STUDIES OF CELLULAR IMMUNITY IN CHILDREN WITH MEASLES

by

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Being a dissertation presented in fulfilment of the requirements governing the degree of Master of Science in the School of Medicine, University of the Witwatersrand.

JOHANNESBURG

September 1978

This is to certify that the dissertation 'STUDIES OF CELLULAR IMMUNITY IN CHILDREN WITH MEASLES' presented for the degree of Master of Science at the University of the Witwatersrand, Johannesburg, is my own work and has not been presented at any other University.

MAX JOFFE

Parts of this dissertation have been published in the following papers :

1. Papova Virus in Human Lymphocyte Cultures.

   Lecatsas, G., Schoub, B.D., Rabson, A.R. and Joffe, M.I., Lancet ii, 907, 1976.

2. Dissociation of Lymphokine Production and Blastogenesis in Children with Measles Infections.

   Joffe, M.I., and Rabson, A.R., Clin. Immunol. and Immunopath. 10, 335, 1978.

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5. The research on which this dissertation is based was commenced in August 1975, and completed in March 1978.

ACKNOWLEDGEMENTS

I thank Prof. J. Metz, Director of the South African Institute for Medical Research, Johannesburg, for provision of the facilities for the studies included in this dissertation. I sincerely thank Prof. A.R. Rabson, Department of Immunology, South African Institute for Medical Research and School of Pathology, University of the Witwatersrand, Johannesburg for his constant help, guidance and advice. I am deeply indebted to Dr. R. Sher of the Department of Immunology for providing the clinical cases used in this study. I also wish to thank Prof. W. Prozesky and Dr. B. Schoub of the National Institute of Virology, Johannesburg, for their collaboration and co-operation in the virological studies. I am deeply indebted to Miss Vivien Green for the typing of the manuscript. I also thank Mrs. M. Ulrich of the Photography Unit, for all the photographs in this dissertation.

ABBREVIATIONS

cpm	:	counts per minute
con A	:	Concanavalin A
CRP	:	C-Reactive Protein
FCS	:	Fetal calf serum
g	:	Gravity
HBSS	:	Hank's Balanced Salt Solution
hepes	:	2-(4-(2-hydroxyethyl)-1-piperazinyl ethanesulfonic acid
HSV	:	Herpes simplex virus
LIF	:	Leucocyte inhibition factor
LMS	:	Levamisole
M	:	Molar
MEM	:	Minimal essential medium
mg	:	Milligram
µCi	:	Microcurie
µg	:	Microgram
MLC	:	Mixed lymphocyte culture
MI	:	Migration index
MIF	:	Macrophage inhibition factor
ml	:	Milliliter
µl	:	Microliter
MN	:	Mononuclear cell
PEC	:	Peritoneal exudate cells
PHA	:	Phytohaemagglutinin
PML	:	Progressive multifocal leucoencephalopathy
PMN	:	Polymorphonuclear cell
PPD	:	Purified protein derivative
S.E.M.	:	Standard error of the mean
S.S.P.E.	:	Subacute sclerosing panecephalitis

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SUMMARY

There have long been two clinical indications of the significance of cell-mediated immunity to measles, namely, the cutaneous delayed hypersensitivity reaction to tuberculin is greatly depressed or even absent during infection and that individuals with hypogammaglobulinemia recover normally from measles.

The experimental work presented in this dissertation basically involved the study of the cellular immune status of children acutely infected with measles virus. Lymphocyte blastogenisis and lymphokine production, two techniques that have been previously employed in investigations of clinical conditions known to be associated with cellular immune defects, were adapted for use in the present study.

Lymphocyte transformation studies of measles MN cells revealed the existence of elevated unstimulated or 'spontaneous' incorporation of ^{3}H-thymidine after overnight incubation, later decreasing to lower levels. The significance of the latter is speculative but most probably reflects *in vivo* activation of MN cells by measles antigen. PHA activation of measles MN cells was essentially normal over a range of different mitogen concentrations and no inhibition of normal lymphocyte transformation was apparent using measles MN cell supernatants or acute serum. In contrast, allogeneic stimulation of measles MN cells in the two-way MLC, resulted in decreased ^{3}H-thymidine incorporation. Not only did measles MN

cells respond poorly to allogeneic activation, but when mitomycin C-treated, they also failed to adequately stimulate control responding cells. Although no ready explanation for this puzzling finding is apparent the possibility exists that different lymphocyte subpopulations are adversely affected by measles virus infection.

To assess lymphokine production a two-stage migration assay was utilized. The first stage consisted of pulsing MN cells with PHA to induce lymphokine production and the second stage involved the incubation of indicator cells with and without lymphokine-containing supernatants. Using an agarose migration system, it was shown that measles MN cells failed to produce LIF while the capillary tube migration system using guinea-pig PEC, demonstrated a lack of MIF activity too.

In contrast to the results obtained by measuring ^{3}H-thymidine incorporation after 18 hours of incubation, measles MN cells did not produce LIF 'spontaneously'. In addition, supernatants from unstimulated measles MN cells did not inhibit the production of LIF by normal PHA-pulsed MN cells.

These results suggest that a specific lymphokine-producing cell population may be adversely affected by measles infection. An attempt at correcting this defect by treatment of measles MN cells *in vitro* with levamisole was however, unsuccessful at the concentration used in this study.

Virological examination of measles MN cell culture supernatants

demonstrated the presence of papovavirus particles in a small number of patients. Specific immunofluorescent staining for the virus in MN cell preparations was however unsuccessful. These results suggest either reactivation of a latent virus infection during the course of measles or simply contamination of specimens during processing in a virological laboratory.

The effect of measles on monocyte function was assessed *in vitro* using a modified Boyden chamber technique. The monocytes of measles patients were shown to migrate adequately to the chemoattractant casein. Although the migration of measles PMN cells *in vivo* was found to be depressed as assessed by the Rebuck skin-window technique, measles monocytes were found to migrate in sufficient numbers after 24 hours.

These results imply that the adverse effects of measles infection on cellular immunity is probably confined to lymphocytes.

I N D E X

P A R T I

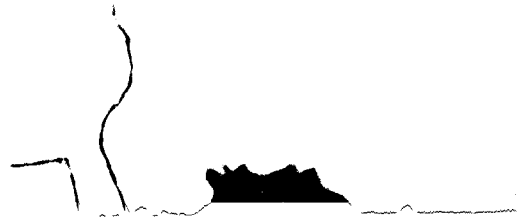
I N T R O D U C T I O N

PART II

EXPERIMENTAL

PART I

INTRODUCTION



CHAPTER I

Historical Review

INTRODUCTION

HISTORICAL REVIEW

Measles (Rubeola) is a highly contagious, acute disease of childhood characterised by fever, catarrhal symptoms, cough and a general maculopapular eruption. The first description of measles has been accredited to the Persian physician, Rhazes in the year A.D. 850. The disease was often confused with smallpox, until the 17th century English physician Thomas Sydenham, first delineated the two diseases and was able to establish measles as a separate clinical entity (Parish, 1968). Its antiquity is accented by the fact that the first attempt to immunize against measles, preceded by nearly half a century Jenner's discovery of smallpox vaccine. This first attempt was made in 1758 by Francis Home who transmitted the disease to humans by applying cotton pledgets soaked in blood from patients with acute cases to the scarified skin of susceptibles (Enders, 1961). The gravity of the disease in those times is well exemplified by what to us today appears to be a rather gross understatement by Holmes of an epidemic in Edinburgh, namely 'that the measles were in general of a mild sort, and not above the twelfth part died of those who were attacked'. In descriptions of former outbreaks, he also noted the incidence of serious complications, especially that of pneumonia, as well as the higher mortality.

In Europe for a thousand years measles was thought to arise from the 'bad' blood of menstruation. As this disappeared during pregnancy, it was believed that it entered the fetus and reappeared after birth as the rash of measles. The sudden appearance of the rash has also led to many beliefs about the disease. Usually the temperature comes down with the appearance of the rash and for this reason in many countries, people tried to 'bring the rash out' with strange customs, such as wrapping the child in a red blanket or even beating the child with nettles.

Measles and the nutritional status of the child form a complex interrelationship In his study of measles in Nigerian children, Morley (1962) observed a striking weight loss provoked by an attack of the disease in the malnourished child, in some children sufficient to precipitate kwashiorkor with its ensuing complications. As a result, the rash darkens to a deep red and violet colour and desquamation ensues. The difference in the type of rash was recognised in antiquity, and Rhazes wrote 'Measles which are of a deep red and violet colour are a bad and fatal kind'. Equivalent changes on other epithelial surfaces, e.g., respiratory and intestinal tracts, are probably responsible for the so called complications of this disease, particularly bronchopneumonia and diarrhea. Thus protein-calorie malnutrition predisposes to severe measles and is itself precipitated into a severe form by the attack of measles to which it has predisposed the child. The local custom of restricting food intake to a minimum of carbohydrate in children with measles may also have influenced

this outcome (Morley, 1969). Given these beliefs, ancient proverbs often reflected the respect in which measles was held (Morley, 1969) :

'Count your children after the measles has passed'

Arabic

'Smallpox will make your child blind, measles will send him to his grave'

Parsee

Measles has influenced the history of many isolated or 'virgin' communities, but few can have been affected so severely as during the Fiji epidemic. The virus was first introduced there in 1875 resulting in an almost 100% attack rate with a 25% mortality. Measles has also influenced the course of history in many countries during the last few centuries. For example, during the American Civil War, the Confederates were sorely pressed when 2 000 of their troops died, out of 40 000 sick with measles. When the Paraguayans declared war on Brazil in 1865, they were defeated because one-fifth of their army died from measles (Davies, 1967). It is also likely that measles epidemics will occur in 'virgin' populations. For example, it is possible that the early American pioneers may inadvertently have introduced measles to the unexposed Red Indian population, thus causing widespread decimation of certain tribes. Similarly, in the early days of the Cape colony, the Hottentot community was also devestated by epidemics of measles introduced by the

early Cape settlers.

With the advent of successful live attenuated measles vaccines in recent times, the incidence of the disease has been greatly curtailed wherever the vaccine has been widely used. In the U.S.A.; successful vaccination programmes have led to a significant drop in the incidence of measles infection. In 1963, following the introduction of live measles virus vaccine in the United States, the number of reported cases of measles decreased from about 400 000 in that year to about 22 000 in 1968. Recent statistics show however, an alarming increase amongst older children and even young adults (Center for Disease Control, 1977). Perhaps the most important cause for the latter, is the gradual accumulation of susceptibles over the years during which measles transmission has been suppressed by vaccination. Theoretically, eradication of measles on a national scale is a feasible objective, but apathy and ignorance allow the virus to persist in immunized 'pockets' of susceptible children living in certain areas of urban and rural poverty where immunization services are lacking.

As recently as 1960, 1 000 Black mineworkers in South Africa required hospitalisation for measles (Dick, 1975) and despite the ever-increasing use of the vaccine by many local authorities here, outbreaks of almost epidemiological proportions are still not uncommon. The large number of hospitalised cases seen in the Johannesburg area in 1976 thus provided an excellent opportunity to institute a study of some of the immunological mechanisms involved in the anergy associated with measles infections.

C H A P T E R 2

The Measles Virus

THE MEASLES VIRUS

Biological Characteristics

Nearly all that is known about the measles virus has been learnt since 1954. Before that time, in spite of a considerable amount of knowledge concerning the clinical features and the natural history of the disease, it was known only that measles was caused by a transmissable micro-organism, and that this did not grow in conventional bacteriological media and was filterable. Although the disease was transferred to monkeys in 1911, (Anderson & Goldberger) it was not until 1954 that the virus, through persistent research by Enders and Peebles (1954) was consistently isolated from clinical materials in primary cultures of human and monkey kidney. This enormous advance opened up the possibilities for investigating the biological and molecular characteristics of the virus leading eventually to the development of vaccines.

The most informative single technique for the study of measles virus has proven to be electron microscopic examination of the virus or of measles-infected cells, prepared by the technique of negative staining (Waterson, 1974) (Fig. 1). Measles virus belongs to a group of viruses (the paramyxoviruses) with a common and characteristic morphology. All measles strains so far studied belong to a single antigenic type. Serologically, the measles, rinderpest and distemper viruses are closely related to each other (See Table 1).

TABLE 1

BIOLOGICAL PROPERTIES OF SOME PARAMYXOVIRUSES

Similar morphology	Cell Fusion	Mumps Parainfluenzas Newcastle disease Measles Rinderpest Distemper	Neuraminidase	Haemagglutination

All the viruses listed have a common morphology, and share the property of fusing cells. Measles differs from rinderpest and distemper in agglutinating simian erythrocytes at 37°C.

TABLE 1

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Figure 1 Electron micrograph of a typical measles virus negatively stained with 3% phosphotungstic acid (x 260,000).

In electron micrographs measles virus appears as roughly spherical particles, 120-250nm in diameter (Fig. 1). In the disrupted particles, and seen spilling out of them, is the inner component consisting of ribonucleoprotein, with the protein units arranged spirally to surround and enclose the ribonucleic acid (RNA). This structure is known as the nucleocapsid. It contains the nucleic acid of the virus but the function of its protein is unknown. Infectivity of free nucleocapsid has not been demonstrated. The nucleocapsid is coiled within an outer envelope some 10-20nm thick, consisting of protein and lipoprotein bearing short projections on its surface. The envelope is believed to be necessary for infectivity. The envelope is responsible for the haemagglutinating (HA), haemolysing (HL) and cell-fusing activities of the virus. It is the carrier of the serological specificity of the outer surface of the virus, i.e. of the antigens to which neutralizing, HA-inhibiting, HL-inhibiting and complement fixing (CF) antibodies are made. Unlike other paramyxoviruses, the measles virus HA will only agglutinate erythrocytes of various primate species at 37^{o}C (Waterson, 1965). The envelope is thus responsible for stimulating the production of haemagglutination-inhibiting, haemolysing-inhibiting and neutralizing antibodies as well as being able to fix complement (Waterson, 1965). Although the precise antigenic inter-relations have not been defined, the separated envelope components would thus prove an acceptable antigenic preparation for the artificial production of humoral immunity.

The envelope of the virus is cell-derived and the virus acquires this envelope by pinching or budding off the cytoplasmic membrane of the host cell. The envelope contains lipid, which is derived from cellular elements, and protein, which is viral in origin and carbohydrate in the form of glycoprotein and glycolipid. Measles virus does not cause extensive cell destruction, and fusion of cells to form syncytia or giant-cells is a regular feature both *in vivo* and *in vitro*.

Measles virus replicates slowly in cell cultures. The latent period of the initial cycle is 9-12 hours and virus is produced at a maximum rate until about 30 hours after infection. Viral RNA and protein appear to be synthesized solely in the cytoplasm, but intact viral particles can be detected only at the cell surface (Davis *et al*, 1969). The virus is readily isolated in primary cell cultures of human or simian kidney and can be propogated in a variety of continuous cell lines. It is rapidly inactivated by various agents, including ether, trypsin, formalin and ultraviolet light. Activity is maintained for several weeks at refrigeration temperature and its half-life at 37^{o}C is about 2 hours.

The occurrence of chromosome breakages in leucocytes during infection by naturally occurring or attenuated measles virus has been reported by Nichols *et al*, (1962). The exact significance of this phenomenon has however, not been fully assessed.

The Pathogenesis and Pathology of Measles Infection (see Fig. 2)

Measles is one of the most highly contagious diseases. It is predominantly a childhood affliction which occurs mainly in epidemics during the winter and spring and is more or less endemic in highly populated urban areas and where hygienic and economic conditions are poor.

Prior to the isolation of measles virus by Enders and Peebles in 1954, knowledge of the etiological agent was meagre. In 1911, Anderson and Goldberger demonstrated that a filterable agent from humans with the disease could produce measles in the monkey. Measles virus produces a fatal disease upon intracerebral passage in suckling mice and hamsters (Imagawa, 1958).

Measles virus is ubiquitous and nearly all of the adult population in most countries with endemic infection have circulating measles neutralizing antibodies. An appreciable antibody titer is transferred to the newborn infant in whom antibody levels are detectable for about 6 to 9 months. As a rule, a primary attack results in lifelong immunity and although varying somewhat from country to country, measles infection results in antibody titers in most children during the early school years.

Spread

As far as is known, no animal reservoirs for measles virus exist and man and monkeys are the only natural hosts. The

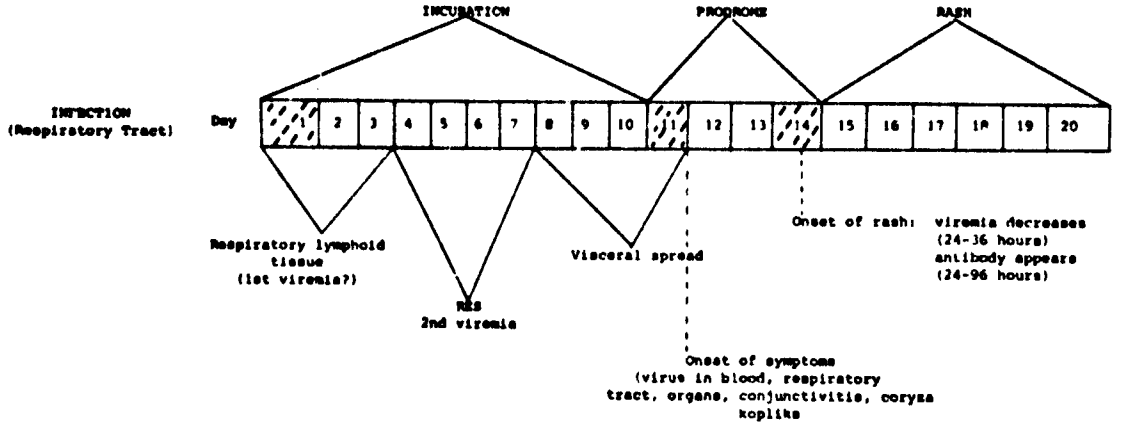

Figure 2 The pathogenesis of measles virus infection.

presence of respiratory sumptoms make it likely that acrosols of virus are projected into the environment and constitute a major source of infection for the susceptible individual. Entry into the host probably occurs as a result of deposition of virus onto the upper respiratory epithelium, including perhaps the conjunctivae (Kempe and Fulginiti, 1964). Neither a carrier state nor third-party transmission have been demonstrated.

Incubation Period

The incubation period of measles virus usually lasts about ten days, with generally no symptoms apparent, although a slight rise in temperature may sometimes be apparent on the sixth or seventh day. Although direct evidence in man for the development of measles infection in the first few days is lacking, a comparison can be made with data obtained from monkeys (Sergiev *et al*, 1960). The simian disease has an incubation period of 6 days and the virus is observed in low titers on the third day, declining on the fifth or sixth day, and increasing markedly on the seventh day. In the susceptible human, it is assumed that a virus particle entering the respiratory tract initiates a symptomless local lesion from which virus passes to the local lymph nodes. Following intense viral multiplication there (1st - 2nd day), virus is transferred to the entire reticuloendothelial system and hence appears in the blood on the 5th and 7th day as it spreads to the viscera.

Measles neutralizing antibody (pooled adult human gammaglobulin) administered early in the incubation period, can modify or even prevent the illness, depending on the quantity as well as the timing of antibody administration. There is, however, a point at which clinical manifestations cannot be suppressed by exogenous antibody, suggesting that the virus has achieved an insurmountable titer or is in a state which cannot be influenced by antibody (Kempe and Fulginiti, 1964). Although little data is available in humans concerning the morphologic alterations associated with measles virus development during the incubation period, some degree of generalised lymphoid hyperplasia and giant-cell formation has been noted in monkeys. (Sergiev *et al*, 1960).

The Prodrome

The early clinical phase of recognizable measles infection is heralded by a 2 to 4 day period of fever and increasing respiratory symptoms. Viremia is present during this period and respiratory secretions yield large amounts of virus and both epithelial and lymphoid giant-cells. Conjunctival inflammation is also a characteristic of the prodrome. The apparence of small irregular bluish-white lesions in the buccal mucosa (Koplik's spots) is one of the few absolutely indicative signs of the disease at this stage.

Onset of the rash

The rash or exanthem traditionally fixes the onset of the illness.

It appears first on the head, then spreads progressively over the chest, trunk and limbs. The characteristic maculopapular rash progresses for approximately three days with systemic and respiratory symptoms reaching a climax during the progression of the rash. Koplik spots and skin lesions show similar pathological changes, but the inflammatory response is more intense in the skin. In both sites, epithelial giant-cells, edema and mononuclear cell infiltration are the predominant features. In electron micrographs, aggregates of viral nucleocapsids and microtubles are seen within the nuclei and cytoplasm of the giant-cells (Suringa *et al*, 1970). Virus can also be demonstrated by immunofluorescence in the skin rash (Olding-Stenkvist and Bjorvatn, 1976). With time viral multiplication decreases and blood and respiratory secretions continue to yield viruses for as long as 46 hours following the onset of the rash (Gresser and Chany, 1963). With the appearance of antibody (first to fourth day of rash), and as titers increase, virus becomes undetectable.

Complications

Measles predisposes a victim to infection by a secondary bacterial invader, particularly in infancy and early childhood. Complications are common and sometimes severe and most frequently include otitis media and pneumonia. Purely viral complications include croup and bronchitis. The most feared complication of measles is encephalomyelitis, which develop in perhaps one out of every 1 000 cases and has a high mortality rate. Subacute sclerosing

panencephalitis (SSPE) is a much rarer and quite different condition, thought to develop some years after the original attack of measles. Electron micrographs of brain tissue reveal particles of a virus morphologically indistinguishable from measles and the patient suffers a progressive loss of cerebral function leading eventually to death.

The Spread of Viruses

In order to gain a better understanding of the immune mechanisms by which the host defends himself against viral infection, it is necessary first to examine the way in which viruses spread from one cell to another.

Basically, viruses can spread by three different routes : extracellularly (Type I spread); from cell-to-contiguous cell (Type II spread), or from parent to progeny cell during cell division (Type III spread) (Notkins, 1974). It is thus clear that different immunological mechanisms are required to control the different modes of viral spread (see Table 2).

