNAIRE / REGISTRATION FORM

REAGENTS

Stock Giemsa stain (Wako pure chemicals Ltd., Tokyo. Japan).

Micro titer Plates-96 well polystyrene plate (12 strips of 8 wells) coated with a murine monoclonal antibody.

Conjugate (polyclonal antibody conjugated to horseradish peroxidase with preservative.

Standard (vial of recombinant human interleukin in a buffered protein base with preservative, lyophilised).

Assay diluent (buffered protein base with preservative).

Calibrator (solution of a buffered protein base with preservative).

Wash buffer concentrate (solution of a 25 fold concentrated buffered surfactant with (preservative).

Color Reagent A (solution of stabilized hydrogen peroxide)

Color Reagent B (solution of stabilized chromogen - tetramethylbenzidine.

Stop solution (2N sulphuric Acid)

Plate covers-(Adhesive strips).

EQUIPMENT

Microtiter Plate Reader (Spectrophotometer) capable of measurement at 450nm and capable of using dual wavelength correction.

Pipettes: 50µl, 100µl, 200µl, and adjustable 1ml-10ml.

IgM + IgG Kits (Denka Seiken Co. Ltd, Tokyo, Japan. Cytokine Kits (Quantikine ™ R+D Systems, Inc. Minn. USA.

Giemsa stain (Wako Pure Chemicals) Tokyo Japan.

REGISTRATION FORM

Name of Hospital ----------

Registration Date -------/ ---------/ Reg. No. ----------

Name ---------- ❑ Patient ❑Control

Date of Birth ----------/----------/------ Age ---------- Sex (M. F.)

Mother's Name ---------- Address ----------

Body weight ----------Kg Height ----------cm

Vaccinations ❑BCG ❑Polio ❑DPT ❑Yellow Fever

❑Measles

Clinical Diagnosis ❑ MEASLES ❑ OTHERS(Specify) ----------

Clinical signs

Body Temperature ----------°C

❑Skin rash ❑Koplik's spot ❑Cough ❑Coryza ❑Hyperaemic conjuctiva ❑Others (Please specify) ----------

Date of onset of fever ----------/----------/----------

Date skin rash appeared----------/----------/----------

Date of sample collection----------/----------/----------

Duration ❑ ≤10 days after onset

❑ >10 days after onset

❑ >2 months after onset

Complications

❑ None ❑ Malnutrition ❑ Pnuemonia ❑ Persistent diarrhoea

❑ Septicaemia ❑ Malaria ❑ Others (Please specify)----------

Informed consent : Do you agree to have your child included in this study?

Signature of Mother ----------

Name of Clinician ----------

Date ----------/----------/----------