Type 1 spread is usually halted by the neutralization of extracellular virus by antiviral antibody. An example of this type of spread is provided by poliovirus. Although the humoral immune response to Type 1 spread has been studied the most extensively, many questions still remain unanswered. For example, it is still not clear why under certain circumstances, the interaction of antibody with virus fails to result in neutralization. The class of immunoglobulin involved in viral neutralization

TABLE 2

THE ROUTE OF VIRUS SPREAD AND IMMUNE CONTROL MECHANISMS

Type	Route of Spread	Example	Mechanism by which immune response stops viral spread	Components of immune system
I	Extracellular	Polio-virus	Neutralization of extracellular virus	Humoral
II	Cell to cell	Measles Herpes Simplex	Destruction of infected cells and/or breaking of cell-to-cell	Cellular, Antibody dependent lysis
III	Parent cell to progeny cells	SV40	Destruction of cells carrying viral genome	Cellular? Humoral?

depends on the site of the infection. IgA antibody, secreted by the mucous surface of the body provides the first line of defence, localizing the infection to the epithelial surfaces and is thus particularly important in combating infections of the respiratory and digestive tracts. If the virus gets into the bloodstream, the predominant immunoglobulins in the blood, IgG and IgM are of importance in neutralizing the virus with or without the help of complement and other factors.

The host's defence against Type II spread is more complex. The virus is protected from neutralizing antibody because it is able to spread from cell to-contiguous-cell as a result of virus-induced cell fusion (Notkins, 1974). This type of spread is best illustrated by members of the herpes virus and the measles virus groups. The virus is protected from extracellular antibody probably by passage to contiguous cells through 'intercellular bridges' formed as a result of cell fusion. Direct 'budding' virus into adjacent cells or pinocytosis of virus contained in vacuoles covered by host membranes may also take place in certain cases. If the virus induces new antigen on the surface of the infected cell, recognition of these antigens by specific antiviral antibody and complement or immune lymphocytes can result in cell destruction. Cell mediated immunity seems to play a more important role in controlling Type II than Type I spread,

although evidence for this still remains somewhat indirect and the concepts are patterned after the known action of cell mediated immunity in allograft and tumour rejection.

How the host controls Type III spread is even less clearly understood. It is thought that the viral genome may be integrated into the host-cell genome and during cell division, the viral genome is passed on to progency cells (i.e. SV40 and other papova viruses, or viruses may establish a nonlytic infection in the cytoplasm of the host cell, i.e. rabies). This type of infection differs from the previous two in that fully infectious virus is neither assembled nor released from the host cell. The host cell then appears to become transformed owing to some alteration in cellular regulatory mechamisms and the virus specifies the production of new antigens, including viral specific and tumour specific antigens on the cell surface (i.e. T-antigen produced by papova viruses such as SV40). To stop Type III spread it is necessary either to destroy the infected cell or to stop cell division. Whether the immune response is successful in destroying the infected cell depends in part upon whether or not the virus induces a sufficient amount of new antigens on the surface of the cell so that the cell is recognized as foreign by immune lymphocytes or by antibody and complement.

CHAPTER 3

Measles Virus and the Immune Response

MEASLES VIRUS AND THE IMMUNE RESPONSE

Evidence for a Role of Cell-Mediated Immunity in Resistance to Measles Infection

It is perhaps ironic that cell-mediated immunity is the least understood component of the immune response to viral infection, yet the first description of the delayed-type hypersensitivity reaction was the response to a virus. In the 18th century, Jenner first described the extensive inflammation produced by variol tion of a milkmaid with cowpox :

'It is remarkable that variolus matter, when the system is disposed to reject it, should excite inflammation more speedily than when it produces the Small Pox'.

For many years, measles was regarded as the classical model of an infectious disease followed by lasting immunity. This was borne out by the study of Panum, during an outbreak of measles on the Faroe Islands in 1846, when it was noted that protection from reinfection existed among those who had contracted the disease during the last epidemics up to 63 years previously (Panum, 1940). Since that time, clinical and epidemiological studies have disclosed the clear correlation between the presence of neutralizing antibodies in the serum and protection against subsequent attacks of measles (Ruckle and Rogers, 1957). Furthermore, convalescent human serum, presumed to contain high-titer antibody was effective in

preventing or attenuating the symptoms of measles if administered to children within the first few days after their contact with the virus (Kemp and Fulginiti, 1964).

Despite the strength of the evidence in favour of a humoral form of protection against measles, conflicting information eventually led Burnet (1968) to hypothesize that the cell-associated or thymus-dependent cellular reaction was mounted against cells with measles antigen expressed on their surfaces. According to Burnet, lymphocytes, within which measles virus are multiplying and carrying antigen in the cell membrane, are widely disseminated by the circulation. Reaction between sensitized T cells and virus containing 'measles cells' results in proliferation of such T cells to form a clone of similar cells which move to other lymph-nodes. Thus, two populations of cells build up in the body : 1) cells carrying measles antigens and 2) thymus-dependent immunocytes capable of reacting with measles antigen. By some as yet unknown mechanism, reaction between the sensitized T cells and virus-containing 'measles cells' takes on a damaging intensity, rupturing the measles cells and liberating virus and soluble measles antigens. The combination of antigen, sensitized T cells and mitogenic lymphokines ensures stimulation of B cells, which proliferate and produce IgM and IgG antibodies. Virus, some free but most still associated with mobile cells, pass into the blood to lodge in skin and mucous membranes. The accumulation

there of sensitized T cells and lymphokines liberated locally will then account for the character of the rashes. The explosive interaction, producing rash and fever, is apparently sufficient to eliminate the infection in a few days. With regard to the well known disappearance of a positive tuberculin reaction and other types of delayed cutaneous hypersensitivity for a few weeks after measles infection, Burnet suggests that there is discharge and exhaustion of all those local cells which contribute pharmacologically to the local reaction. He also assumes that the regions from which large numbers of sensitized lymphocytes are liberated have been temporarily exhausted, thus perhaps explaining the loss of reactivity of lymphocytes to other antigens such as tuberculin, *in vitro*.

Burnet's hypothesis rested strongly on the findings of Good and Zak (1956), that children with congenital agammaglobulinemia developed a normal clinical response following measles infection and recovered fully, acquiring seemingly lasting immunity. Other evidence included the fatal outcome of measles in children with leukemia who were being treated with cortisone (Enders *et al*, 1959). They developed fatal giant-cell pneumonia, no neutralizing antibodies and no associated exanthem following exposure to the virus despite the ability to mount humoral responses to other viral and bacterial antigens. Nahmias *et al.* (1967) also reported the occurrence of fatal giant-cell measles pneumonia in an infant who had no identifiable thymus. A

similar disease occurred in a sibling who had the same type of immunological defect.

Equally intriguing were the cases mentioned by Katz *et al.* (1962) in an early report on the Edmonston attenuated vaccine. They noted a small percentage of patients with no detectable neutralizing antibody against measles in whom they could not induce antibody formation by immunization Exposure of these patients to natural measles infection resulted in neither appearance of antibody nor production of clinical disease. Ruckdeschel *et al,* (1975) provided comparable evidence by describing cellular responsiveness to a measles complement fixation antigen *in vitro* in two pediatric residents who had negative hemagglutination inhibition antibody (HIA) titers and who had neither had clinical measles nor atypical measles but were frequently exposed to the agent.

In animal models it has been possible to demonstrate that thymic impairment, either by neonatal thymectomy or treatment with anti-thymus globulin can markedly increase susceptibility to a virus such as herpes simplex virus (HSV) (Zisman *et al,* 1969) while having no effect whatsoever on susceptibility to enteroviruses such as coxsackie B3 (Zisman *et al,* 1971). It has been possible in some instances to restore immune competence and promote recovery from infection by transfer of immune T-cells, as in the case of ectromelia (Blanden, 1971).

As can be seen in Fig. 3 control of infection by 'budding' viruses

can follow a number of classical pathways. Interaction of virus-infected cells with sensitized lymphocytes may lead to the death of the cells. The T-cells must recognise :

(a) virally modified histocompatibility antigen

(b) a complex of histocompatibility antigen with virally associated antigen or

(c) both virally associated and self-histocompatibility antigens (Roitt, 1977).

This direct attack on the cell will effectively limit the infection if the surface antigen changes appear before full replication of the virus, otherwise the organism will spread by two major routes. The first, involving free infectious viral particles released by budding from the surface can be checked by humoral antibody. The second, which depends upon the passage of virus from one cell to another, cannot be influenced by antibody but is countered by cell mediated immunity.

Sensitized cells proliferate after re-exposure to the sensitizing viral antigen. This blastogenic response serves to increase the number of virus-sensitized cells. In addition, sensitized cells may produce a number of soluble mediators or lymphokines which directly influence other cells. For example, chemotactic factors attract phagocytic cells to the area of viral replication; migration inhibitory factors keep these cells in the area of infection while activating substances induce macrophages to produce interferon which will render the contiguous cells non-permissive for the replication of any virus acquired by intercellular-transfer.

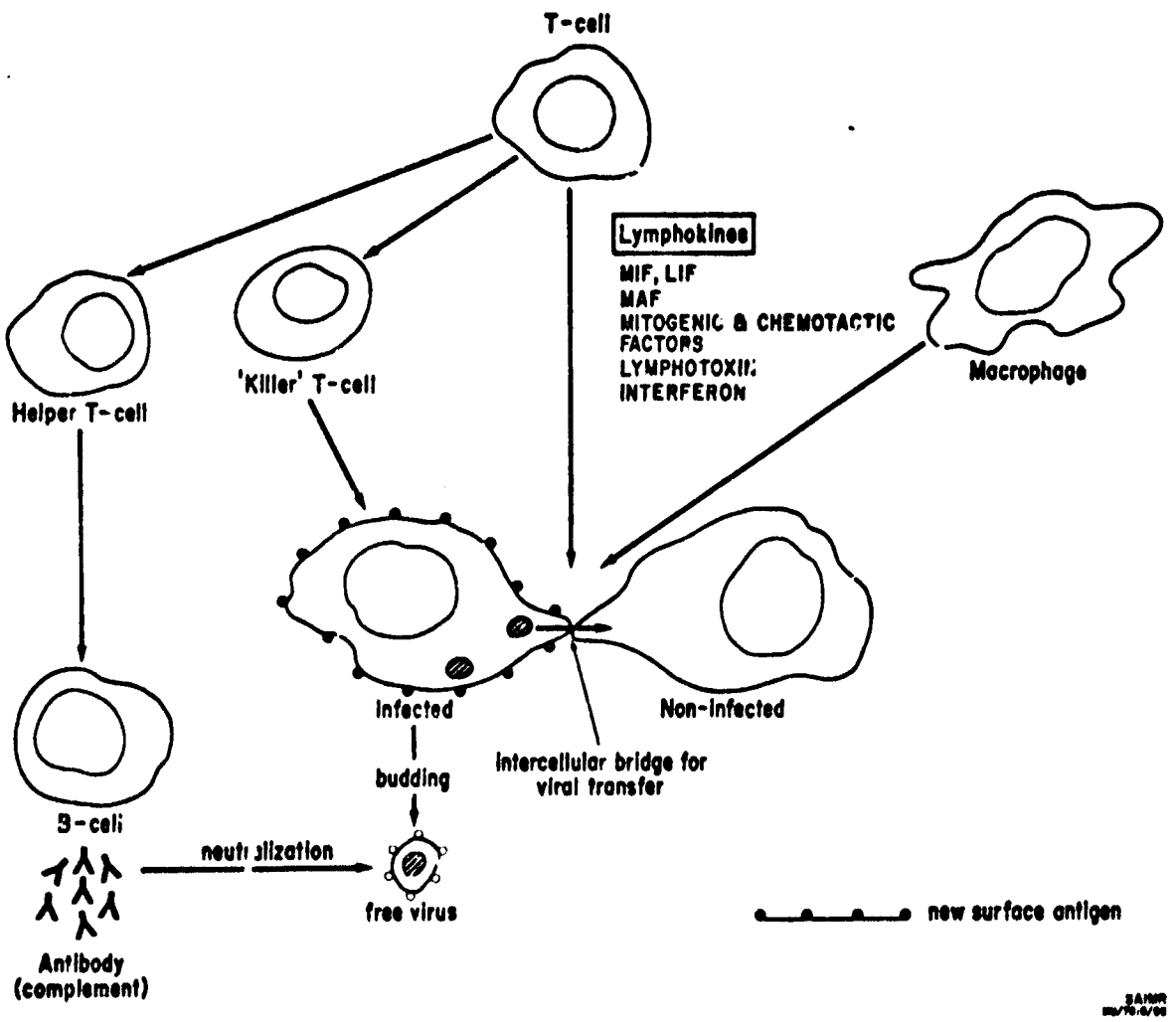

Figure 3 A schematic presentation of the immune control mechanisms involved against 'budding' viruses.

Association of Measles Virus with Lymphocytes

Whole blood and washed leucocytes from measles patients have been shown to produce measles when inoculated into susceptible subject (Hektoen, 1904; Papp, 1937), and the first isolation of measles virus *in vitro* was made from whole blood and from a throat swab (Enders and Peebles, 1954). Measles virus was also isolated *in vitro* from washed peripheral blood leucocytes from measles patients (Gresser and Chany, 1963).

Histopathological changes attributable to measles virus have been found in lymphoid tissues of infected man and monkey (White and Boyd, 1973; Yamanouchi *et al*, 1973). These are particularly striking in the thymus after fatal cases of measles (White and Boyd, 1973) where aggregation and formation of large syncytiae of thymocytes ('giant-cells') was found. The cytopathic changes mimic those observed in cell culture *in vitro*. Formation of multi-nucleated giant cells can be induced by culturing lymphocytes from measles patients *in vitro*, in the presence of phytohaemagglutinin (PHA) (Osunkoya *et al*, 1973). In addition, structures resembling measles virus nucleocapsids have been observed by electron microscopy in lymphocytes from children with measles (Narang, 1973).

In order to explain the depression of cutaneous delayed hypersensitivity reactions to the *in situ* aggregative

destruction of thymocytes, White and Boyd (1973) examined thymus-derived cells already in the peripheral blood. Although occasional giant cells and generalised lymphoid depletion were found, the picture of abundant giant cells (as in the thymus) was not seen. It might be anticipated that these destructive changes seen in the thymus could lead to a decreased lymphocyte count in the peripheral blood. Although leucopenia is common in early measles, it is generally accepted that this is predominantly a neutropenia (Whitby & Britton, 1969). However, thymectomy in the experimental mouse or genetically determined thymus deficiency in man, as in DiGeorge's syndrome, are not necessarily followed by lymphopenia in the peripheral blood. Aiuti (1973) found normal T lymphocyte numbers in the blood of children with measles and Kantor (1975) also found normal T lymphocyte numbers in the circulation of subjects vaccinated with measles virus. Hicks *et al*, (1977) using monkeys infected with measles virus found that the numbers of circulating or lymphatic T cells, B cells, or total mononuclear cells were not significantly altered.

Replication of Measles Virus in Human Leucocytes

A. Infection of leucocyte subtypes with measles virus

One of the factors which may determine whether or not a cell is susceptible to a particular virus is the presence of receptors or binding sites for that virus in the cell

membrane. In some viral systems, infectibility is apparently limited to selected leucocyte subclasses due in part to a differential distribution of surface viral receptors among the leucocyte subclasses (Woodruff and Woodruff, 1974; Jondal and Klein, 1973). Indeed, the work of Jondal and Klein (1973) strongly suggests that the Epstein-Barr virus is B lymphocyte trophic and that receptors for the virus are not present on the surface of thymic-derived (T) lymphocytes. In contrast, replication of 17D yellow-fever virus in human monocytes has been demonstrated by Wheelock and Edelman (1969).

Several different lines of evidence support the view that measles virus can replicate in human leucocytes. Papp (1937) first demonstrated that measles virus could be transferred to susceptible individuals by washed buffy coat cells from patients with naturally acquired measles infection. Gresser and Chaney (1961) also reported that circulating leucocytes from patients with measles infection could transfer infectivity to cultured cell monolayers, while Berg and Rosenthal (1963) first demonstrated that peripheral blood leucocytes from normal adults and from patients with lymphoblastic leukemia could support measles virus replication *in vitro*. Gresser and Chaney (1963) also reported that circulating leucocytes from patients with measles infection could transfer infectivity to cultured lymphoid cell monolayers. Recently, in experiments

conducted with human and simian lymphocytes, a receptor for measles virus was found (Valdimarsson *et al*, 1975). Using measles-infected fibroblasts, these workers showed that lymphocytes seemed to cluster or rosette around the infected carrier cells regardless of the donor's previous immune status to measles. Most of the carrier cell-attached lymphocytes could in turn be rosetted by sheep red cells (E-rosettes). Furthermore, using a T lymphocyte enriched population, almost all lymphocytes bound to the carrier cells whereas a B lymphocyte enriched population showed a reduced number of adherent cells.

Studies were performed which examined the susceptibility of human peripheral blood mononuclear cells to measles virus infection and replication in an attempt to define an *in vitro* model to correlate with clinical observations (Sullivan *et al*, 1975; Joseph *et al*, 1975). Mixed populations of circulating mononuclear cells (i.e. lymphocytes and monocytes) obtained from either measles-immune or non-immune human adults supported measles virus replication to a very limited extent. However, prestimulation of such cells with mitogens increased the efficiency of measles virus replication as assessed by virus-plaque assay of culture supernatants. PHA, a potent T cell stimulator, was associated with the

highest percentage of infectious cells, followed by concanavalin A (Con A), also a potent T cell stimulator. The percentage of cells stimulated by pokeweed mitogen (PWM), a predominantly B cell mitogen, led to smaller amounts of replicated virus. Allogeneic stimulation of lymphocytes was also associated with a marked increase in cells replicating measles virus. Selective rosetting techniques additionally indicated that E-rosette enriched cells (96% T cells) and E-rosette depleted cells forming EAC3 rosettes (85% B cells) could each replicate infectious virus after prestimulation with PWM (Joseph *et al*, 1975). In addition, measles virus infection of continuously cultured human lymphoblastoid cell lines indicated that both T (Molt 4) or B (RPMI-8866) subtypes proved capable of replicating measles virus. This latter finding was further substantiated by Barry *et al*, (1976), who demonstrated that the susceptibility of lymphoblastoid lines to persistent infection was independent of either the T or B characteristics of the cells or whether they were originally derived from patients who had malignant disease or who were normal.

Although certain viruses, i.e. yellow-fever virus can replicate in monocytes (Wheelock and Edelman, 1969), monocytes from adults were found to replicate measles virus at a very low level (Sullivan *et al*, 1975; Joseph *et al*, 1975). Monocytes obtained from neonates, however,

were capable of replicating measles virus in the range observed for mitogen stimulated lymphocytes (Sullivan *et al*, 1975).

Polymorphonuclear cells (PMN) infected with measles virus and sampled at various time periods and subjected to ultrastructural analysis, showed no evidence of viral replication throughout a 72 hour sampling period (Joseph *et al*, 1975).

B. The *in vitro* effect of measles virus on lymphocyte reactivity

In an attempt to delineate possible mechanisms for measles virus suppression of cell-mediated immunity (CMI), Sullivan *et al*, (1975a) investigated the effect of the virus on PHA induced stimulation of human peripheral blood mononuclear cells. Using a partially purified live measles virus preparation, marked inhibition of tritiated thymidine incorporation by PHA-stimulated lymphocytes was observed within 72 hours.

Lucas *et al*, (1977) have thoroughly investigated the conditions and mechanisms by which measles virus inhibits lymphocyte stimulation. The inhibition by live measles virus was observed in all four different systems of lymphocyte stimulation which were tested, i.e. stimulation by the mitogens PHA and PWM, stimulation by irradiated allogeneic cells, and the *in vitro* response to antigen

(PPD). It was concluded that the observed effects were therefore probably not due to an interaction between measles virus and the stimulating agents or the interactions of the latter with the lymphocyte membrane, because it seemed unlikely that the virus should have the same effect on all four stimulants. In agreement with Sullivan *et al*, (1975a), it was also found that the strongest inhibition of lymphocyte stimulation occurred when the measles virus was added together with or shortly after PHA addition. When the virus was added 48 hours after the addition of PHA, no inhibition occurred. A similar picture was seen when virus was added 96 hours after the addition of PWM, PPD, or allogeneic cells. Presumably the absence of measles virus induced inhibition of lymphocyte reactivity after addition of virus at a later stage could be a combination of lower efficiency of infection and lack of time for viral replication (Lucas *et al*, 1977). A least three parameters of stimulation were also affected when lymphocytes were cultured in the presence of measles virus. Cell numbers tended to be lower as compared to noninfected cultures although the viability of the cells was not significantly different, and morphologically, fewer larger transformed cells or blasts occurred. Measles virus also lost its inhibiting activity after

inactivation by ultraviolet light or after heating the virus at 56°C for one hour, thus confirming the findings of Sullivan and co-workers (1975a). However, these results differed from Zweiman and Miller (1974) who found that not only live measles virus, but also autoclaved virus was able to suppress PHA-induced lymphocyte transformation. This latter finding was presumably due to cell-derived inhibitory factors in their unpurified virus preparation.

An important factor that affected virus-induced inhibition of lymphocyte reactivity, was the serum source used for culturing lymphocytes. When lymphocytes were cultured in medium containing human serum instead of fetal bovine serum, no inhibition by measles virus was seen (Lucas *et al*, 1977). Measles antibodies present in the human serum presumably bound to the virus and thus counteracted the virus infection and prevented extensive spreading of the virus. The addition of various dilutions of IgG from the serum of a patient with SSPE (high measles antibody titers) showed that the inhibiting effect of measles virus could be blocked by specific IgG antibodies to measles virus. When serum was tested which had been obtained from a child without a measles history and without detectable measles virus antibody, no influence on the inhibition

was seen. By adding pooled human serum at different times after the addition of virus to lymphocytes, it was shown that even after 24 hours, the serum still neutralized the deleterious effect of the measles virus.

Finally, in order to determine whether virus replication was essential for the observed inhibition of lymphocyte stimulation, lymphocytes were cultured at 39°C after they had been infected with virus. At that temperature (non-permissive temperature), measles virus replication was slower, and much less inhibition of lymphocyte stimulation took place than at 37°C (Lucas *et al*, 1977).

In vitro at least, these phenomena present a remarkable situation since stimulation of lymphocytes either by mitogen or antigen seem to be an essential prerequisite for the replication of measles virus. Thus, in order to be able to suppress lymphocyte reactivity, measles virus first requires an activated cell. Lucas *et al*, (1977) hypothesized that initial infection may lead to a small number of infected cells which, after stimulation, produce sufficient infectious virus to increase the percentage of infected cells considerably. In similar vein, Joseph *et al*, (1975) speculated that replication of measles virus in leucocytes turned on by specific antigenic stimuli might permit preferential infection

of tuberculin-sensitive lymphocytes in patients with quiescent tuberculosis who had acquired a measles virus infection. Thus, flareups and dissemination of tuberculosis in patients subjected to measles virus infection might be related to a narrow, rather than a broad, impairment of cell-mediated immunity. Sullivan *et al*, (1975b) also speculated on the possibility that measles virus infection of children during infancy might result in increased replication in immature macrophages resulting in increased dissemination of the virus, particularly to the lungs and central nervous system. These findings may then explain the increased severity of measles infection seen in children less than one year of age and may also be a factor in patients who subsequently develop SSPE. Similarly, infection of lymphocytes *in vivo* by measles virus may allow for continued latent virus infection in the host with periodic activation, providing antigenic stimulation for long term immunity.

CHAPTER 4

In vitro Studies on Cell-Mediated Immunity to Measles Virus

IN VITRO STUDIES ON CELL-MEDIATED IMMUNITY TO MEASLES VIRUS

A. Lymphocyte transformation to measles antigen

Viral infections in humans has been demonstrated to have a variety of effects on specific and non-specific host defense mechanisms (Wheelock and Toy, 1973; Notkins *et al*, 1970). A review of the current literature on measles virus infection has revealed conflicting effects on cell-mediated immunity. This apparent disparity in results may reflect the heterogeneity of conditions in which the naturally occurring disease was studied, as well as differences in methodology among investigators. One of the greatest problems in the study of lymphocyte responsiveness to measles virus resides in the material being used as antigen.

Many of the conflicting results obtained in measuring lymphocyte reactivity to measles virus antigen may be the result of the physical characteristics of the antigenic stimulus itself. Measles complement-fixation antigen (CFA) is usually prepared by ultracentrifuging freeze-thawed remnants of measles virus infected monkey kidney cells (Vero). The resuspended pellet consists of both live virus and substantial membrane fragments, presumably with some membrane-bound or membrane-associated viral antigens.

On the other hand, vaccine or live virus preparations are usually prepared from the supernatant of the original infected tissue rather than from the cell pellet material. Methods of removing cells (e.g. millipore filtration) are such that nearly all membrane fragments are removed as well. Sullivan *et al*, (1975), propogated the measles wild strain as well as a vaccine strain in a continuous Vero cell line. Suspensions of virus were harvested by collecting medium or homogenizing infected cell sheets at the time of maximal cytopathic effect. Concentration and partial purification of the virus was attained by sedimenting the crude virus pools at 78,000 X G and resuspending the pellet in one-tenth the original volume of culture medium. Control fluids were obtained by harvesting supernatant media of uninfected Vero monolayers or by homogenization of uninfected cell sheets. Using tissue culture fluid from infected and non-infected Vero cells, significant inhibition of PHA-induced ^{3}H-thymidine incorporation was demonstrated. The inhibitory capacities of these preparations were not diminished by ultraviolet irradiation or heat inactivation. However, partial purification of the measles virus preparation by high speed ultracentrifugation, enabled Sullivan and co-workers to separate the inhibitory effect of the cell-derived factors from the marked inhibition induced by measles virus alone. Only live and not UV-irradiated or heat

inactivated virus was associated with significant inhibition of ^{3}H-thymidine incorporation.

Most observers have been unable to demonstrate lymphocyte blastogenesis in response to various preparations of measles virus or measles antigen. For example, Saunders *et al* (1969) reported 'a striking increase' in lymphocyte transformation to measles antigen, but review of the data showed the stimulation ratio (dpm experimental/dpm control) to be less than 2 in nearly every instance. The findings of Smith *et al*, (1973) that rubella CFA could produce *in vitro* lymphocyte responsiveness with a dose response and kinetic pattern identical to that obtained with specific antigens, e.g. PPD, suggested that *in vitro* phenomena, such as antigen preparation, could account for the suppression seen in other studies. In line with the work carried out with rubella virus, Graziano *et al*, (1975a) demonstrated specific lymphocyte reactivity to a measles CFA. The measles CFA and measles control antigens were obtained commercially. This antigen was prepared as a concentrated cell block antigen by centrifugation, freeze-thawing of the pelleted material, ultra-centrifugation, and resuspension of the pellet in tissue culture medium. The antigen did not undergo any clarification or purification and contained substantial amounts of cell membrane material. Control fluid was prepared in an identical manner except for the

absence of measles virus. Seventeen patients with positive histories of measles as children demonstrated *in vitro* lymphocyte responsiveness using this measles CFA. Kinetic data disclosed maximal responsiveness on day 7, and viral inactivation experiments disclosed that live virus was neither necessary for nor inhibitory to the reaction. In a further study, Ruckdeschel *et al*, (1975b), tested two pediatric residents who had negative haemagglutination inhibition antibody (HIA) titers against measles and who were frequently exposed to measles patients. Neither resident had had clinical measles nor atypical measles syndrome and both displayed strong *in vitro* cellular responsiveness to measles CFA as measured by ^{3}H-thymidine incorporation. Dunmire *et al*, (1975) investigated the effects of measles CFA on *in vitro* PPD responsiveness. They demonstrated that numerous dose combinations of measles and PPD could result in significant enhancement or suppression of the *in vitro* response to PPD alone, a fact contradicting the widely held belief that the influence of measles virus was uniformly suppressive of *in vitro* lymphocyte responsiveness to PPD. These results could be possibly explained in terms of the interaction of two antigens added concurrently to lymphocyte cultures, rather than any purported deleterious effect of measles on the lymphocytes themselves. The differential release of

suppressor and amplifier substances in response to concurrently added ant.gens might explain the pattern of inhibition and enhancement obtained in these results.

Despite the previous workers demonstration of cellular immunity against measles virus using the lymphocyte transformation test with CFA, experiments performed by Arstila *et al*, (1977) demonstrated that a purified measles antigen clearly inhibited the responses of human lymphocytes to PHA, PPD, and to allogeneic cells (MLC). The antigen consisted of measles virus grown in Vero cells that was purified by subsequent centrifugations, with a final linear sucrose gradient centrifugation and heat inactivation. The purity of the virus preparation was confirmed by polyacrylamide-gel electrophoresis and protein other than viral polypeptides were not found. No control mock-viral preparation was, however, concurrently prepared, and it is thus impossible to determine whether the phenomenon observed was a viral-specified one or one related to the method of preparing the antigen.

Kreeftenberg and Loggen (1977) felt that it could not be inferred that the stimulation measured by incubating lymphocytes with measles CFA (Graziano *et al*, 1975; Ruckdeschel *et al*, 1975 a & b) was specific for measles virus antigens. These workers felt that stimulation could be caused by a non-specific mitogen or an antigen

which was not specific for measles virus. Children were vaccinated with live measles vaccine and their lymphocyte transformation was determined using measles CFA. An important finding of this study was that only certain lots of the commercially obtainable measles CFA stimulated lymphocytes at all. Using an antigen preparation that did stimulate adult donor lymphocytes, it was demonstrated that the non-vaccinated childrens lymphocytes could not be stimulated although normal mitogen stimulation was attainable. Furthermore, measles antibody could not be demonstrated in the serum of these children. In contrast, four to six weeks after vaccination, lymphocytes from vaccinated children, responded to measles CFA and measles antibodies were detected in the sera. These results strongly suggested that with the antigen used in these experiments, a specific cellular immune reaction against measles could be demonstrated. Because of the age differences between the vaccinated and non-vaccinated children (12 months as compared to 1 - 7 months of age), it was felt that sensitization in the period between 2 and 12 months against an antigen which was present in the measles preparation used for lymphocyte stimulation, but not specific for measles virus, was, therefore, still theoretically possible (Kreeftenberg and Loggen, 1977).

B. Lymphocyte transformation to mitogens and soluble antigens

The first report by von Pirquet in 1908 on the unfavourable interaction of concurrent measles infection with clinical tuberculosis was followed by reports that measles infection or vaccination deminished or eliminated the delayed hypersensitivity-like response to intradermal tuberculin (Mellman and Wetton, 1963; Starr and Berkovich, 1964). A series of reports subsequently attempted to examine *in vitro* correlates of this clinical phenomenon.

Smithwick and Berkovich (1966), first noted that live measles virus added to PPD-sensitized lymphocytes reduced the response of those lymphocytes to PPD. They noted no effect on the response of one patient to PHA stimulation however. Fireman *et al*, (1969) demonstrated that immunization with the live attenuated measles vaccine diminished the *in vivo* cutaneous delayed hypersensitivity reaction to PPD, candida and ragweed antigen for 4 weeks. A modest depression of total leucocyte counts was noted for a period of one to three weeks, and at the same time, the capacity of lymphocytes from vaccinated patients to respond *in vitro* to stimulation with PPD, candida and ragweed antigens was also suppressed without a decrease in their *in vitro* response to PHA. The mechanism of the suppression of delayed hypersensitivity by live measles vaccine appeared dependent on a viable virus since formaldehyde inactivated measles

virus vaccine had no demonstrable effect on pre-existing cutaneous delayed hypersensitivity or on the *in vitro* lymphocyte responses.

Kadowaki *et al*, (1970) also observed that PHA-induced transformation was not depressed in the lymphocytes of 30 subjects given attenuated measles vaccine. However, they demonstrated suppression of PHA responsiveness during the first 4 - 5 days of the measles rash, using a technique to determine the absolute mitotic cell number and lymphocyte blastic transformation rate. None of the aforementioned authors made use of the radioisotopic method to assess lymphocyte reactivity. Using a radio-labelling technique, Zweiman and co-workers (1969, 1971) showed reduced responsiveness of lymphocytes to PPD and PHA after administration of live vaccine or addition of live or autoclaved virus to the lymphocyte cultures *in vitro*. The results of these studies were used to suggest that the *in vivo* effect of measles infection on clinical tuberculosis or the delayed hypersensitivity reaction to tuberculin could be explained by the toxic effect of measles virus on lymphocytes seen in their *in vitro* assays.

Finkel and Dent (1973) examined the *in vitro* proliferative behaviour of lymphocytes from seven children with classical measles and three children with atypical measles. Impaired *in vitro* lymphocyte responses to PHA stimulation was observed in both groups of patients, but the impairment

was only evident at suboptimal concentrations of PHA, and disappeared in convalescence. Interestingly, elevated spontaneous DNA synthesis in the peripheral blood leucocytes of both groups of patients was observed. In an effort to explain this quantitative depression of PHA responsiveness in the lymphocytes of patients with acute measles infection, these workers quoted the findings of Oppenheim *et al*, (1968), who found that the *in vitro* blastogenic response of purified lymphocytes to specific antigens and to low doses of PHA was impaired as compared to that of unpurified (macrophage-containing) lymphocytes. The response to optimal concentrations of PHA was not impaired in cultures of purified lymphocytes. Thus, the finding of abnormal PHA responses at suboptimal concentrations of stimulant with normal responses at the optimal concentrations would suggest that the measles virus had a primary effect on the function of the phagocytic cells in the peripheral blood and would also explain the preferential effect of the virus on the lymphocyte response to specific antigens rather than to PHA stimulation (Smithwick and Berkovich, 1966; Fireman *et al*, 1969; Zweiman *et al*, 1971). Alternatively, the proliferative stimulus of a primary measles infection or the hypersensitivity reaction induced by measles vaccine could be of such extraordinary magnitude that the majority of competent lymphocytes are involved in a response to measles antigen, precluding their response to other antigens or non-specific mitogens. The markedly elevated spontaneous DNA

synthesis in circulating lymphocytes would favour this hypothesis (Finkel and Dent, 1973). A major criticism of this work is the small number of patients studied as well as the fact that a whole-blood culture transformation method was employed which does not really permit analysis of the response of equal numbers of lymphocytes per culture.

Whittle *et al*, (1973) investigated the effects of measles infection on the immune system of young Nigerian children. At the time of the rash, skin tests to PPD, candida and streptococcal antigens were negative. Although the PHA response of lymphocytes from patients with measles was generally lower than that of the response of healthy controls, the difference was not statistically significant (both morphological assessment of blast cells as well as radioisotope incorporation after PHA stimulated were carried out). Hypothesizing on these findings, these workers suggested that the function of thymus-dependent memory cells may be selectively impaired due to changes in antigen receptors on the cell surface induced by measles virus. In a study undertaken by Coovadia *et al*, (1974), PHA-induced lymphocyte blastogenisis was studied in African children with severe measles. Of 24 children, 19 (79%) had depressed PHA stimulation at varying intervals after the appearance of the rash. Although 86% of the children with measles were not undernourished (as judged

by the serum albumin levels), most of them had secondary infections i.e. pneumonia, and thus their depressed PHA responses could have been a result of the secondary infection and not the measles infection.

Kantor (1975) studied human T cells in the anergic state induced by vaccination with a combination of measles, mumps and rubella vaccine. Lymphocytes were removed at various intervals before and after vaccination and stimulated by two mitogens and one antigen and blastogenesis was then assessed by the incorporation of ^{3}H-thymidine. Vaccination alone resulted in increased DNA synthesis at three and five weeks. After stimulation with PHA and PWM, ^{3}H-thymidine incorporation was similar both before and after vaccination, while stimulation with candida antigen was depressed.

In an attempt to define the relative roles of the various components of the immune system in recovery from acute measles infection, Hicks *et al*, (1977) followed various T and B cell functions *in vivo* and *in vitro*, in immunosuppressed rhesus monkeys experimentally infected with measles. Immunosuppression with anti-human thymocyte gammaglobulin caused a predictable delay in viral clearance and a concomitant decrease in circulating and lymph node T cells with resulting T cell mitogen unresponsiveness. Measles infection of non-immunosuppressed monkeys, slightly

suppressed mitogen responsiveness (PHA, Con A and PWM) of circulating lymphocytes in the second and third week when compared to controls, but no suppression of lymph node cells could be demonstrated. Circulating lymphocytes displayed an enhanced response to mitogens during the period of maximal virus replication (the first week of infection), while lymph node lymphocytes, from which maximal virus titers were obtained (on day 7) did not display any suppression. It was felt that infection of lymphocytes by measles virus as an explanation of depressed mitogen responsiveness during the second and third week of measles, might not be adequate, since mitogen stimulation during viraemia was not suppressed and since the virus had been cleared before any observed suppression. Rather, non-specific binding of measles to T cells (Valdimarsson *et al*, 1975) may alter the circulating pattern of lymphocytes and transiently deplete lymphocyte sub-populations in the circulation, without affecting mitogen responsiveness of centrally located T cells.

C. Migration tests

Generation of lymphocyte-effector molecules (lymphokines) *in vitro* by human lymphocytes stimulated by mitogens and antigens has provided an important tool in the evaluation of the cellular immune response. Rocklin *et al*, (1974)

demonstrated that lymphokines may be produced independent of DNA synthesis and cellular proliferation, and it has thus become relevant to study the response of lymphocytes both in terms of lymphocyte proliferation and lymphokine production. The inability of some workers to demonstrate reproducible lymphocyte blastogenesis using measles antigen preparations, has subsequently led to attempts to establish measles virus-induced migration inhibition of human leucocytes as an *in vitro* test of cell-mediated immunity to the virus. As was the case with measles antigen-induced lymphocyte transformation, controversy also surrounds the use of a satisfactory measles antigen for leucocyte migration studies.

Utermohlen and Zabriskie (1974), demonstrated that patients with multiple sclerosis manifested depressed cellular reactivity to measles antigen, using a direct leucocyte migration inhibition test. The measles antigen was commercially obtained, and used at two dilutions. It was intially prepared by freeze-thawing virus-infected cells followed by gentle centrifugation to remove larger cellular debris. Similar results were obtained by Platz *et al*, (1974) using undiluted measles and parainfluenza antigens. These workers suggested decreased cellular immunity against measles virus, thus leading to persistent virus infection with measles or closely related viruses as a possible cause of multiple sclerosis.

Using the direct leucocyte migration test and a measles antigen preparation, Dossetor *et al*, (1977) measured the cellular immune status of both malnourished and well-nourished children with measles. The antigen was a haemagglutinating antigen treated with Tween-ether and was obtained commercially. The malnourished children showed significantly less leucocyte inhibition to measles antigen than the controls, probably the combined result of the immunosuppressive effects of their malnutrition and the measles infection. In unpublished results, it was also claimed that non-immune cord blood lymphocytes showed significantly less inhibition than immune lymphocytes.

The concept of measles virus-induced migration inhibition of human leucocytes as an expression of cell-mediated immunity, was thoroughly investigated by Nordal *et al*, (1976). Leucocytes from healthy donors previously exposed to measles virus (confirmed by the demonstration of measles virus-specific antibodies in their sera) and leucocytes from 10 children without a clinical history or serological evidence of measles, were used in the leucocyte migration test. Two different sources of measles antigen were used throughout the experiments. Both were prepared similarly by freeze-thawing and sonicating infected Vero cell cultures, and using the cell-associated virus material as antigen. In agreement with other reports

(Utermohlen and Zabriskie, 1974; Platz *et al*, 1974), material from both measles virus-infected cell cultures inhibited the migration of human leucocytes *in vitro*. However, the children without any clinical or serological evidence of previous measles virus infection showed the same inhibition of migration as that of leucocytes from children immunized by natural exposure to the virus. Various explanations for the lack of discrimination between donors with and without evidence of measles immunity were put forward by Nordal and co-workers. One possibility was that viral structures present in the measles antigen had effects other than antigenic on migrating buffy coat cells. Valdimarsson *et al*, (1975), reported that most human T lymphocytes have receptors for measles virus, and that on binding to measles-infected cells, may be cytotoxic, irrespective of the donor's previous experience of measles infection. There is also evidence indicating that previous immunization to measles virus does not prevent the infection of human leucocytes by this virus *in vitro* (Joseph *et al*, 1975). Another strong possibility is that leucocyte agglutination by the virus might explain the migration inhibition seen when using human buffy coat cells (Utermohlen *et al*, 1975).

CHAPTER 5

The Humoral Immune Response to Measles

THE HUMORAL IMMUNE RESPONSE TO MEASLES

Introduction

Measles is a membrane-associated virus that acquires its envelope by budding off the cytoplasmic membrane of the host cell (See Page 8). The cytoplasmic membrane of the infected host cell acquires the capability of fusing easily with an adjacent uninfected host cell, thus enabling the virus to pass from cell to cell without exposure to extra-cellular fluid (Agnarsdottir, 1977). As antibodies are not capable of penetrating cells, the virus may, therefore, remain inaccessible to their action except when it is released on budding or after the death of the host cell. Thus, the primary role of antibody is in protecting against infection and, to an extent, restricting the spread of extracellular virus, in contrast to the cell-mediated response of eliminating or restricting virus-infected cells.

Being a highly contagious childhood illness, few individuals reach adulthood without being exposed to the virus, and thereafter immunity is lifelong, with antibody persisting in the circulation for many years. In commenting on reasons for this long-lasting measles-specific antibody, Enders-Ruckle (1965) considered two mechanisms which could provide an explanation. One is re-infection by the same or by cross-reacting viruses, the other is persistence of infection accompanied by the production of virus or of sub-units of the virus. In support of the second possibility she

quotes the isolation of four virus strains recovered from the lymph nodes of two patients who had had measles several weeks before.

The Appearance of Antibodies during Measles Infection

With most acute viral infections, clinical recovery is often preceded by the eradication of the infectious virus from the blood stream. In measles, antibody certainly plays an important part in 'mopping' up extra-cellular virus particles. Following infection or vaccination, the bone marrow-derived lymphocytes (B cells) and their descendants the plasma cells, synthesize and secrete antigen-specific antibodies with affinity to the corresponding antigenic determinants of the virus. Viral antibodies so formed belong to three immunoglobulin classes : IgM, IgG and IgA.

The IgM (19S) antibody represents the initial 'acute phase' antibody response and IgG (7S) constitutes the major source of specific antibody in later life and after the disappearance of IgM antibody (Schleuderberg, 1965). The humoral immune response following measles vaccination is also similar to that induced by clinical infection; IgM antibody is present initially, followed later by IgG.

In a study undertaken by Ruckle and Rogers (1957), twenty-five cases of measles were investigated for the presence of virus as well as serum antibody at different times during illness and convalescence. Virus could only be isolated from those

patients whose blood specimens and throat secretions were collected between 48 hours before and 32 hours after onset of rash. Furthermore, there was a correlation between the presence of virus in throat washings and blood specimens and the absence of neutralizing antibodies in the serum. When virus was isolated, antibodies were absent; when antibodies were present, virus was not isolated. Antibodies able to inhibit the cytopathic capacity of measles virus and capable of fixing complement appeared in all patients during the convalescent period. However, the time of initial appearance varied in individual patients. The earliest observation of antibody was approximately 24 hours after onset of rash, and 13 sera obtained during the period from 24 to 96 hours after onset of rash showed detectable antibodies. Convalescent sera, 144 - 192 hours after onset of rash demonstrated neutralizing antibody titers of 27 to 64 and complement-fixing titers from 64 to 512. In serum specimens obtained 3 to 4 months after illness, approximately the same neutralizing antibody titers were present as that in the first week after onset of rash. However, after 11 months, the antibody titer decreased significantly when compared to titers obtained during the first week of illness. The results obtained with the complement fixation tests suggested a similar trend in the antibody pattern. In addition, adults from various parts of the world who had contracted measles as children, demonstrated neutralizing antibody titers ranging from < 4 to 27, and complement-fixing antibody titers from 4 to 128.

The Humoral Response to Antigen during Measles Infection

B cells stimulated by antigen differentiate into antibody-secreting cells. T cells act directly in cellular immune reactions and also exert a helper effect on antibody production even though they do not themselves become plasma cells. Yamanouchi *et al*, (1974) demonstrated that rinderpest virus, a paramyxovirus related to measles, exerted a suppressive effect on the primary antibody response of rabbits to sheep erythrocytes, which was most marked in animals immunized within 3 days after virus infection. In other experiments, evidence was obtained that a mouse-adapted strain of measles virus impaired T cell helper function without affecting B cell activity (McFarland, 1974). Thus, restoration of antibody formation could not be achieved in x-irradiated mice infused with hapten-primed spleen cells from non-infected donors and carrier-primed spleen cells from donors infected with mouse-adapted measles. In contrast, high levels of responsiveness were observed in recipients of hapten-primed spleen cells from donors infected with mouse-adapted measles. In contrast, high levels of responsiveness were observed in recipients of hapten-primed spleen cells from measles-infected donors and carrier-primed cells from normal donors. This suppression of the anti-hapten response was apparently dependent on the replication of virus in the host since heat inactivated virus did not suppress T helper-cell function.

Whittle *et al*, (1973) investigated the effects of measles infection on the humoral immune system of well nourished children having a rash less than 4 days old. No significant difference was found in the immunoglobulin levels of the measles and control groups on the first day of the investigation. Antibody levels were measured after immunization with tetanus toxoid and *Salmonella typhi* vaccine. Children with measles showed a significantly smaller rise in the titer of antibody to the H and O antigens of *S. typhi* than did the controls. Twenty-seven percent of the patients with measles showed a response to immunization with tetanus vaccine compared with 50% of controls. The difference, however, was not significant. A similar depressed antibody response to typhoid vaccination in children with measles given shortly after the onset of rash was observed by Coovadia *et al*, (1974).

The humoral immune response of children with complicated measles (mostly pneumonia or diarrhoea) was investigated by Wesley *et al*, (1975). Patients were immunized with TAB vaccine within 10 days of onset of rash. Of the 28 measles cases, 22 failed to produce 'O' antibody while none showed any reaction to 'H' antigenic stimulus. In addition, measles antibody was rarely detected in patients on or before day 4 after the appearance of the measles rash and all but one patient demonstrated a rise in antibody titer over the 10 day study period. There was also no correlation between the late appearance of measles antibodies and inadequate antibody responses to TAB vaccine in the same

patients. Although these results are similar to those obtained by Whittle *et al*, (1973), the response of the latter's patients appeared much better than that observed in the present study. The reason for this difference was probably due to the fact that these patients had more severe measles, in that they all had some complication or a superimposed bacterial infection.

Hicks *et al*, (1977) studied the immune response during measles infection of rhesus monkeys immunosuppressed with anti-human thymocyte globulin (ATG). These monkeys were infected with measles and simultaneously inoculated with sheep erythrocytes (SRBC), a thymus-dependent antigen and with pneumococcal polysaccharide type III (SSS-III), a thymus-independent antigen. Measles infection of non-immunosuppressed monkeys ran a typical course with clearance of virus in 10 days and detection of circulating cytolytic and neutralizing antibodies shortly thereafter. In contrast, ATG treatment caused a delay in viral clearance and cytolytic antibodies were 4 fold lower at day 14, but neutralizing antibodies were not significantly depressed. These antibody titers as well as circulating and lymph node T cells rose to normal levels after cessation of ATG treatment, both correlating with viral clearance. Antibody production to SSS-III was similar in all monkeys, unaffected by immunosuppression or by measles infection. In contrast, antibody production to SRBC was significantly depressed in the monkey's receiving ATG. Measles infection alone did not suppress SRBC antibody production. Clearly, measles infection did not suppress

simultaneous T-dependent primary antibody responses *in vitro* to SRBC or to measles virus itself. This is in contrast to the observed depression of secondary antibody responses in mice following intraperitoneal administration of a non-replicating hamster neurotropic measles virus (McFarland, 1974).

Viral Clearance

The expression of viral antigens on the surface of infected cells may trigger immunological attack mechanisms, both cellular and humoral. Although specific cellular immune mechanisms of viral clearance have not been clearly demonstrated, Valdimarsson *et al*, (1975) presented evidence that lymphocytes of both measles-immune and non-immune subjects were toxic to measles-infected cells, indicating that cytotoxicity did not require the presence of measles-specific lymphocyte clones. Joseph and Oldstone (1974) have described what seems to be redistribution of measles virus antigen on the surface of carrier cells which were exposed to measles virus-specific antibody. They found that measles-specific IgG was cytotoxic and, further, that the specific antigen-antibody complex at the cell surface activated the complement system by the alternate pathway (Joseph *et al*, 1975). Perrin *et al*, (1977) characterized the interactions between target cells infected with measles virus and expressing viral antigens on their surfaces, and the immune response. Using a chromium-release cytotoxicity method, it was demonstrated that leucocytes from patients with chronic measles virus infection

(SSPE) or from immune adults, were cytotoxic for the target cells. Maximal killing in the absence of added antibodies to measles virus was usually detected only after 15 to 18 hours of incubation and with a high leucocyte to target ratio. When antibody to measles virus was added, cytotoxicity was not blocked, and instead, killing was enhanced with maximal lysis occurring with fewer leucocytes and a shorter incubation time (6 hours). Depletion of either glass-adhering or E rosette-forming cells did not reduce cytotoxicity in either system, while removal of EAC-rosette-forming cells or cells bearing Fc receptors, significantly abrogated killing of the virus-infected cells. These latter findings, suggesting that the cytotoxic effector cells belonged to a population of Fc-receptor-bearing cytotoxic cells or K cells, were substantiated by Kreth and Wiegand (1977). In experiments using lymphocytes from SSPE patients, measles sero-positive controls, and patients with acute measles, Fc-receptor bearing cells were removed by adsorption onto immune complex monolayers with the resulting loss of about 75% of measles specific cytotoxicity. In addition, measles-specific cell-mediated cytotoxicity was not enhanced by the addition of measles antibodies after removal of K-cells. The importance of measles antibody in the lysis of measles-infected target cells was emphasized by the fact that all experiments were set up in fetal calf-serum-containing tissue culture medium. To inves the possibility that lymphoid cells might have been contam with serum antibodies, the cells were treated with pronase ..

in order to strip all exogenous material from their membranes. After a 24-hour recovery in culture, these cells were tested together with freshly prepared lymphoid cells from the same donor. The cultured lymphoid cells were no longer cytotoxic against measles infected target cells when compared to freshly isolated lymphocytes. These findings, therefore, suggested that lymphocytes were contaminated with serum antibodies and that in culture, these antibodies dissociated and sensitized target cells having membrane-bound measles virus antigens on their surface. Alternatively, it was suggested that K-cells might have picked up measles immune complexes from the circulation which are known to exist in SSPE and probably also in patients with acute measles infection (Kreth and Wiegand, 1977).

The inability to detect T lymphocyte killing in measles virus-infected targets using mononuclear cells from patients with active SSPE or from measles-immune donors, may be technical, but may also reflect difficulties in obtaining T cell cytotoxic responses from lymphocytes a long time after the initial viral infection. Perrin *et al*, (1977) thus felt that cytotoxic T cells were generated over short time periods, usually during the first 5 to 8 days after initiation of infection. In contrast, experimental evidence, although only limited to two patients clearly points to K cells rather than cytotoxic T cells being involved in measles-specific cytotoxicity in the patients with acute measles studied by Kreth and Wiegand (1977). Furthermore, Lucas *et al*, (1978) investigating the killing capacity of lymphocytes infected with measles virus, demonstrated that the activity of K cells in killing sensitized target cells was not impaired.

PART II

EXPERIMENTAL

CHAPTER 6

Lymphocyte Transformation as an *in vitro* Assay for Cell-Mediated Immunity in Children with Measles

LYMPHOCYTE TRANSFORMATION AS AN *IN VITRO* ASSAY FOR CELL-MEDIATED IMMUNITY IN CHILDREN WITH MEASLES

INTRODUCTION

It is more than 15 years since Nowell (1960) showed that PHA extracted from the bean, *Phaseolus vulgaris*, stimulated the proliferation of lymphocytes in culture. This observation showed that the small lymphocyte could proliferate and disproved the contention that this cell was terminally differentiated. Similarly, concanavalin A (Con A) and pokeweed mitogen (PWM) are examples of a growing list of plant lectins that nonspecifically stimulate the proliferation of leucocytes from humans or animals. These molecules differ from antigens which stimulate the proliferation of lymphocytes obtained from subjects previously immunized with the test antigen. Similarly, lymphocytes from one person can also be stimulated to divide when cultured with lymphocytes from a histoin[illegible] individual, the so called mixed lymphocyte culture (MLC).

Efforts to determine the mechanism(s) responsible for the depression of delayed cutaneous hypersensitivity seen during acute measles have frequently involved the measurement [illegible]ymphocyte transformation or blastogenisis induced by nonspecific mitogens. Previous studies employing lymphocytes obtained from individuals during the acute stage of measles infection have shown in some instances a depression of PHA stimulation (Kadowaki *et al*, 197C; Finkel and Dent, 1973) whereas others have reported normal responses (Fireman *et al*, 1969;

Osunkoya *et al*, 1974a). In view of these findings, mitogen-induced lymphocyte transformation was investigated in this study using MN cells obtained from children with acute measles infection. In addition, the possibility of measles MN cells spontaneously incorporating tritiated thymidine in the absence of any stimulus was also investigated following the discovery in this laboratory of spontaneously activated cells in Hodgkin's disease (Golding, 1976).

MATERIALS & METHODS

Patients and Controls

Studies were performed on 37 well-nourished children with measles, ranging in age from 8 months to 6,5 years (the mean age being 1,10 years old). These children were from infant homes or orphanages and were hospitalised for social and epidemiological reasons. All children had uncomplicated measles as assessed by clinical examination, chest roentgenogram and absence of pyrexia after the rash had reached its maximum intensity, and those with evidence of secondary bronchopneumonia were excluded. Only a mild analgesic (paracetemol) was administered to the children. Tests were performed during the acute phase of the disease, either on the day of the appearance of the rash or within 2 days thereafter. To obviate the problem of obtaining blood from healthy children, normal adult laboratory workers served as daily controls. In previous studies from this laboratory (Lomnitizer *et al* 1977), it has been shown that the cellular immune status of normal children of corresponding age to the measles patients, was essentially similar to adult responses.

Cell Separation Procedures

Preparation of Ficoll-Hypaque Gradients for blood separation

Ficoll-Hypaque gradients were prepared by mixing 24 parts of Ficoll to 10 parts of Hypaque according to the method of Böyum (1968) :

1. A nine percent solution of Ficoll was prepared in distilled water by dissolving with a magnetic stirrer.
2. Ampoules containing 65% Hypaque were diluted with 19.0 ml of distilled water.

3. The Ficoll and Hypaque were mixed together in a measuring cylinder at a ratio of 24:10.

4. The specific gravity (SG) of the mixture was adjusted to between 1.076 and 1.078 at room temperature by means of a hydrometer. Distilled water was used to lower the SG and undiluted Hypaque to raise the SG.

5. The mixture was then dispensed into aliquots and sterilised by autoclaving (20 mins. at 15 lb/in^2) and then stored at 4^oC.

Isolation of Mononuclear Cells (MN)

Approximately 10-15 ml of venous blood from patients and controls was collected in haparinized bottles (10 units/ml, preservative-free heparin). 15 ml of Ficoll-Hypaque was dispensed into 50 ml stic disposable tubes and the temperature of the solution was adjusted to room temperature before use. Blood was layered gently onto the mixture by tilting the tubes to an angle of about 45^o and pouring the blood slowly down the wall of the tubes. Care was taken not to disturb the blood-Ficoll interphase when layering the blood onto the gradient. Using a swing-out head centrifuge, tubes were centrifuged at room temperature for 30 minutes at a s i of 400g. The MN cell fraction at the plasma-Ficoll-Hypaque interphase was collected by means of a sterile Pasteur-pipette; diluted about 50% with Hank's Balanced Salt Solution (HBSS) and collected each time by centrifuging for 10 minutes at 250g. This cell fraction consisted of 60 - 70% lymphocytes, 15-20% monocytes and 10% polymorphonuclear cells (PMN). After the final wash,

the cells were resuspended in MEM buffered with 20 mM hepes and containing 2 mM L-glutamine, 10% FCS, 100 units/ml of penicillin, 100 ug/ml of streptomycin and 2.5 ug/ml of amphotericin B. The buffered supplemented medium will be referred to as complete MEM.

Isolation of Polymorphonuclear Cells (PMN)

PMN cells for use in migration assays were always obtained from the Ficoll-Hypaque separation of normal blood. The pellet at the bottom of the centrifuged gradient, containing PMN and erythrocytes was mixed with 0.83% cold ammonium chloride in a ratio of 1:10 v/v and kept at $4^{o}C$ for 10 - 15 minutes to lyse the erthrocytes. The remaining PMN were centrifuged at 250g for 5 minutes and washed three times with HBSS. The resultant suspension contained 98-99% PMN.

Counting of Leucocytes

Following Ficoll-Hypaque separation and washing, leucocytes were resuspended carefully in a small volume of complete MEM. Using sterile procedures, a 0,1 ml aliquot of leucocytes was then added to 0,9ml of Turk's counting fluid. The composition of the latter consisted of :

Glacial Acetic Acid	74ml
Gentian Violet	0.2g
Distilled Water	926ml

Following thorough mixing on a vortex mixer, a small aliquot of the Turk's-leucocyte mixture was placed in a hemocytometer (Neubauer).

Nuclei were stained blue while any residual erythrocytes were lysed. Duplicate cell counts were made and an average count utilised.

Assessment of Leucocyte Viability

Before utilization of leucocytes, viability was always assessed by mixing equal volumes of a leucocyte aliquot and a 1% trypan blue solution at room temperature for 5 minutes. An aliquot of this mixture was introduced into a hemocytometer and the viable (unstained) and non-viable (blue-staining) leucocytes counted.

Spontaneous Transformation

MN cells of measles patients and controls, obtained from Ficoll-Hypaque gradients, were washed three times in HBSS and suspended in complete MEM. The cells were then cultured in triplicate in round-bottom microtiter plates; 2×10^5 cells/well for 18 hours in the presence of ^{3}H-thymidine (1μCi/well), at 37°C in 5% CO_2 in 95% humidified air. Cells were then harvested with a multiple automatic sample harvester (MASH II) onto filters. The filters were washed with phosphate buffered saline and allowed to dry in scintillation vials; 5 ml of scintillation fluid was then added (Insta-Gel) and thymidine incorporation measured in a Packard tri-carb liquid scintillation counter.

Mitogen Activation

MN cells in complete MEM were cultured in round bottomed microtiter plates at a concentration of 2×10^5 cells/well. All cultures were performed in triplicate. In some experiments (when cell numbers

permitted), three dilutions of PHA were added to MN cells (either 2.5, 5.0 or 10 µg) in order to determine the dose response effects of PHA stimulation on measles MN cells. However, in most experiments, a fixed concentration (10 µg) of PHA was used, a dose that has previously been shown to stimulate adequately. Cells were cultured for 72 hours at 37°C in 5% CO_2 in 95% humidified air. Tritiated thymidine was added for the last 18 hours of culture. The cells were then harvested as described above. A stimulation index was calculated as follows :

$$SI = \frac{\text{cpm of PHA stimulated cultures}}{\text{cpm of control cultures}}$$

The effect of supernatants from unstimulated lymphocyte cultures on normal lymphocyte transformation

MN cells from normal donors were suspended in a 1 : 1 mixture of complete MEM and either normal or patient's control supernatant. Cells were cultured in microtiter plates (2×10^5/well) in triplicate for 72 hours in the presence or absence of PHA (10 µg) at 37°C in 5% CO_2 in humidified air. Tritiated thymidine was added for the last 18 hours of culture and the cells were harvested as previously described.

The effect of measles serum on normal lymphocyte transformation

MN cells from normal donors were suspended in MEM containing 10% measles serum. 2×10^5 cells were cultured in microtiter plates in the presence or absence of PHA (10 µg) for 72 hours at 37°C in 5% CO_2 in humidified air. Tritiated thymidine was added for the last 18 hours of culture and the cells harvested as previously described.

Mixed Lymphocyte Culture (MLC)

MN cells from normal donors and measles patients were suspended in complete MEM. Two-way mixed lymphocyte cultures were performed by mixing equal numbers of patient and normal MN cells. 2×10^5 cells of the resulting cell suspension were dispensed into wells of a microtiter plate and ^{3}H-thymidine incorporation was assessed after 120 hours incubation at 37°C in 5% CO_2 in humidified air. Mixtures of cells from two unrelated normal volunteers served as controls.

In one-way MLC, either patient or control MN cells were exposed to 25 µg/ml Mitomycin C for 30 minutes at 37°C, washed three times in HBSS and mixed with an equal number of untreated patient or control responder cells. This mixture of cells (2×10^5) was then cultured in microtiter plates for 120 hours at 37°C in 5% CO_2 in humidified air and the incorporation of ^{3}H-thymidine assessed as previously described.

Statistical Analysis

In all studies, statistical significance was determined by the Student's t-test. A p value greater than 0.05 was considered not significant.

RESULTS

Spontaneous Transformation

MN cells from measles patients demonstrated spontaneous ^{3}H-thymidine incorporation after 18 hours of incubation, without prior stimulation. The mean uptake of ^{3}H-thymidine by MN cells from healthy donors was 1,526 + 713 cpm whereas from measles patients this was significantly raised (mean 13,156 ± 770 cpm) ($p < 0.01$). When MN cells from measles patients were cultured for a period of 72 hours, however, the raised spontaneous ^{3}H-thymidine uptake was substantially reduced (Fig. 4), although cells remained viable as assessed by trypan blue exclusion.

Mitogen Activation

In preliminary studies a number of PHA dose-response studies were performed using 2.5, 5.0 or 10 µg of PHA. Adequate stimulation by measles MN cells was observed at most doses (Fig. 5) and as can be seen in Fig. 6, MN cells from children with measles responded adequately to 10 µg PHA as determined by the incorporation of ^{3}H-thymidine after 72 hours.

The effect of supernatants from unstimulated lymphocyte cultures on normal lymphocyte transformation

The ability of unstimulated measles MN supernatant to inhibit PHA-induced transformation on normal lymphocytes was studied (Table 3a). No inhibitory effect on normal MN cell activation by PHA as measured by ^{3}H-thymidine incorporation was observed.

The effect of measles serum on normal lymphocyte transformation

As can be seen in Table 3(b), measles acute-sera did not inhibit the PHA-induced transformation of normal lymphocytes.

Response to al ogeneic lymphocytes

When measles MN cells were incubated with control MN cells in a two-way MLC, significantly less ^{3}H-thymidine incorporation was observed after 120 hours incubation than in cultures containing MN cells from two different control donors ($p < 0.005$) (Fig. 7a). Not only did measles MN cells respond poorly to allogeneic activation, but when mitomycin treated, they also failed to adequately stimulate control responding cells ($p < 0.005$. (Fig. 7b).

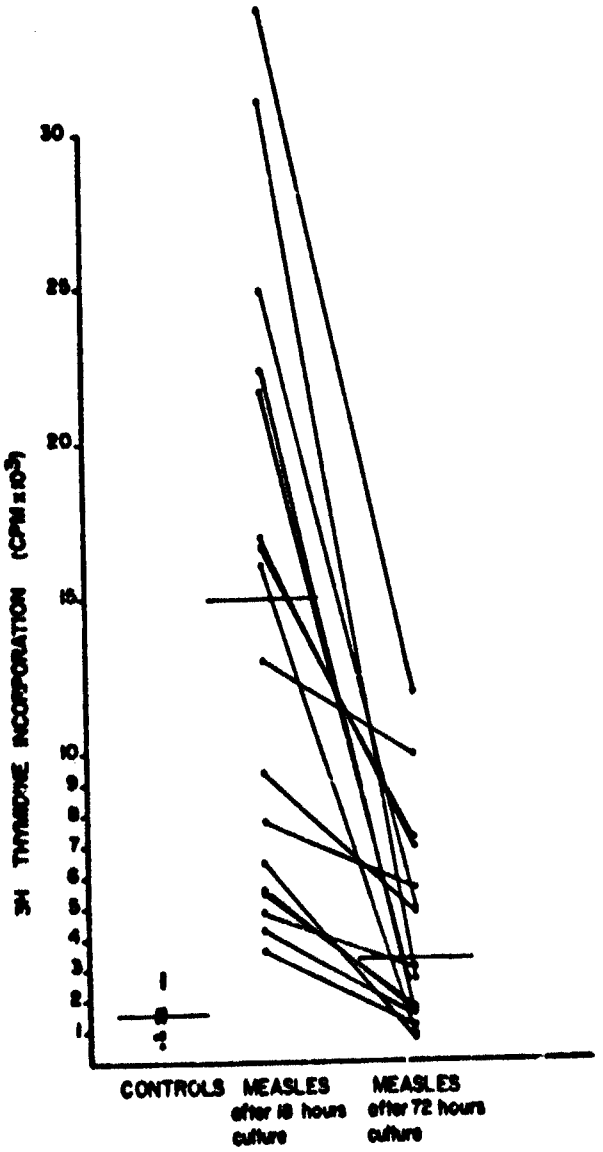

Figure 4 Spontaneous uptake of ^{3}H-thymidine by control and measles MN cells after 18 and 72 hours culture.

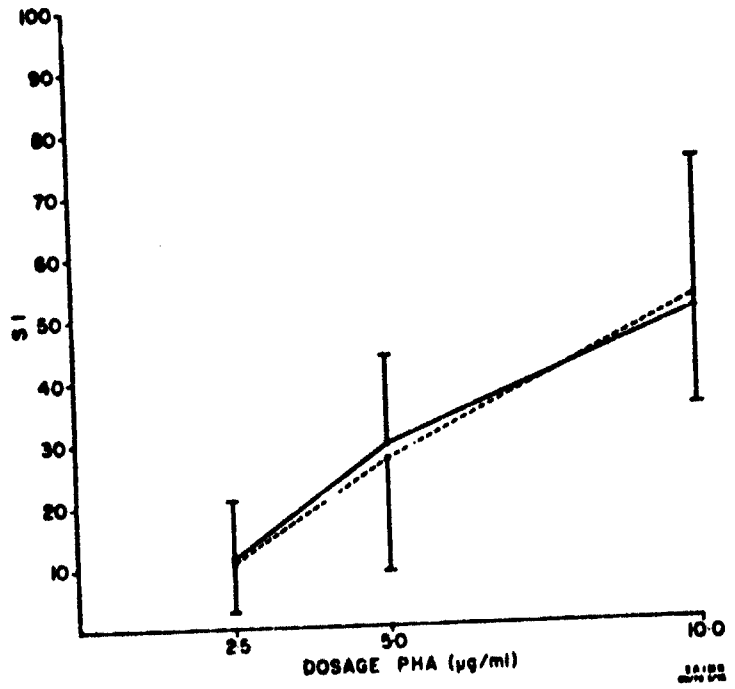

Figure 5 Activation of control (——) and measles MN (---) cells by different concentrations of PHA (Mean ± S.E.M. of 8 experiments).

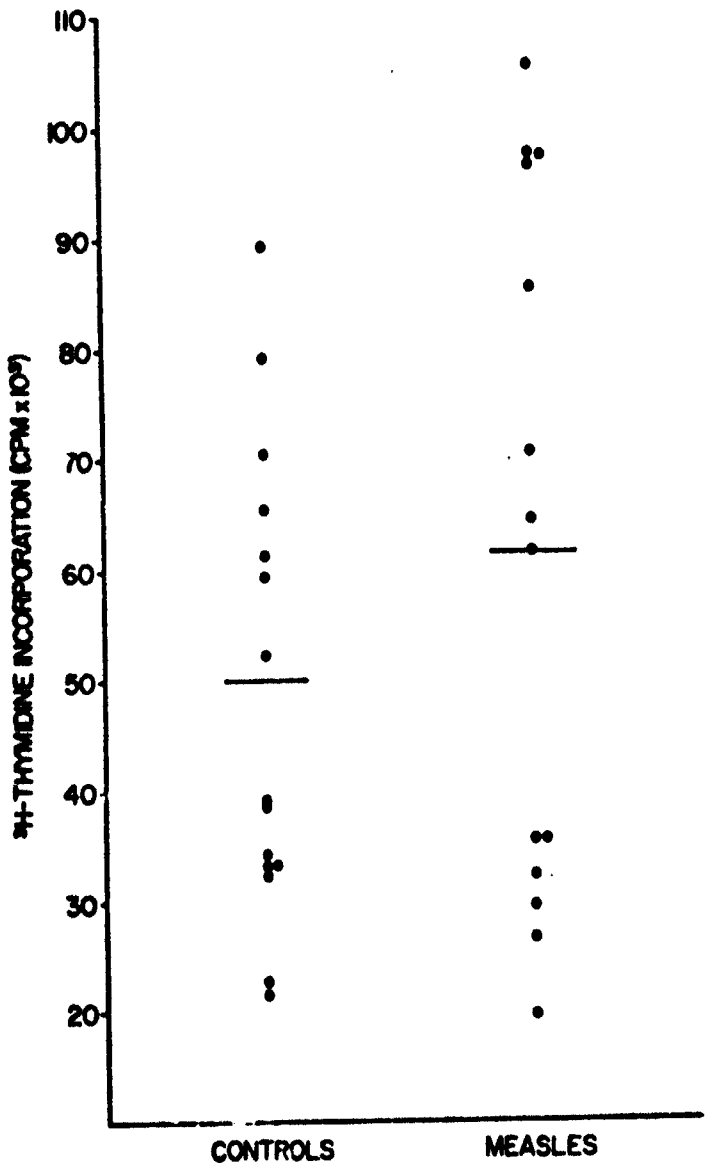

Figure 6 ^{3}H-thymidine incorporation of control and measles MN cells 72 hours after activation with PHA (10µg).

TABLE 3a

THE EFFECT OF UNSTIMULATED MN CELL CULTURE SUPERNATANTS ON NORMAL LYMPHOCYTE TRANSFORMATION

	Mean ± SEM (cpm x 10^3)	P value
Normal Unstimulated Supernatant (n = 7)	33 738 ± 8107	n.s.
Measles Unstimulated Supernatant (n = 10)	30 954 ± 3404	

TABLE 3b

THE EFFECT OF MEASLES SERUM ON NORMAL LYMPHOCYTE TRANSFORMATION

	Mean ± SEM (cpm x 10^3)	P value
Normal Serum (n = 7)	83 490 ± 6002	n.s.
Measles Serum (n = 7)	87 935 ± 4562	

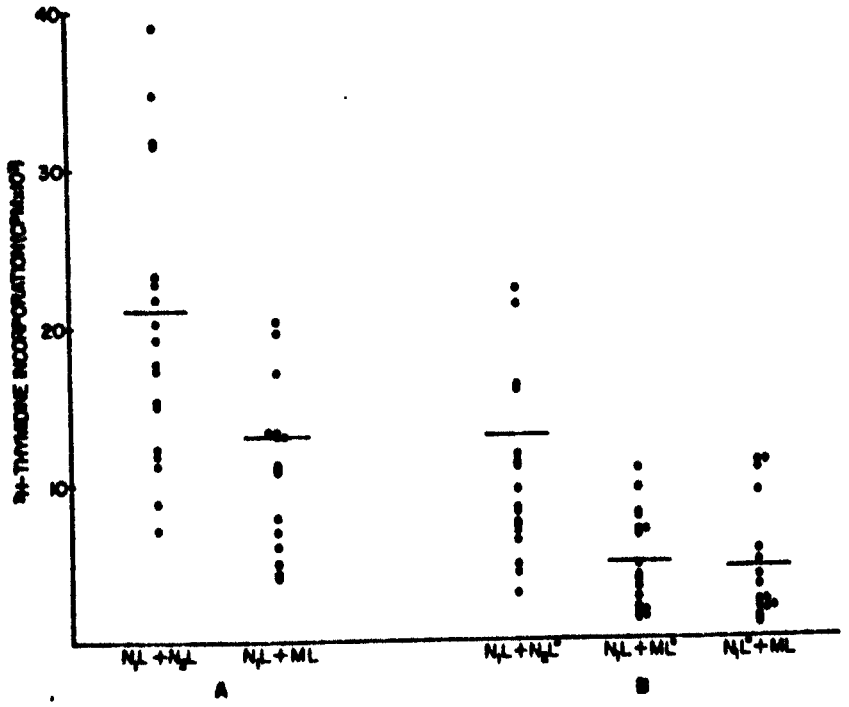

Figure 7 Two-way MLC between two different control MN cell populations (N_1L and N_2L) and between measles MN ells (ML) and one of the control populations (A).

One-way MLC between various combinations of mitomycin (*) and untreated normal and measles MN cells (B).

Figure 8 Typical 'blast'-like MN cells from measles blood. (Giemsa preparation x 1000).

DISCUSSION

The differing results obtained by various workers investigating the effect of PHA activation of MN cells from children with measles (see Chapter 4 page 37) can possibly be explained by differences of technique, doses of mitogen and even stages of clinical disease.

Although the assessment of lymphocyte blastogenesis by specific antigen, namely measles antigen, would have ideally been desired, problems in antigen preparation and batch variability (see page 31) as well as the possibility of reactivity to non-virion components and contaminating cellular antigens in crude virus preparations, would have resulted in added confusion. In a number of trial experiments using a measles complement-fixation antigen obtained commercially, no lymphocyte reactivity to a wide range of antigen dilutions was demonstrated in this laboratory, using various measles-immune blood donors. Consequently, lymphocyte transformation was subsequently assessed after mitogenic stimulation of MN cells. The PHA induced transformation of measles MN cells were found to be adequate, and at sub-optimal doses of mitogen, normal ^{3}H-thymidine incorporation was generally demonstrated. It must be emphasised that all patients used in this study were free of secondary infections and all were tested during the first three days of appearance of the rash. Because of the suppressive

effects of protein calorie malnutrition on cell- mediated immunity, as demonstrated by depressed mitogen induced lymphocyte transformation *in vitro* (Burgess *et al* 1974), it was important to exclude any of the latter type of children from this study.

Results similar to this study were obtained by Finkel and Dent (1973). Peripheral blood was, however, cultured without separation and then stimulated with an optimal concentration of PHA (40 µg/ml) as well as a sub-optimal PHA concentration (2 µg/ml). The average response at optimal PHA concentration in acute measles did not differ significantly from the response of normal controls, while at sub-optimal PHA concentration, the response was 20% of normal. These authors felt that the quantitative depression of PHA-responsiveness in the lymphocytes of patients with acute measles infection was perhaps a result of th effect of the measles virus on the function of the monocytes, as a previous study (Oppenheim *et al* 1968) reported that the *in vitro* blastogenic response of monocyte depleted lymphocytes to specific antigens and to low doses of PHA was impaired as compared to that of unpurified lymphocytes, while the response to optimal concentrations of PHA was not impaired in cultures of purified lymphocytes. This latter explanation, cannot, however be supported by the findings of Sullivan *et al*, (1976b), who showed that only less than one percent of monocytes from measles-immune and non-immune MN cells could be infected with measles virus *in vitro*. Furthermore, monocytes that differentiated into

macrophages also did not show any striking difference in their ability to replicate measles virus.

Hicks *et al* (1977) found that measles infection of rhesus monkeys, slightly suppressed mitogen responsiveness of circulating lymphocytes in the second and third week when compared to non-infected controls, but that no suppression of lymph node cells could be demonstrated. Circulating lymphocytes displayed an enhanced response to mitogens during the period of maximal virus replication, and lymph node lymphocytes, from which maximal viral titers were obtained, did not display any suppression. Suppression of responses to mitogens has indeed been shown when lymphocytes are infected *in vitro* with much higher titer of measles virus that occurs in *in vivo*. (Sullivan *et al* 1975a). The fact that mitogen stimulation during viremia was not suppressed seems to indicate that measles virus may alter the circulatory pattern of lymphocytes by some unknown mechanism and transiently deplete lymphocyte subpopulations in the circulation without affecting mitogen responsiveness of circulating T cells.

One of the most puzzling findings of this study was the failure of measles MN cells to respond normally after allogeneic cell stimulation. Not only did measles MN cells function as poor responder cells, but their stimulatory capacity on normal allogeneic MN cells was also depressed. Mangi *et al* (1974) found depressed lymphocyte reactivity *in vitro* to PHA as well as to allogeneic lymphocytes during the acute phase of infectious mononucleosis (IM), while Twomey (1974) found normal lymphocyte PHA responsiveness but

hyporesponsiveness in the mixed lymphocyte reaction of 10 patients with IM. This depression of the mixed lymphocyte reaction seen in patients with IM was explained by the granulocytopenia seen in these patients, being accompanied by a lack of monocyte precursors among the cultured leucocytes. This monocytopenia could have been of sufficient magnitude to interfere with the macrophage-dependent lymphocyte responses *in vitro*. In general, however, it has been shown that the numbers of all major cell types decrease during acute measles infection (Black and Sheridon, 1967). Morphological assessment of Ficoll-Hypaque measles MN cells generally showed adequate numbers of monocytes to be present, thus excluding the possibility that insufficient numbers of monocytes was the cause of the depressed MLC reactions observed in this study.

Lucas *et al* (1978) studied the role of monocytes on the measles-induced suppression of lymphocyte functions *in vitro*. Lymphocytes were infected with measles virus and stimulated with irradiated allogeneic cells in the presence of human serum containing antibodies against the virus. This serum thus prevented spreading of the infection to the monocytes present in the suspension of stimulator cells. Under these conditions the mixed lymphocyte reaction was still inhibited considerably, demonstrating that the presence of irradiated non-infected monocytes did not counteract the inhibitory activity of the measles virus. Similarly, lymphocytes which were infected with virus and then themselves irradiated were tested

for their stimulatory capacity with different unrelated responding cell suspensions. The stimulatory capacities of infected lymphocytes were once again diminished in agreement with the results obtained in the present study. Although no ready explanation for this phenomenon is available, it can nevertheless be concluded that B lymphocytes can become infected with measles virus, as it has been demonstrated that only B lymphocytes can serve as stimulator cells in the MLC (Oers and Zeijlemaker, 1977).

A more likely explanation for the observations seen in the present study is that put forward by Dorkin *et al* (1975) who suggested that different sub-populations of lymphocytes responded to mitogenic and allogeneic stimuli in human immunodeficiency disease. Furthermore, measles virus has been detected in unstimulated MN cell supernatants from patients with measles (see Chaper page). It is possible, therefore, that infection of the normal responding populations could have occurred during the period of culture. Cytoplasmic membranes of measles infected cells have the capability of fusing easily with adjacent uninfected cells, thus enabling the virus to pass from cell to cell (Agnarsdottir, 1977). Another possibility was that the virus might somehow obscure serologically defined human histocompatibility antigens (HLA), thus accounting for the observations that measles MN cells were both poor responders and stimulators in the one-way MLC. This explanation was, however, discounted by the elegant experiments of Haspel *et al* (1977) who studied the interaction of HLA antigens with measles virus antigens expressed

on the surface of a variety of cultured human lymphoblastoid cells. During the course of measles virus infection, no quantitative or qualitative alterations in surface HLA antigens were observed. In contrast, infection with polio virus type 1 or vesicular stomatitis virus, or treatment with puromycin resulted in a significant decrease in surface HLA, suggesting that an inhibition of host protein synthesis rather than the insertion of virus-specified antigens into the membrane results in a net decrease in amounts of this cell surface antigen. The HLA antigens also appeared to be both functionally and structually distinct from measles virus surface antigens. Furthermore, pretreatment of cells with HLA-directed antibody did not prevent the infection of these cells by measles virus, thus proving that HLA antigens were unrelated to the measles virus receptor site on the plasma membrane. Electron microscopic studies also revealed that measles virus maturation occurred at membrane sites devoid of demonstrable HLA, and HLA antigens could also not be detected on the surface of mature infectious virions.

During the course of experiments to determine the response of measles MN cells to PHA activation, it was noted that increased spontaneous uptake of ^{3}H-thymidine occurred. Similar increased spontaneous transformation has been reported by others as well. In accordance with the results of this study, Finkel and Dant (1973) found that MN cells from all patients with classical measles

had showed a markedly elevated spontaneous ^{3}H-thymidine uptake. A significant decrease in spontaneous DNA synthesis was observed in patients in convalescence (8 - 63 days after the onset of the rash) although this was still significantly higher than normal control values. Mangi *et al* (1974) found that during the first two weeks of acute IM, when cutaneous anergy was present, there was a significant increase of spontaneous *in vitro* DNA synthesis of peripheral blood lymphocytes. The level of ^{3}H-thymidine incorporation of unstimulated cells returned to normal over a period of three weeks. Kantor (1975) demonstrated that following measles vaccination of children, peripheral blood MN cells spontaneously incorporated increased levels of ^{3}H-thymidine. At the same time, both *in vivo* and *in vitro* unresponsiveness to Candida antigen occurred.

It would appear from the preceding discussion that DNA-synthesizing cells may enter the blood in response to an antigenic stimulation, namely measles virus infection. It is likely that the elevated spontaneous ^{3}H-thymidine uptake represents an *in vivo* lymphocyte response to membrane associated measles antigen. These dividing cells are now capable of replicating virus explaining the *in vitro* finding of measles virus in 24-hour MN cell culture supernatants (see Chaper page). After 72 hours in culture, the decrease in ^{3}H-thymidine uptake observed may be interpreted to mean that these lymphoblasts are now completely infected with measles virus, and such infection will nowinhibit DNA synthesis by a non-cytolytic mechanism (Sullivan *et al* 1975a).

Numerous blast-like cells were observed in 24 hour cultures of measles MN cells (Fig. 8) similar to the 'atypical lymphocytes' seen by Horwitz *et al*, (1970) who studied spontaneous DNA-synthesising cells by autoradiographic methods in various viral and inflammatory diseases. Although the spontaneously transforming cells in measles have not been positively identified, similar cells in IM had sheep erythrocyte receptors as well as human T-lymphocyte specific antigens and also lacked B-cell markers. (Pattengale *et al*, 1974). Furthermore, Huber *et al*, (1975) identified the spontaneously transforming cells found in Hodgkin's disease as T-lymphocytes, by combining both autoradiography after *in vitro* incubation with ^{3}H-thymidine, and spontaneous rosette formation with unsensitized sheep erythrocytes. Kantor (1975), on the basis of experiments performed with lymphocytes from anergic patients and experimental animals, suggested a feed back suppression by lymphocyte products as an explanation for anergy. He proposed that due to an antigenic stimulus, an immune reaction in the host is induced, followed by stimulation of suppressor T cells which then release a substance(s) that inhibit cell-mediated immunity. The results presented in this study cannot support this theory, however. Unstimulated measles MN cell supernatants were incapable of suppressing ^{3}H-thymidine incorporation by normal lymphocytes confirming the results of Sullivan *et al*, (1975a) who indicated that lymphocytes infected *in vitro* with measles virus, did not release viral induced inhibitors. Results which will be presented in the next chapter indicate that unstimulated measles MN cell supernatants also did not suppress lymphokine production.

SUMMARY

Mononuclear cells from children with acute measles were investigated for their ability to incorporate ^{3}H-thymidine after activation with PHA and after allogeneic activation in mixed lymphocyte culture. Measles MN cells incorporated elevated levels of ^{3}H-thymidine without prior stimulation over a period of 18 hours. Further culture for 72 hours, however, resulted in substantially less ^{3}H-thymidine incorporation. PHA activation of measles MN cells resulted in adequate stimulation over a number of mitogen concentrations. In contrast, allogeneic stimulation of measles MN cells by control MN cells in the two-way MLC, resulted in decreased ^{3}H-thymidine incorporation. Further experiments revealed that not only were measles MN cells poor responders, but also poor stimulators in the one-way MLC.

The presence of inhibitors of PHA-induced lymphocyte transformation were not found in both the serum or culture supernatants of measles patients.

CHAPTER 7

Lymphokine Production by Measles Mononuclear Cells as a Correlate of Cell-Mediated Immunity

A. Lymphokine Production by measles mononuclear cells as a correlate of cell-mediated immunity

Introduction

The technique of packing cells in capillary tubes and observing the effect of a test material on their migration out of the tube was introduced by George and Vaughan in 1962. Using this technique considerable information has accumulated, which has enhanced the understanding of the test mechanism and has contributed to the exploration of the mediator field. David (1966) showed that a soluble factor was elaborated by sensitized lymphocytes upon stimulation by specific antigen. This factor (MIF) inhibited the migration of normal guinea-pig peritoneal exudate cells out of capillary tubes and its production closely correlated with the *in vivo* state of cellular hypersensitivity of the host. Søborg and Bendixen (1967) adopted the capillary migration technique for the studies of human peripheral leucocyte migration. According to their method human peripheral blood leucocytes are collected after spontaneous sedimentation of the blood. The washed cells are aspirated into capillary tubes which are sealed, centrifuged, cut at the cell-media interface and placed in chambers. The chambers are filled with the various media, sealed and incubated for 24 hours at 37°C. The migration areas are then projected, traced and measured by planimetry. In

their initial studies, these authors reported that a particulate preparation of Brucella antigen inhibited the migration of leucocytes obtained from subjects with delayed hypersensitivity to Brucella, as indicated by a positive skin test.

For assessing cell-mediated immunity employing cell migration techniques, two systems are available, the direct, one-stage method and the indirect, two-stage technique. The direct method relies upon the presence of antigen or mitogen together with lymphocytes and indicator migrating cell in the same system. Mediator production and its effect on migration are assessed simultaneously. In the indirect assay, lymphocytes are first cultured with antigen or mitogen to induce mediator production, cell free supernatants are collected and there are assayed separately on the appropriate indicator cells. For both systems, 20% inhibition or greater is usually statistically significant and indicative of LIF or MIF production. The main advantage of the direct test is the short time needed (18- 24 hours) to obtain results and the relatively small volumes of blood required. The disadvantages are numerous. The cell population used is heterogenous, the relative ratio between the different cell types varies from person to person and standardization is difficult. Dissecting the test into two distinct parts has overcome most of the abovementioned difficulties. The use of distinct cell populations in each step enables a more

accurate determination of defects and makes standardization easier. The disadvantages associated with the two-stage test are mainly technical. It requires larger quantities of blood, is time consuming and calls for strict sterility.

An agarose plate method introduced by Clausen (1972), has the advantage of requiring smaller amounts of cells and antigens and of being highly reproducible and simple to perform. In this technique, the cells are incubated for 30 minutes with or without the antigen or with control and active supernatants and then inserted into wells punched in an agarose gel layer in a Petri dish. The cells migrate between the gel and the bottom of the Petri dish. After 24 hours incubation at 37^{o}C the area of migration is projected and traced and inhibition of migration is calculated. Until 1973, most migration experiments involved the use of guinea-pig peritoneal exudate cells as indicator cells but it was soon apparent that human buffy-coat cells could similarly be used. Recently it has been shown that PHA-induced inhibition of peripheral blood leucocytes was a useful indicator of cell-mediated immunity. (Morison, 1974). Lomnitzer *et al*, 1975 developed a modified two-stage migration system using supernatants obtained from mitogen activated lymphocytes. Lymphokine-containing culture supernatants were obtained following a 2 hour pulse with PHA (see following section) and cultured for 72 hours. These

supernatants inhibited the migration out of capillary tubes of both human PMN and guinea-pig PEC, but failed to inhibit human MN cells. Using ultra-filtration techniques it was shown that the active supernatant contained two inhibiting factors. The one with a molecular weight between 50 000 and 75 000 inhibited PMN but not PEC, whereas the other with a molecular weight of between 15 000 and 50 000 was specific for guinea-pig PEC and failed to inhibit PMN. These results confirmed those of Rocklin (1974) who used antigen-stimulated supernatants to demonstrate that MIF was distinct from LIF.

Migration assays have been applied by a number of authors to assess cell-mediated immunity in various viral diseases. Not withstanding the doubts expressed concerning the significance of migration tests using measles antigens (see chapter 4, page 42), Utermohlen and Zabriskie (1974), and Utermohlen *et al*, (1975) have used this test to show sensitivity to measles antigen in multiple sclerosis. Anders and Natvig (1976) used the indirect agarose technique to measure the response of human MN cells to several viruses and demonstrated in the case of mumps virus and PPD, close correlations between the degree of migration inhibition and the size of the skin reactions. Cell-mediated immunity to Epstein-Barr virus (EBV) was found by Lai *et al*, (1977) to be absent or depressed in patients with acute IM while O'Reilly *et al*, (1977) found that LIF production in patients

with recurrent herpes simplex virus (HSV) infection was depressed at the time of and immediately before the viral - induced vesicular eruptions.

Most *in vitro* studies of cell-mediated immunity in measles involved the measuring of mitogen and specific antigen induced proliferation of measles patients lymphocytes. The study of soluble mediator production which also reflects the state of cellular immunity was to some extent neglected, largely because of the lack of a reproducible micro-migration inhibition technique that would overcome the difficultyof harvesting sufficient MN cells from the blood of small children. In the present study, such a micro-migration inhibition technique was perfected and applied to measles patients in order to supplement the results obtained initially using a micro-lymphocyte transformation test. In addition, where cell numbers allowed, the migration inhibition of guinea-pig PEC (MIF) was measured.

MATERIALS & METHODS

Patients and Controls

Peripheral blood MN cells were obtained from Ficoll-Hypaque gradients as previously described (page 62) and resuspended at a final cell concentration of 2×10^6/ml in complete MEM.

Preparation of Indicator Cells

Guinea-pig Peritoneal Exudate Cells (PEC)

Normal, randomly bred guinea-pigs weighing more than 500g were injected with 25ml of light sterile paraffin oil. Three days later they were killed by cardiac puncture and PEC were harvested by peritoneal lavage with cold HBSS. The PEC were then resuspended in MEM + 15% FCS at a concentration of 3×10^7 cells/ml.

Human Polymorphonuclear Cells

Human PMN were obtained from a Ficoll-Hypaque gradient as previously described (page 63). A final cell concentration containing 13 - 15 $\times 10^6$ PMN cells/ml in MEM was prepared.

Lymphokine Production

Two million peripheral blood MN cells were incubated in the presence of (active supernatant) and in the absence (control supernatant) of 10 µg/ml PHA in plastic tissue culture tubes for 2 hours at 37°C. After the 2-hour pulse, the cells of

both the active and control cultures were washed three times in MEM and then resuspended to their original volume in complete MEM. The cultures were then incubated at 37°C in 5% CO_2 in air for 72 hours. The cell-free supernatants from unstimulated and PHA-pulsed lymphocyte cultures were obtained by centrifugation at 250g for 10 mins. and the supernatants stored at -20°C until assayed for lymphokine activity.

To assess LIF production during MLC, 1 x 10^6 measles MN cells were incubated with an equal number of mitomycin C - treated (30 minutes at 37°C) control MN cells for 120 hours at 37°C in 5% CO_2 in air. As a control, two histoincompatible adult donors were used; the one donor's MN cells being similarly treated with mitomycin C. After incubation, supernatants were collected and stored as described before.

Leucocyte Inhibitory Factor Assay (LIF)

A. Preparation of agarose medium :

LIF was assayed using a modified agarose gel technique (Clausen, 1972). (See Fig. 9). The agarose plates were prepared as follows :

1. 0.75g agarose was dissolved in 8ml of sterile water by boiling for about 15 minutes.

2. The dissolved agarose solution was allowed to cool to 45°C in a waterbath.

3. The following supplements were added to 8ml of agarose :

   (a) 1 ml horse serum

   (b) 1 ml 10 times concentrated TC-199 tissue culture medium and

(c) 0.1 ml 4.4% sodium bicarbonate solution.

4. After addition to the dissolved agarose, 5ml volumes of the complete agarose solution were transferred to 60 x 15 mm plastic Petri dishes on a level surface.

5. The agarose was allowed to gel at room temperature for about 20 minutes followed by a 10 minute period at 4°C.

6. 2.5 mm diameter wells were punched in the gel by means of a gel-cutter and plastic template (12 wells per dish). Care must be taken not to score the bottom of the dishes with the punch otherwise cell migration will be inhibited. The agarose plugs are then removed with a 26-gauge hypodermic needle, carefully avoiding distortion of the wells.

7. Before use the dishes are incubated at 37°C in a humidified atmosphere of 5% CO_2 in air thus allowing the pH of the agarose to adjust to between 7.2 and 7.4

8. 5 minutes before filling the wells with the PMN cell suspension, the agarose dishes are removed from the incubator and any condensation sucked from the wells by means of a small capillary tube attached to a mouth pipettor.

Agarose dishes may be stored for up to one week before use at 4°C. Prior to use, these dishes are incubated in a CO_2 incubator at 37°C for at least one hour in order to adjust the pH to 7.4.

B. Assay of Control & LIF Supernatants

Purified PMN cell suspensions (13 - 15 x 10^6) are gently centrifuged (500 rpm); the supernatants discarded and the cells resuspended in 0.05 ml of control or active supernatants. The cell suspensions are incubated in a 37°C waterbath for 30 minutes with occasional shaking and 0.005 ml aliquots distributed in triplicate into wells cut in the agarose.

C. Determination of migration inhibition

After 16 - 18 hours of incubation at 37°C in 5% CO_2 in humidified air, migration areas were fixed to the plastic by either of two methods :

1. the agarose plates were flooded with about 2ml of methanol and allowed to incubate at room temperature for about 30 minutes. The methanol was gently poured off and replaced with 2ml of 35% formaldehyde for another 30 minutes. The formaldehyde was then discarded and the agarose layer removed by vigorously flicking the plate in a downward movement. If the migration pattern could not be fixed immediately, dishes were flooded with 2ml methanol and left at 4°C and then treated as above.

2. water in a beaker was heated to between 80-90°C; an agarose dish is floated on the surface of the water for 5 minutes, and the dish is then immersed in the

water, removed from the beaker and allowed to stand for 5 mins and then dipped in cold water so that the agarose will float off the plastic.

After fixing, the migration areas are stained with 0.5% crystal violet for 5 minutes and then washed with tap water. Migration patterns are then projected (see Fig. 9) traced on to paper and the area of migration measured by planimetry. The migration index (MI) was calculated as follows :

$$MI = \frac{\text{average area of migration in the presence of LIF containing supernatant}}{\text{average area of migration in the presence of control supernatant}}$$

Migration index of less than 0.80 was considered to demonstrate *in vitro* LIF production.

Macrophage Migration Inhibition Assay (MIF)

Fifty-microliters of the cell suspension to be assayed was drawn into siliconized capillary tubes which were then sealed with plasticin and centrifuged at 300g for 5 minutes. The tubes were cut at the cell-fluid interphase and the capillary stumps containing the packed cells were placed on the floor of a migration chamber and held in place by a drop of silicon grease. For testing the effect of supernatants on the migration pattern

of the indicator PEC, 0.5 ml control and active supernatants were used to fill the chambers. The chambers were then sealed with sterile glass coverslips and incubated at 37°C in 5% CO_2 in air for 40-42 hours. The migration pattern (see Fig. 12) was projected and traced and the area of migration measured by planimetry. The migration index was calculated as described for LIF activity.

Spontaneous LIF production

Unstimulated measles and control MN cells were cultured in plastic tissue culture tubes for 18 hours at 37°C in 5% CO_2 in air. The supernatants were removed by centrifugation and tested for LIF activity as previously described. Results were expressed as follows :

$$MI = \frac{\text{Area of migration in presence of 18 hour unstimulated measles supernatant}}{\text{Area of migration in presence of 18 hour unstimulated normal supernatant}}$$

MN cells from normal donors were suspended in complete MEM, pulsed for 2 hours with PHA, washed 3 times in HBSS and cultured at 37°C for 72 hours in a 1 : 1 mixture of complete MEM and either normal or patient's control supernatant. Supernatants were then collected and tested for LIF activity as previously described.

Measurement of C-Reactive Protein (CRP)

Equal volumes of patient serum or PHA activated MN cell supernatants and latex CRP reagent were mixed at room temperature and the agglutination pattern read after 5 minutes. Both positive and

negative CRP sera were used as controls.

Statistical analysis

In all studies statistical significance was determined by the Student's t-test. A p value greater than 0.05 was considered not significant.

RESULTS

Response to PHA

In 31 out of 37 cases, MN cells from children with measles, failed to produce adequate amounts of MIF (See Fig. 10a). Whereas the mean MI for the control group was 0.69 ± 0.08, for the measles group it was 0.91 ± 0.11 ($p < 0.001$).

Supernatants derived from PHA-activated MN cells were tested for additional lymphokine activity using a two-stage MIF assay. As can be seen in Table 4, supernatants from measles MN cell cultures did not inhibit the migration of non-sensitized guinea-pig PEC.

Response to allogeneic lymphocytes

When LIF assays were performed on the supernatants of one-way mixed lymphocyte cultures, minimal LIF activity was detectable in cultures of measles MN cells responding to mitomycin-treated control cells (Fig. 11). The mean MI of supernatants from control responding cells was 0.73 ± 0.05, whereas from measles cells it was 0.99 ± 0.12 ($p < 0.001$).

Spontaneous LIF production

Although unstimulated measles MN cells incorporated increased amounts of ^{3}H-thymidine they did not simultaneously produce LIF after 18 hours culture (Table 5(a)).

The effect of unstimulated MN cell supernatants on LIF production by Control MN cells

Control supernatants (unstimulated) from measles MN cells had

no inhibitory effect on normal MN cell activation by PHA as measured by LIF production (Table 5(b)).

C-Reactive Protein

Of the 20 measles sera tested for CRP, only 1 showed any appreciable agglutination. None of the PHA activated measles MN cell supernatants showed any evidence of CRP.

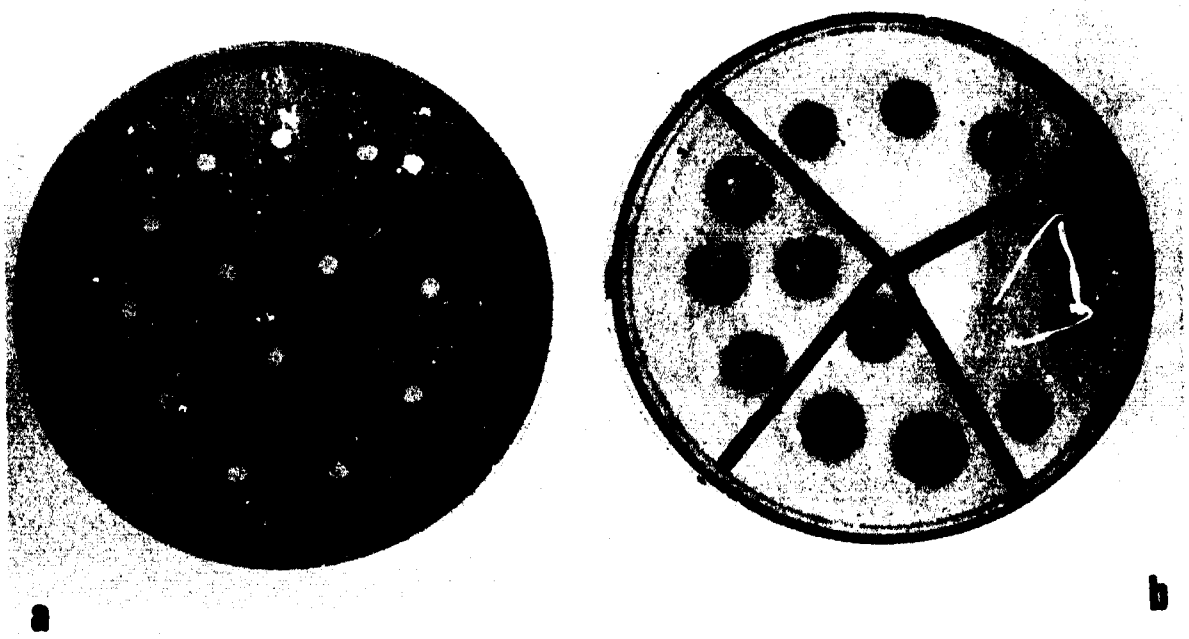

Figure 9 Agarose migration plates used for the determination of LIF. (A) wells cut in the agarose gel and (B) PMN cells migrating in the presence of control and active supernatants.

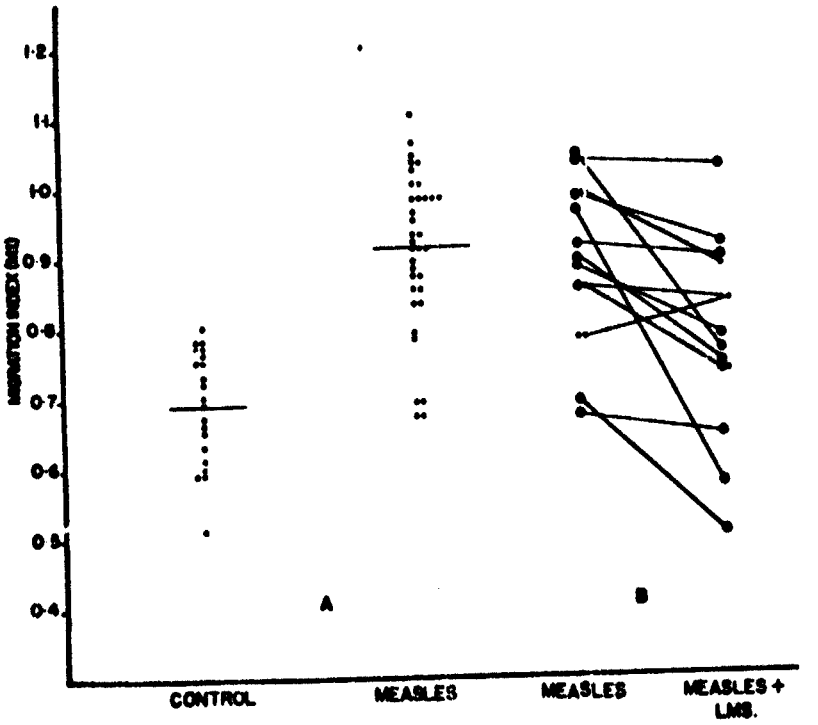

Figure 10 Production of LIF by control and measles MN cells after PHA activation (A). Before and after *in vitro* levamisole (10^{-3}M) treatment (B).

TABLE 4

PRODUCTION OF MIGRATION INHIBITION FACTOR (MIF)
BY CONTROL AND MEASLES MN CELLS

	Migration Index (M.I.) ± SEM	P value
Measles (n = 10)	0.87 ± 0.13	p< 0.005
Controls (n = 10)	0.66 ± 0.08	

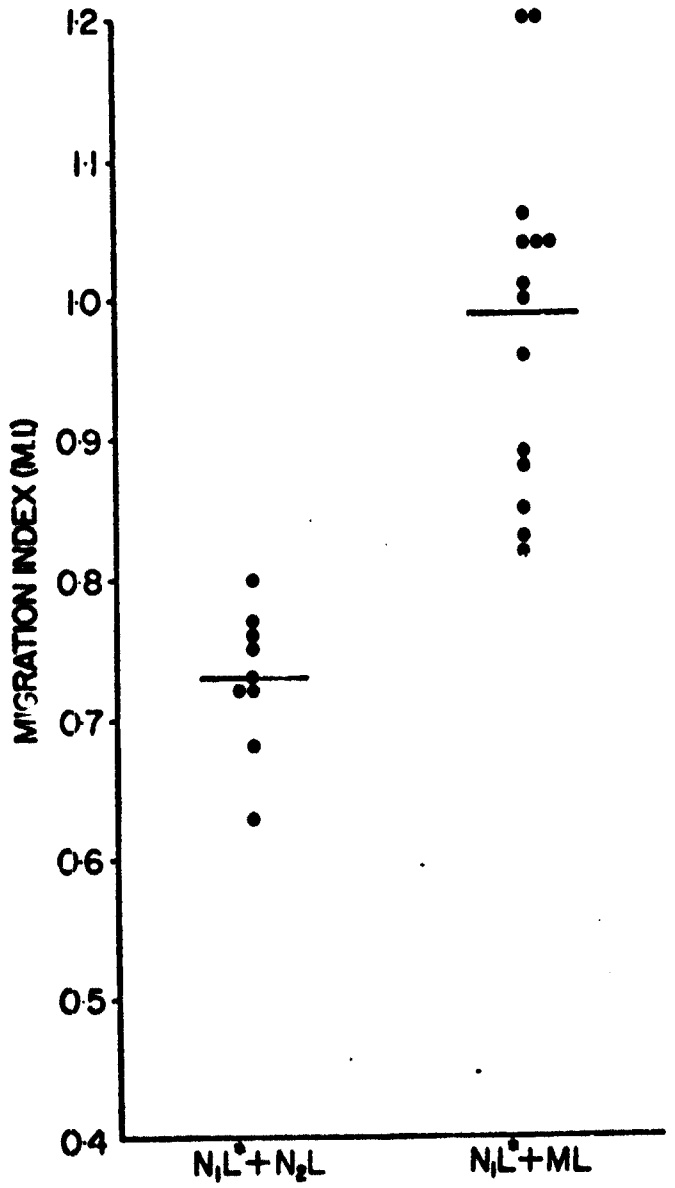

Figure 11 Production of LIF by control and measles MN cells activated by mitomycin-treated (*) allogeneic cells.

TABLE 5a

THE ABILITY OF UNSTIMULATED MEASLES MN CELLS TO PRODUCE LIF AFTER 18 HOURS INCUBATION

Patient	Unstimulated LIF Production Migration index (MI)
V.S.	0.97
K.C.	1.12
S.A.	0.91
A.vS.	0.89
B.B.	1.00
R.B.	0.91
D.P.	0.97
G.H.	0.97
A.F.	0.90
P.S.	0.90

TABLE 5b

THE EFFECT OF UNSTIMULATED MN CELL SUPERNATANTS ON LIF PRODUCTION BY PHA PULSED CONTROL MN CELLS

	Mean M.I. ± SEM*	P value
Normal unstimulated (control) supernatant (n = 7)	0.65 ± 0.02	N.S.
Measles unstimulated (control) supernatant (n = 10)	0.69 ± 0.03	

* MI $\leq$ 0.80 constitutes a positive LIF assay

Figure 12 Migration of guinea-pig PEC in the presence of control (A) and active (B) supernatants from capillary tubes.

DISCUSSION

Generation of lymphocyte effector molecules (lymphokines) *in vitro* by human lymphocytes provides an important tool in the immunological evaluation of patients with defective cellular immunity. Rocklin (unpublished data) observed that MIF and LIF production *in vitro* correlated qualitatively well with *in vivo* skin tests but not quantitatively. That is, the amount of inhibition of migration did not correlate with the size of the skin test. Despite the finding of adequate ^{3}H-thymidine incorporation following PHA activation (see Chapter 6 page 67), MN cells from children with measles were incapable of producing the lymphokines LIF and MIF, neither after PHA stimulation, nor in MLC. Previous workers have suggested that lymphokines may be produced independent of DNA synthesis and cellular proliferation. Rocklin and Ratcliff (1972) presented evidence for two lymphocyte populations, one producing MIF and another proliferating in response to antigen, while Rühl *et al* (1974) demonstrated that human peripheral blood lymphocytes stimulated *in vitro* with PHA produced a monocyte chemotactic factor that preceded the blastogenic response. Górski *et al* (1976) studied Con A-induced LIF production in patients with primary and secondary immuno-deficiency disease. Although in most cases immunological abnormalities were manifested by both depression of lymphocyte transformation and mediator production, there were situations in which lymphocytes could

produce LIF in the presence of poor blastogenesis or *vice versa*. Moreover, in some cases, mitogen-induced LIF responses differed from the MLC-LIF response. This data would then support the view of Dorkin *et al* (1975) that different cell subsets respond with DNA synthesis and lymphokine production. It was, therefore, relevant to study the response of lymphocytes in terms of lymphokine production in parallel with the evaluation of the proliferative response to gain a better understanding of the mechanism of anergy found in measles.

With regard to viral diseases, O'Reilly *et al* (1977) described a dissociation between lymphocyte proliferation and lymphokine production in patients with recurrent herpes simplex infections. Using an ultra-violet inactivated herpes simplex virus antigen (obtained from the supernatant of infected Vero cells), virus-specific lymphoproliferative responses were regularly detected in patients with recurrent infection irrespective of the clinical stage of infection. In contrast, transient deficiencies in herpes-specific LIF and interferon production was regularly documentated at the time of and immediately before herpes simplex-induced vesicular eruptions. LIF production was assayed by both the direct and indirect agarose migration methods. The production of LIF from blood lymphocytes was also studied in patients with infectious mononucleosis (Palit *et al* 1978).

In 16 out of 17 patients, the production of LIF in response to stimulation by Con A was absent or diminished. Furthermore, in one-half of the patients, spontaneous LIF production was

demonstrated. No correlation was found between spontaneous LIF production and the number and proportion of activated lymphocytes in the blood, but at a later stage, when blood lymphocyte morphology was normal, spontaneous LIF production had practically ceased.

In view of the previously discussed difficulties experienced by various workers using measles antigen, it was decided to employ a PHA-pulse method of LIF production in the present study as the technique has proven to be both consistent and easy to perform (Lomnitzer *et al* 1975). Using a variety of commercially available viral and control antigens, i.e. mumps, herpes simplex type 1, cytomegalovirus and adenovirus, Anders and Natvig, (1977) found that only with the mumps antigen, was there reasonable correlation between migration inhibition, lymphocyte transformation and size of the skin reaction. Furthermore, in the few cases where measles antigen has been used in migration tests some evidence suggests that inhibition of migration by measles virus may be due to a leucoagglutinating effect and not truly reflect the state of cell-mediated immunity to measles. (Nordal *et al* 1976).

The children with measles investigated in this study showed a clear-cut differentiation between lymphokine production and ^{3}H-thymidine incorporation after PHA activation. Although the reasons for this disparity are not totally clear, a number of hypotheses can be afforded :

1. Specific inactivation or destruction of lymphokine-producing cells by measles virus is an attractive possibility in view of what has previously been discussed concerning the dissociation between lymphocyte proliferation and lymphokine production (Rocklin and Ratcliff, 1972, Gorski *et al* 1976, O'Reilly *et al* 1977). Osunkoya *et al* (1974a) observed measles-specific giant-cells in PHA-stimulated lymphocyte cultures obtained from children up to 7 days after the onset of measles rash. This latter finding provides evidence that during acute measles infection, lymphocytes are infected and virus can persist within leucocytes for at least a week after the appearance of neutralizing antibodies. Since stimulation of lymphocytes appears to be an essential prerequisite for the replication of measles virus *in vitro* (Sullivan *et al* 1975a) it can be hypothesized that the virus first requires a stimulated cell to suppress lymphocyte reactivity. It is conceivable, particularly in a young child whose immune system is highly activated through frequent encounters with various environmental pathogens, that the virus selectively infects and multiplies in an activated sub-population of T-lymphocytes, thus with time causing a specific unresponsiveness, manifested by the classical anergy demonstrated in children with measles. Osunkoya *et al* (1974b) found that after PHA stimulation of measles MN cells (obtained one to seven days after the appearance of the rash), giant cells containing many nuclei

were seen in the leucocytes of most patients. While viral antigen was detectable by immunoflourescence on these cells, only a minority of apparently normal blast cells were positive, indicative perhaps of a selective infection of subpopulations of circulating lymphocytes by the virus. Furthermore, it has been suggested by White and Boyd (1973) that the suppressive effects of measles virus is a result of the widespread aggregative destruction of lymphoid cells as seen in the thymus in fatal cases of measles. These aggregative cell masses in the cortex of thymuses of children who died of severe measles were similar to the giant cells in measles PHA leucocyte cultures (Osunkoya *et al* 1974b), thus leading the latter to speculate that a depletion of effective lymphoid cell populations may explain the immunosuppressive effects of measles virus. McFarland (1974) has clearly demonstrated in a mouse model that measles virus infection produces a suppression of the anti-hapten response, due predominantly to the effect of the virus on a specific lymphocyte sub-population, namely the T-helper cell. Such a mechanism might be important if certain clones of helper or suppressor cells were permanently affected by measles, for the resulting imbalance of immunological control might cause abnormally high or abnormally low responses to measles virus to persist even in the absence of further infection.

2. Selective margination or migration of virus-sensitized lymphocytes capable of producing lymphokines from the circulation to the site of infection is another possibility. If such migration does occur in measles, it is selective, since antigen-responsive lymphocytes, as assessed by proliferative responses are still detected in the circulation during the acute illness. Lymphocyte recirculation through lymphoid tissue is believed to play an important role in the induction of immunity by promoting interaction between immunologically competent cells and antigen. Interactions between effector T-cells with antigen in tissues initiates cellular immune lesions and evokes inflammation through recruitment of mononuclear cells from the bloodstream. Thus, disturbance in lymphocyte traffic could play a role in suppressing responsiveness, particularly delayed cutaneous hypersensitivity reactivity. Hicks *et al* (1977) noted that in experiments with measles virus infected monkeys, mitogen stimulation (^{3}H-thymidine uptake) during viremia was not suppressed and it was suggested that infection of lymphocytes per se by measles virus as an explanation for diminished cell-mediated immunity was not adequate. It was felt that nonspecific binding of measles to T-cells as suggested by Valdimarsson *et al*, (1975) may alter the circulatory pattern of lymphocytes and transiently deplete lymphocyte subpopulations in the

circulation, without affecting mitogen responsiveness or T-helper cell functioning of centrally located T cells. This concept has been demonstrated with Newcastle disease virus (a paramyxovirus) where traffic of recirculating lymphocytes was profoundly affected in rats inoculated with the virus. (Woodruff and Woodruff, 1974). Alterations in lymphocyte distribution were mediated by attachment of virus to the cell surface and were the same as those induced by incubating lymphocytes with neuraminidase. Although the measles virus envelope does not contain neuraminidase (see page 6), a similar alteration or destruction of lymphocyte surface components by some other mechanism may lead to abnormal distribution *in vivo*.

3. Suppression of lymphokine producing populations by virus-induced suppressor cells or suppressor factors generated during viral infection could also occur. Kantor (1975) on the basis of experiments performed with lymphocytes from anergic patients and experimental animals, suggested a feedback suppression by lymphocyte products as an explanation for anergy. Due to antigenic stimulation, an immune reaction in the host is induced, followed by stimulation of suppressor T-cells which will then release a substance(s) that inhibits cell-mediated immunity. The stimulus may be directly on the T cells or else on macrophages which will then stimulate suppressor cells either by direct contact or via a soluble mediator. The data which has been presented in this study

do not support this theory. Unstimulated measles MN cell supernatants were incapable of suppressing both ^{3}H-thymidine incorporation and LIF production by normal lymphocytes thus confirming the work of Sullivan *et al*, (1975b), who demonstrated that lymphocytes infected *in vitro* with measles virus did not release a viral-induced inhibitor since supernatants from infected PHA-stimulated cultures did not abrogate ^{3}H-thymidine incorporation by fresh PHA-stimulated autologous lymphocytes. Furthermore, no serum inhibitors were found to be present when measles serum was used in transformation experiments thus confirming the findings of Finkel and Dent (1973) who also found that the serum of measles patients did not inhibit the incorporation of ^{3}H-thymidine after PHA stimulation.

C-reactive protein (CRP), a serum constituent which rapidly increases during the acute stage of the inflammatory response, has been reported to contribute to impaired lymphocyte responsiveness *in vitro* (Mortensen *et al*, 1977). Purified human CRP was added to human lymphocytes and the results obtained were analogous to those obtained in the present study, namely, that mitogen-induced lymphokine production was inhibited with no effect on mitogen-induced proliferation. The general absence of CRP in measles sera and PHA activated measles MN cell supernatants, however, preclude this factor

as the cause of leucocyte dysfunction in measles patients.

Although the aforementioned hypotheses may not offer completely satisfactory explanations for the mechanisms of depressed lymphokine production by measles MN cells *in vitro*, the results of this study stress the importance of a full evaluation of lymphocyte function *in vitro*. Because of the abundant evidence to suggest that proliferative responses and mediator production are functions of different sub-populations of cells, only a partial answer regarding cellular immune function will be obtained if either lymphocyte transformation or lymphokine production alone is employed. Furthermore, since there can be a dissociation between lymphokine production and proliferation, one may obtain a false impression if only one test is utilised. For example, Rocklin (unpublished data) found that some anergic patients with sarcoidosis have intact lymphocyte transformation responses to mitogens and antigens but their MN cells do not produce MIF.

In view of these results and reports suggesting that the antehelminthic drug, levamisole, has a corrective effect on conditions associated with depressed cellular immunity, a study was commenced with a view to investigating the *in vitro* effect of this drug on the depressed LIF production by measles MN cells.

SUMMARY

Lymphokine production by PHA-pulsed and allogeneic activated measles MN cells was investigated. LIF production was assessed using a two-stage agarose migration technique and it was found that production of this lymphokine was grossly impaired when measles MN cells were either pulsed with PHA or stimulated by mitomycin C-treated control cells. MIF activity was assessed using guinea-pig peritoneal exudate cells in a two-stage capillary tube migration system. PHA-pulsed measles MN cells failed to produce adequate amounts of MIF.

In contrast to the elevated 'spontaneous' uptake of ^{3}H-thymidine by measles MN cells, no simultaneous production of 'spontaneous' LIF was noted after 18 hours of culture. Furthermore, supernatants of unstimulated measles MN cells did not inhibit LIF production by PHA-pulsed control MN cells.

In view of this demonstration of defective lymphokine production and reports suggesting that the antehelminthic drug, levamisole, has a corrective effect on conditions associated with depressed cellular immunity, a study was initiated to investigate the *in vitro* effect of this drug on depressed active LIF production.

B. The *in vitro* effect of Levamisole (LMS) on LIF production by measles MN cells

INTRODUCTION

Levamisole is the non-proprietary name for the hydrochloride of the laevorotatory isomer of 2, 3, 5, 6-tetrahydro-6-phenylimidazo (2, 1-b) thiazole. It is a stable white crystalline powder, soluble in water, with a molecular weight of 240.75. It has been extensively used in nematodal infestations of humans and animals with few side effects (Gatti *et al*, 1972). The capacity of LMS to stimulate cellular immune responses was demonstrated in patients with solid tumours by Tripodi *et al*, (1973). Lieberman and Hsu (1976) evaluated the potential of LMS to modulate the immune response *in vitro* in patients with congenital and acquired immunologic disorders. Enhancement of leucocyte migration inhibition to recall antigens was consistently observed in all patients. Dau *et al*, (1976) found that following one week of treatment with LMS, a group of multiple sclerosis patients was found to have increased lymphocyte stimulation responses towards four virus cell-associated antigens and increased delayed hypersensitivity responses towards a battery of skin test antigens. Furthermore, two out of four patients with SSPE who had low E-rosette numbers and who were subsequently treated with LMS, showed significantly

increased E-rosette numbers accompanied by clinical improvement (Verhaegen *et al*, 1977). Golding *et al*, (1976) have shown that LMS *in vitro* may reverse the inability of non-responding MN cells from patients with Hodgkin's Disease and other non-responsive conditions to produce LIF, and suggested that the drug could restrict the production of inhibiting factors.

In line with aforementioned findings, a study was, therefore, undertaken to assess the effect of LMS *in vitro* on measles MN cells in order to determine whether lymphokine production could be restored to normal levels.

MATERIALS AND METHODS

Patients

Fifteen of the 37 patients participating in the original studies were investigated. The control group consisted of healthy adult donors as previously described (see page 61).

LIF production by Levamisole treated and untreated MN cells

MN cells were separated from whole blood by centrifugation on a Ficoll-Hypaque gradient as previously described. The cells were washed three times in MEM and the cell count adjusted to 2×10^6/ml in complete MEM. MN cells were first incubated for 1 hour in the presence or absence of 100 µg/ml (4×10^{-4}M) of LMS at 37°C and were then washed three times in MEM and resuspended to the original volume with complete MEM. The MN cells were pulsed with 10 µg/ml of PHA for 2 hours, washed three times with MEM and resuspended to the original volume. The cell suspensions were further incubated for 72 hours. Cell free supernatants were obtained by centrifugation.

LIF Assay

LIF assay was performed as previously described (page 89).

Statistical Analysis

Statistical significance of the effect of LMS was determined by the Students t-test. A p value higher than 0.05 was considered not significant.

RESULTS

LIF production by PHA activated MN cells before and after *in vitro* LMS treatment are summarized in Fig.10b. In only 4 out of 15 measles patients, was the ability to produce significant quantities of LIF restored. These results are not statistically significant.

DISCUSSION

Elucidation of the mechanism by which LMS stimulates the immune response may have practical application in the management of a variety of immune deficient states, as well as contributing to a better understanding of the immune response. Among the possible modes of action, stimulation of either or both macrophage or lymphocyte function seems most logical. LMS can stimulate the immune responsiveness by affecting cell proliferation and/or augmenting the production of lymphocyte mediators. Most *in vitro* studies on the effect of LMS on cell-mediated immunity have concentrated on lymphocyte transformation and few studies assessing lymphokine production under the same circumstances have been performed. It was of interest, therefore, to study the effect of the drug on mediator production in anergic measles patients.

Most of the patients studied in this survey exhibited depressed cell mediated immunity as determined by impaired LIF production following PHA stimulation. A one hour pulse of the MN cells with LMS (4×10^{-4}M) prior to the PHA pulse, did not significantly restore LIF production even though similar treatment of MN cells from anergic Hodgkin's Disease patients with depressed LIF production, resulted in a marked increase and normalisation of the latter response. (Golding *et al* (1976). No significant augmentation of LIF production by MN cells of controls with initially adequate cell-mediated immunity occurred after LMS

treatment. Furthermore supernatants from MN cell cultures which were pulsed only with LMS (without further PHA pulse) did not contain LIF. Lieberman and Hsu (1976) evaluated the potential of LMS to modulate the immune response *in vitro* in patients with congenital and acquired immunologic disorders using a direct leucocyte migration test. By studying the response to antigen alone and antigen plus LMS, before and after LMS treatment, they found that only in the presence of LMS *in vitro* did they produce inhibition of leucocyte migration. Interesting results were also reported by Whitcomb *et al* (1976) who found that LMS augmented MIF and MAF (Macrophage Activating Factor) production by human lymphocytes stimulated with suboptimal concentrations of Con A, while no effect on proliferation could be detected. LMS on its own did not induce inhibitory activity and the concentration which was most effective (100 µg/ml) is in accordance with the amount used in the present study.

Golding *et al* (1976) explained the *in vitro* reversal of anergy in Hodgkin's disease and other related anergic conditions by LMS, as a selective impairment of immunoregulatory suppressor factors. MN cells from measles patients could not be significantly activated after LMS treatment *in vitro* implying a different mechanism for the immune unresponsiveness. Although the *in vitro* effect of LMS on measles MN cells have proved to be disappointing, more positive results may be shown to

occur after *in vivo* treatment. O'Reilly *et al* (1977) found that patients with recurrent herpes progenitalis on LMS treatment reported a decrease in the frequency of recurrences. Herpes-antigen-induced production of LIF *in vitro* was similarly enhanced in these individuals. In four patients reporting no improvement, virus-specific LIF generating responses were either depressed or unchanged.

SUMMARY

LIF production by PHA activated measles MN cells before and after *in vitro* treatment with levamisole, demonstrated that only in 4 out of 15 patients, was the ability to produce significant quantities of LIF restored.

Whether the restoration of lymphokine activity in the unresponsive cells of a small number of patients was a direct result of the levamisole treatment or an unassociated phenomenon, remains to be established in a larger investigation.

CHAPTER 8

Papovavirus in Measles Mononuclear Cell Culture Supernatants

Papovavirus in Measles Mononuclear Cell Culture Supernatants

INTRODUCTION

Evidence has accumulated which suggests that specific defects in cell-mediated immunity may be responsible for the appearance of human papovaviruses in detectable quantities in certain patients. In this respect papovaviruses have been reported in clinical cases of lymphoma (Zu Rhein and Varakis, 1974), leukemia (Reese *et al*, 1975), and in Wiskott-Aldrich syndrome (Takemoto *et al*, 1974) and also in a high proportion of renal transplant recipients (Lecatsas *et al*, 1973). The human papovaviruses (BK and JC) cross-react antigenically with the simian SV40 papovavirus. An important characteristic of some members of the papovavirus group is their potential for inducing tumors in certain hosts. The SV40 and to a certain extent the human papovaviruses, induce tumors following intracerebral inoculation of newborn hamsters, with the production of T (tumor) antigens (Gardner, 1971).

During the routine examination of supernatants from 24 hour measles MN cell cultures for the presence of measles virus by electron microscopy, it was noticed that an additional virus (papova) was present in the supernatants, (Lecatsas *et al*, 1976) thus prompting an investigation of this phenomenon. In addition, an attempt to demonstrate this virus in measles MN cells by means of a flourescent technique was carried out.

MATERIALS AND METHODS

Patients and Controls

Fifteen well nourished children with measles and an equal number of normal controls (as described previously) were used in this study.

Mononuclear cell culture

Control and measles MN cells were harvested from Ficoll-Hypaque gradients and washed three times in MEM. Cells were then suspended in complete MEM (2×10^6/ml) in plastic tissue culture tubes and incubated for 24 hours at 37°C in 5% CO_2 in air.

Electron Microscopy

After incubation, supernatants were separated by low speed centrifugation (1 000 rpm) and the cell pellets disrupted in distilled water. After clarification by low speed centrifugation, the cell pellet suspensions together with the original culture supernatants were centrifuged at 105 000g for 2 hours and examined for virus by negative staining electron microscopy using 3% phosphotungstic acid. Grids were viewed in a Philips EM300 electron microscope at an instrumental magnification of 42 000 and an accelerating voltage of 60 kV.

Direct fluorescent staining of measles mononuclear cells for the presence of Papovavirus T-antigen

Measles and control MN cells (0.5×10^5) were seeded on polytetrafluoroenthylene-coated multispot slides, air-dried and fixed in 95% methanol for 1 minutes and treated as follows :

1. A 1:4 dilution of hamster anti-SV40-T-antigen antiserum (kindly supplied by Professor O.W. Prozesky) was added to the cells for 30 minutes at room temperature.
2. Slides were washed twice in phosphate buffered saline (PBS) for 10 minutes.
3. A rabbit anti-hamster antiserum was then added to the cells at a dilution of 1:5 for 30 minutes at room temperature.
4. The slides were washed twice in PBS for 10 minutes.
5. Finally, fluorescent sheep anti-rabbit antiserum (diluted 1:10) was added to the cells for 30 minutes at room temperature.
6. The slides were washed again in PBS and mounted in a PBS-glycerol mixture (1:1) and viewed under a high power fluorescent microscope (Ortholux 2).

A positive control, consisting of human foetal fibroblasts infected with papovavirus and expressing the T-antigen (supplied by Professor O.W. Prozesky) was handled in a similar fashion.

RESULTS

Lymphocyte culture supernatants from 15 measles patients and normal controls were studied. Typical 43 nm papovavirus particles were detected by electron microscopy (Fig. 13) in two of the measles patients. The abundance of particles in both the cell suspension and the supernatant fluid suggested that they represented progeny virus and not contaminants from the patients serum.

Fluorescent staining of both measles and normal MN cells consistently failed to demonstrate papova T-antigen although the positive control (infected fetal fibroblasts) displayed the characteristic nuclear fluorescence of the papova T-antigen. It must be stressed, however, that the anti-T antiserum was only acquired some time after the demonstration of papovavirus in measles MN cell suspensions by electron microscopy and it was, therefore, not possible to simultaneously stain the MN cells from the two papovavirus positive patients with the anti-T antiserum.

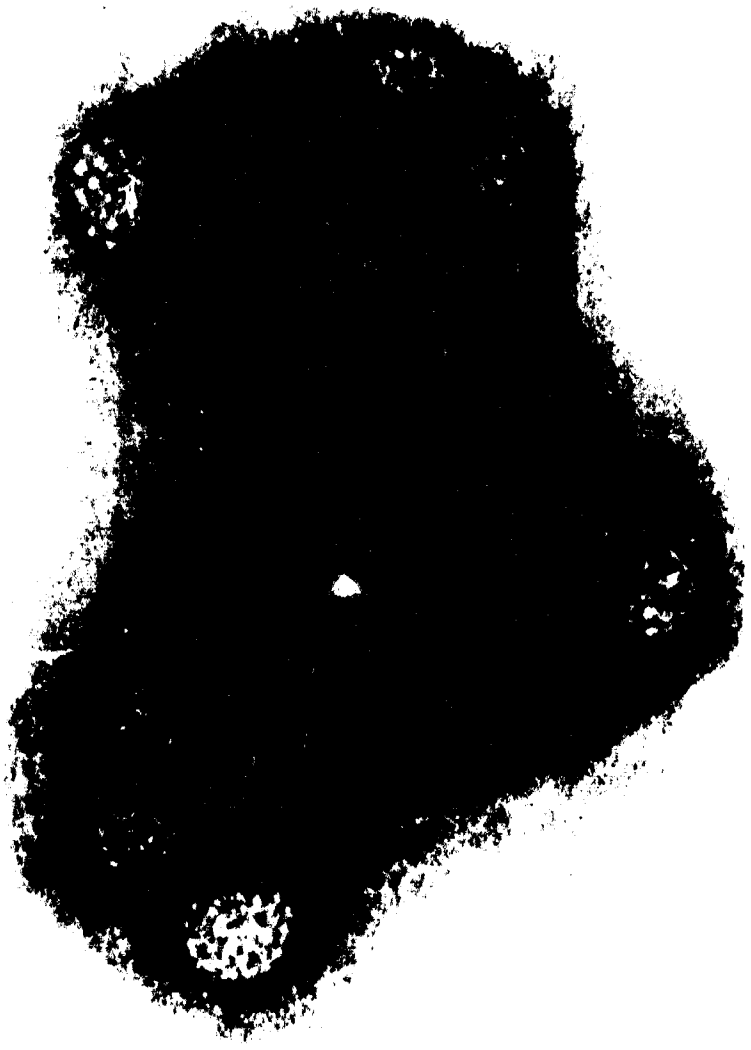

Figure 13 Papovavirus from measles MN cell culture supernatants negatively stained with 3% phosphotungstic acid (x 300,000).

DISCUSSION

Interest in the human papovaviruses was kindled in 1965 when ZuRhein and Chou described papovaviruses observed in glial nuclei displayed in ultrathin sections from the brain of a patient with the rare demyelinating disease progressive multifocal leucoencephalopathy (PML). Koprowski *et al*, (1970) discussed the finding of papova-like particles in the cytoplasm of a SSPE human brain culture as well as in Vero cells infected with virus resembling measles virus. Papova-like virus particles in Vero cultures were seen only in cells forming syncytia. It was postulated that the papova-like particles were present as unobtrusive symbionts in human tissue, particularly brain tissue, incapable of causing signs of illness *per se*, and also unable to spread to other cells of the organism in normal physiological conditions. Cell fusion engendered by viruses belonging to the paramyxovirus group and found in cells containing the papova-like agent enables the latter to spread from cell to cell and when a sufficient number of brain cells become involved in this process, the disease syndrome manifests itself. In the light of these statements, the possibility exists that the two papovavirus positive measles patients found in the present study might similarly fit in with this hypothesis, namely that the papovavirus is harboured in the lymphocytes in a repressed form, and, under suitable conditions (measles infection)

may lead to transformation and production of virus particles. Lymphocytes certainly provide an efficient means of virus spread in the body. In this respect, the demonstration of virus in kidney and brain and the fact that the PML associated papovavirus can be excreted in the urine, suggests that efficient movement of the virus occurs in the body within circulating cells. Serological studies have shown that antibody to papovavirus is common in the general population and it is probable that the majority of primary infections occur in childhood. Such infections have not yet been associated with clinical illness nor has the virus been isolated from normal subjects. All the strains recovered have been from patients who are in some way, either as a result of disease or the nature of the treatment they are receiving, immunologically abnormal. Thus, Lecatsas *et al* (1973) demonstrated that a number of renal transplant patients became chronic virus excretors, suggesting that they had a specific cell-mediated immunodeficiency to papovavirus which may have been enhanced by immunosuppressive therapy. In addition, the occurance of virus DNA in DNA obtained from certain human tumors has recently been reported (Fiori and Di Mayorca, 1976).

Although the findings of the present study show for the first time an association between papovavirus and peripheral blood lymphocytes, the nagging possibility exists that these results may indeed be an artifact. The measles MN cell cultures were

processed in a laboratory where studies on papovavirus were being carried out and the possibility of contamination of the cultures (i.e. glassware) cannot be entirely ruled out. Absolute confirmation of these results using the fluorescent T-antigen method as well as careful monitoring of the patients' clinical conditions, must however remain a long term proposition.

SUMMARY

Examination of measles MN cell culture supernatants by electron microscopy demonstrated the presence of papovavirus particles in 2 out of 15 patients studied.

Immunofluorescent staining for the papovavirus T antigen in both control and measles MN cells were unsuccessful.

The implications of these findings are discussed in the context of the relationship between measles virus and depressed cellular immunity.

CHAPTER 9

Monocyte Chemotaxin in Measles

Monocyte Chemotaxis in Measles

INTRODUCTION

Since macrophages are immune effectors that can phagocytose and destroy antigenic material, accumulation of these cells at sites of inflammation is an important event in host defence. Inhibition of the responsiveness of circulating monocytes would be one mechanism by which viruses could depress inflammatory responses.

The term 'chemotaxis', with reference to directed movement of leucocytes, was first used by Leber in 1888 who clearly showed the 'directed' movement of leucocytes towards a number of biologically derived substances, such as bacteria and fungi, animal tissue, putrefaction products and water and alcoholic extracts of *Staphylococcus aureus*. Prior to 1962 literature pertaining to leucocyte chemotaxis was more suggestive than precise. Techniques both *in vivo* and *in vitro* although intriguing, were often highly intricate and difficult to interpret. It was not until 1962 that Boyden described the first accurate technique for the assessment of chemotaxis. The actual test apparatus was fairly simple, comprising a vertically bicompartmental chamber in which the upper and lower chambers were separated by a millipore filter. The

pore size of the filter is determined by cell size such that cells could migrate through actively but not drop passively. A numerically standardized cell suspension was placed in the upper compartment of the chamber and soluble chemotactic substances placed in the lower chamber. The chambers were subsequently incubated and the cells allowed to respond chemotactically. After a suitable time interval the filter was fixed and stained and the lower surface examined microscopically. Chemotaxis was evaluated by counting those cells which had completely traversed the filter.

The inherent principle involved in the Boyden chamber is the establishment of a chemotactic factor concentration gradient. At the outset of an experiment the chemotactic factor may be completely localized in the lower chamber. However, with time, a positive concentration gradient develops. Chemotactic factor diffuses across the micropore filter into the cell compartment interacting with cells and inducing a migratory response. Due to uneconomical cell and chemotactic factor requirements, the Boyden technique was considered unsatisfactory. It was, therefore, decided to adopt a micro-chemotactic technique in the present study.

Zigmond and Hirsch (1973) presented a modified micropore filter technique designed to facilitate the microscopic evaluation of chemotaxis. Once again, as with the Boyden system, a bi-compartmental chamber was used. However, the chamber incubation period was shortened to allow cellular migration into the

filter but not complete filter transpassage by the cells. After a suitable time interval, filters were removed, fixed and stained as described previously. However, the filter was not inverted and the lower side quantitated, but was scored by measuring with the optical micrometer on the fine focus knob of the microscope, the distance from the top of the filter to the furthest focal point which contained at least two cells in focus. In positive controls the distance migrated into the filter by the 'fastest moving cell front' was much greater than that of the negative controls. With this technique, results are expressed in microns travelled and not cells per high power field.

Both the aforementioned methods have certain disadvantages or sources of error. When cells are allowed to migrate through the filters, relatively long incubation times are required during which one might be concerned about cell viability and maintenance of the chemotactic factor gradient. In addition, there is the problem of cells falling off the filter after migration. Measuring the distance of the leading front of cells is more objective and relatively easy to do, however, cellular quantitation may be difficult since a number of cells may migrate into the filter to normal depths, thus masking the inability of others to elicit a chemotactic response. In view of the small amounts of blood available for investigation in the present study as well as other

factors to be explained later, the method of Zigmond and Hirsch (1973) as modified by Russel *et al* (1976) was chose to assess the ability of measles monocytes to react to a chemotactic stimulus *in vitro*. Furthermore, in a small number of cases, the ability of measles patients to respon to an inflammatory stimulus was also assessed *in vivo*.

METHODS AND MATERIALS

Patients and Controls

Ten measles patients and an equal number of healthy adult controls (as described earlier) were investigated.

Separation of blood

MN cells from patients and controls were separated on Ficoll-Hypaque gradients, washed three times in HBSS, and resuspended at a concentration of 5×10^6/ml in HBSS, pH 7.0.

Preparation of chemotactic factors

1. Endotoxin activated serum (EAS)

   Purified bacterial endotoxin was obtained commercially, carefully weighed out prior to each experiment and chemotactic activity generated by the addition of fresh serum (500 µg endotoxin/ml serum). The mixture was incubated at 37°C for 30 minutes to generate chemotactic activity and diluted 1/400 with HBSS.

2. Alkali treated Casein

   Casein (Nach Hammerstein-Merck) was solubilized according to the method of Wilkinson (1972). Commercially available casein was insoluble in HBSS at pH 7.2. However, by gently increasing the pH, by drop-wise addition of 1M NaOH, to pH12, casein solubilized. The solution was carefully brought back to neutrality with $NaH_2PO_4 2H_2O$. The final concentration of casein was 5 mg/ml of HBSS.

For all studies in this presentation the same casein preparation was used at an optimal concentration of 600 µg/ml.

Chemotaxis Assay

1. The Boyden Assay

(a) Assembly and Incubation of Chambers

Monocyte chemotaxis was performed using a modification of the method of Snyderman *et al*, (1972). The chamber used in chemotaxis tests was a simplified and smaller version of Boyden's original chamber. A plastic disposable pipette (for use with Clay Adar . Selectapette) was sawn in two and the end-piece (± 10mm) with the level surface, used to glue a circular 5.0µ nucleopore polycarbonate filter to it. The type of glue used was commercially available ('UHU') and satisfied 3 criteria (Anderson, 1974) :

(a) It was non-toxic to leucocytes.

(b) It was non-chemotactic.

(c) It was methanol soluble.

The lower fluid compartment was constructed as follows :

(a) A 5ml plastic test tube 'push-on' cap was punched with a number 2 cork-borer, and the circular plastic disc removed.

(b) The 'cell-chamber' (pipette and filter) was fitted into this and was tightly held in place, while permitting vertical adjustment.

(c) A 4ml autoanalyser tube was utilized for the chemotactic factor.

(d) The inner chamber freely fitted the outer chamber permitting ease of manipulation of the inner chamber to equalize inner and outer fluid levels during chamber assembly.

In order to eliminate the appearance of air bubbles above or below the filter during the chamber filling, pre-filling of chambers with cell suspension solution was carried out. Chambers were allowed to stand for a few minutes, during which time air bubble formation occurred, then emptied and filled with leucoattractant and cell suspension.

A diagram of the modified chamber is presented in Figure 14.

One milliliter leucoattractant or HBSS was introduced to the lower chamber and a numerically standardized MN cell suspension introduced into the upper chamber, with a long needled 1ml syringe. A cell volume of 0.2ml was carefully layered onto the nucleopore membrane. The inner chamber containing the cells was then fitted into the lower chamber (leucoattractant) and the inner and outer fluid levels equalised. Chambers were incubated at 37°C for 90 minutes in a humidified atmosphere of air.

(b) Filter removal and staining

Subsequent to incubation the inner chamber was carefully

removed and immersed in methanol for 30 seconds. Methanol caused cell fixation and also dissolved the glue. The filter was easily removed with fine forceps and then stained in Diff-Quik and rinsed in water.

(c) Microscopic evaluation of chemotaxis

Following staining, the filters are inverted (so that the bottom side is uppermost), placed on a glass slide, allowed to air-dry and mounted in xylene. Chemotaxis was quantitated by counting the mononuclear cells which had migrated completely through the filter. The MN cells in 10 horizontal and 10 vertical microgrid fields (x 400) were counted and averaged. The chemotactic index is then expressed as cells (monocytes)/ high power field (HPF).

2. The Method of Zigmond and Hirsch

(a) Assembly and incubation of chambers

Monocyte chemotaxis was assessed using the modified method described by Russel *et al*, (1976). Millipore filters (SCWP 01300, 8μm) were glued to 'sawn-off' barrels of tuberculin syringes using UHU glue and the chambers were then assembled as previously described and incubated for 130 minutes at 37°C in a humidified atmosphere of air.

(b) Filter removal and staining

The inner chamber was carefully removed after incubation

and immersed in methanol for 30 seconds. Filters were removed, rinsed in distilled water and stained in haematoxylin for 1 minute and then immersed in 3 changes of ethanol for 2 minute intervals (dehydration) before clearing filters in xylene for 1 minute.

(c) Microscopic evaluation of chemotaxis

Subsequent to staining, the filters are placed on a glass slide and mounted in xylene. Chemotaxis was scored by measuring with the optical micrometer on the fine focus knob of the microscope, the distance from the top of the filter to the furthest focal point which contained at least two cells in focus. Results are expressed in micrometers.

Monocyte migration *in vivo*

Rebuck devised a simple procedure for observing the leucocytic infiltration in an area of inflammation (Rebuck and Crowley, 1955). This technique slightly modified, is as follows :

A small area of skin on the volar forearm is cleansed with alcohol. With the use of a sterile scalpel blade, the epithelium is scraped until the papillary layer of the corium is reached (free bleeding is avoided). A drop of sterile egg-white is applied to the area and a sterile glass coverslip is placed over the area. Tape is then applied so that the glass is pressed firmly against the lesion. The coverslip is changed after 3 hours and after 24 hours; allowed to air-dry, fixed with methanol and stained with Diff Quik and examined microscopically.

RESULTS

1. Monocyte Chemotaxis using the Boyden method

In a preliminary study using normal controls, studies were undertaken to assess the reliability of the Boyden method using the standard 5.0μ polycarbonate Nucleopore filters as suggested by Snyderman *et al*, (1972). As can be seen in Table 6a, a wide range of chemotactic responses was observed using numerous normal controls. Other difficulties encountered with this method were :

(a) being so thin (15μ), considerable skill was needed to focus on the bottom side of the filters in order to enumerate the migration cells. (See fig. 15).

(b) the distribution of poresin the filters are irregular producing 'patches' or clumps of cells resulting in inaccurate cell counts.

(c) it is difficult to standardize different mononuclear cell suspensions to contain equal numbers of monocytes.

(d) filters are difficult to handle during the assembly of the chemotactic chamber.

(e) the counting of numerous different high-power fields is also tedious and time consuming.

Because of these factors, it was therefore decided to use the method of Zigmond and Hirsch to assess monocyte chemotaxis in measles patients.

2. Monocyte Chemotaxis using the method of Zigmond & Hirsch

As can be seen in Table 6b, monocytes of children with measles migrated adequately towards the leucoattractant casein.

3. Rebuck Skin-Windows

Figures 16a and 16b, show typical measles and control skin-windows after 3 hours. A marked reduction in the accumulation of PMN cells was evident. In contrast, adequate numbers of measles monocytes were present in the inflammatory site after 24 hours (Fig. 17a). Although some PMN cells were also present in the contr 1 24 hour skin-window (Fig. 17b), there was little evidence of this in the measles skin-window.

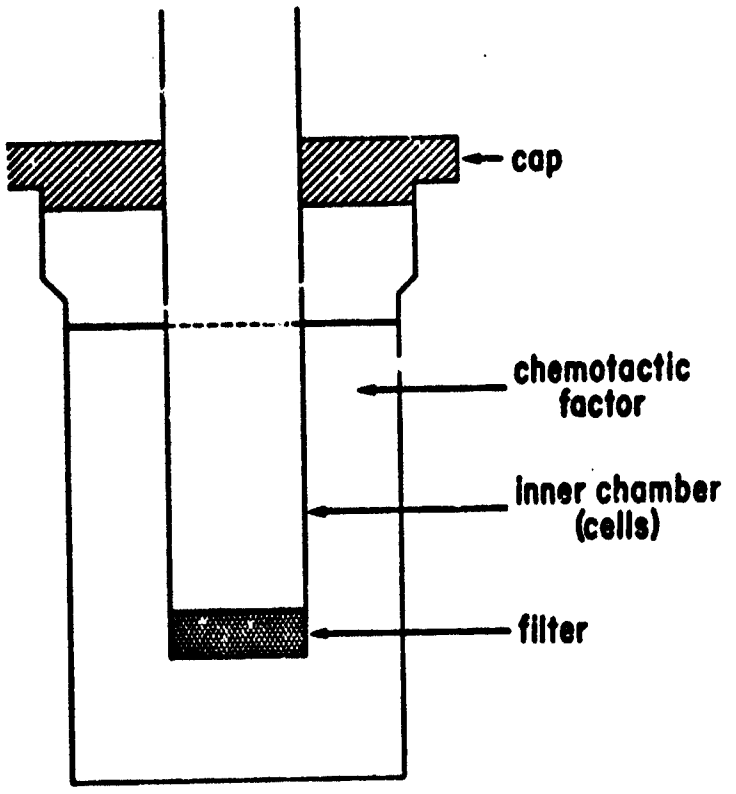

Figure 14 Diagram of a modified Boyden chamber.

TABLE 6a

COMPARISON OF THE NUMBER OF MIGRATING MONOCYTES IN DIFFERENT CONTROLS USING NUCLEOPORE FILTERS

	Monocytes per high power field (HPF)*	
Experiment	Mean	Range
1	59.6	44 - 72
2	48.6	39 - 59
3	27.0	25 - 32
4	33.2	29 - 42
5	35.2	27 - 42
6	32.3	27 - 37
7	37.6	28 - 45
8	34.7	27 - 44
9	37.2	32 - 46
10	25.5	19 - 30
11	42.8	36 - 50
12	38.8	27 - 48
13	26.4	24 - 30
14	29.8	25 - 35

* Each value represents the mean cell count of triplicate filters minus the chemotactic response to media alone (HBSS).

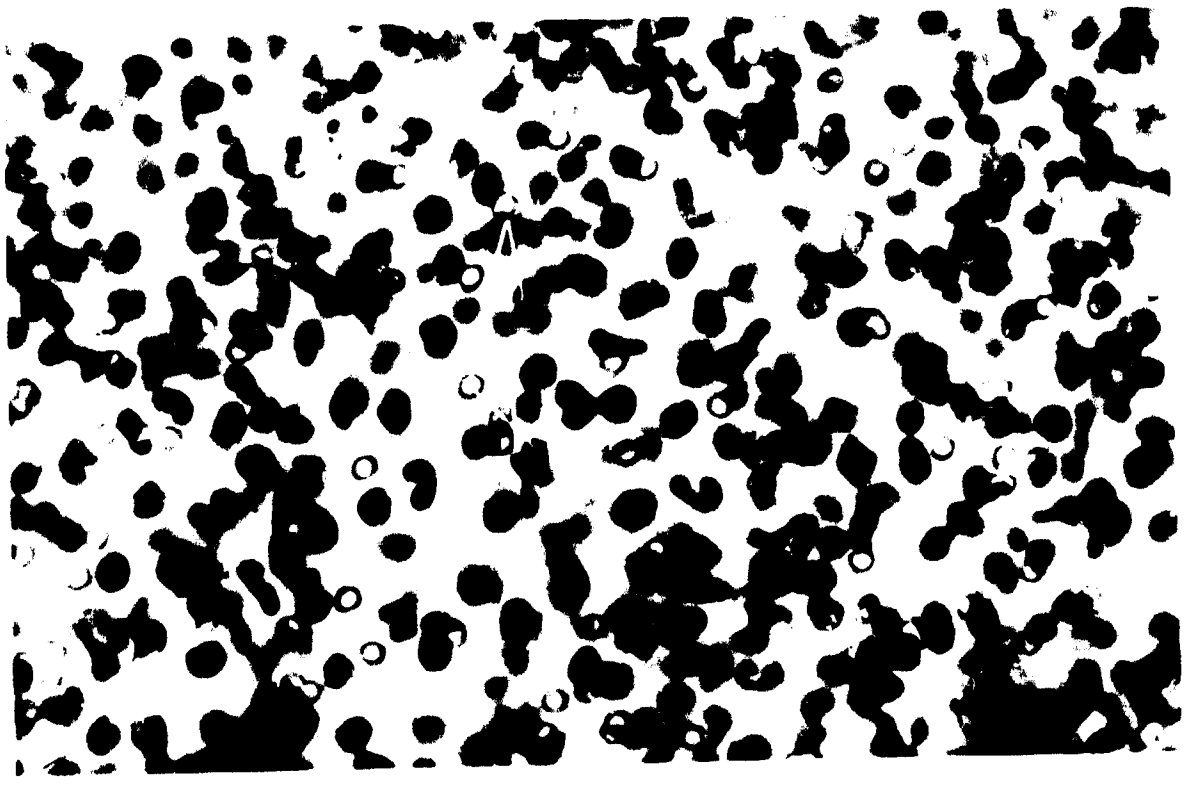

Figure 15 Chemotactic response of human monocytes to endotoxin activated human serum. Cells which have migrated through the nucleopore filter are in focus. Also seen are empty pores and pores with cells in their lumens (x 400).

TABLE 6b

THE CHEMOTACTIC ABILITY OF CONTROL AND MEASLES MONOCYTES AS ASSESSED BY THE LEADING CELL-FRONT TECHNIQUE

Source of monocytes	Filter penetration Mean ± S.E.M.*	P value
Measles (n = 10)	47.3 ± 11.7	N.S.
Controls (n = 10)	53.0 ± 11.1	

* Figures quoted are mean migration distances in micrometers minus the chemotactic response to media alone.

A

B

Figure 16 3-hour Rebuck skin-windows from a patient with measles (A) and from a control (B).

A

B

Figure 17 24-hour Rebuck skin-windows from a patient with measles (A) and from a control (B).

DISCUSSION

Because of the profound neutrophil chemotactic abnormality observed in children with measles (Anderson *et al* 1976), the possibility existed that the absence of cutaneous delayed hypersensitivity responses in these patients could be due to a similar inability of mononuclear cells to move into the inflammatory site. Despite difficulties encountered in an initial study using the Boydon method of chemotaxis, the leading front method of Zigmund and Hirsch as modified by Russel *et al* (1976) was successfully adapted. It might be argued that this method measures a qualitative rather than a quantitative chemotactic response but for the purposes of this study, where many of the patients were leucopenic precluding cell manipulation, the results obtained are still of importance. Anderson *et al*, (1976) demonstrated that the chemotactic ability of PMN from ten patients with uncomplicated measles was grossly impaired when compared to normals. Chemotaxis to endotoxin activated normal serum and to hydrolysed casein was markedly depressed but serum from measles patients, when activated by endotoxin, generated normal chemotactic activity and did not contain leucotactic inhibitors. Similarly, a study of monocyte chemotaxis during acute influenza infection (Kleinerman *et al*, 1975) demonstrated that the acute serum of influenza patients did not contain humoral inhibitors.

The Rebuck skin-window technique provides a simple method for observing the cellular events of acute inflammation *in vivo*. With this technique in normal individuals, PMN appear at the inflammatory site in 1 to 3 hours and constitute 90 - 100% of the initial cellular exudate. By 9 to 12 hours, a mononuclear cell exudate accumulates and by 24 hours, monocytes predominate (Dale and Wolff 1971). Anderson *et al*, (1976) demonstra l a marked reduction in the accumulation of PMN cells in skin-windows of 5 measles patients during the acute phase of their illness. In contrast to the latter findings, adequate monocyte accumulation was observed in the skin-windows of the measles patients after 24 hours. Dale and Wolff (1971) have noted that in patients with neutropenia, PMN accumulation at an inflammatory site was not necessary to initiate a normal mononuclear cell response. Examination of punch-biopsy specimens from the skin rashes of patients with measles further-more show a noticeable mononuclear inflammatory response, presumably against measles virus antigen in surface epithelium and corium (Olding-Stenkvist and Bjorvatn, 1976). Since monocytes accumulate more slowly at an inflammatory site, the frequent secondary infections noted in measles patients may be attributable to the deficient early acute PMN inflammatory response before monocytes can accumulate.

Various workers have demonstrated that measles virus does not replicate to any great extent in human monocytes (Sullivan *et al*, 1975b, Joseph *et al*, 1975, Lucas *et al*, 1978,

and it is, therefore, likely that the major cause of anergy observed in measles patients is the failure of lymphocytes to produce lymphokines. The administration of live measles vaccine gave to Black and Sheridan (1967) an opportunity to follow the cellular changes in some detail and they reported that a 'broad trough of leucopenia was apparent' which began about the 4th day after infection and ended at about the 13th day after inoculation. The declining white cell count sometimes affected lymphocytes on the 3rd day and neutrophils mostly about the 8th day after infection, whilst monocytes were hardly affected. Kleinerman *et al*, (1975) demonstrated that the chemotactic responsiveness of monocytes from patients with acute influenza was significantly depressed *in vitro*. In addition, exposure of normal MN cells to herpes simplex or influenza virus *in vitro*, depressed monocyte chemotaxis as compared to MN cells incubated with non-infectious viruses. Electron microscopy showed that UV-irradiated herpes virus could still attach to monocytes, but since they could not replicate, it was speculated that there was something in the replicating phase of the virus infection that caused inhibition of the chemotactic response. Measles virus, in common with herpes and influenza viruses is an enveloped virus having an outer structure originating from host cell membranes (in contrast to the unenveloped REO and polio viruses and vaccinia virus which does not have host cell membrane derived structures) (Kleinerman *et al*, (1974). However, as mentioned

previously, there is no evidence of significant measles replication in human monocytes to suggest impaired monocyte chemotaxis according to this theory. Furthermore, Lucas *et al*, (1978) provided convincing evidence that monocytes were not implicated in the measles virus induced suppression of lymphocyte functions *in vitro*. In a series of experiments, lymphocytes infected with measles virus were stimulated with irradiated allogeneic cells in the presence of human serum containing antibodies against measles virus, thus preventing the spread of the infection to the monocytes present in the suspension of stimulator cells. Under these conditions, the mixed lymphocyte reaction was still inhibited considerably thus demonstrating that the presence of irradiated noninfected monocytes did not counteract the inhibitory activity of the virus. Anderson *et al*, (1976) further found that only children with acute measles infection had depressed PMN chemotaxis while children with other viral infections (mumps, chicken-pox, infectious hepatitis and meningoecephalitis) had normal PMN chemotactic responses. The fact that PMN chemotaxis is markedly depressed in patients with bacterial infections (Anderson, 1974) while monocyte chemotaxis has been reported to be unaffected in similar patients (Kleinerman *et al*, 1975) lends support to the fact that a similar situation takes place during measles infection. Although the movement of monocytes appeared to be unaffected by measles infection, it still remains to be determined whether a lymphokine that attracts monocytes (lymphocyte-derived chemotactic factor) can be elaborated by lymphocytes from measles patients.

SUMMARY

Monocyte chemotaxis was investigated both *in vivo* and *in vitro*. For the *in vitro* assessment of monocyte chemotaxis, the Boyden chamber method was utilized using nucleopore filters. This method was found to be insensitive due to a number of technical reasons and was subsequently abandoned in favour of a modified version of the method of Zigmond and Hirsch. This method depended on the leading cell front as an index of monocyte movement. Using hydrolysed casein as chemoattractant, it was demonstrated that the ability of measles monocytes to migrate was essentially unimpaired.

In vivo cell migration was assessed by the Rebuck skin-window technique. The skin-windows of measles patients differed from normals in that at 3 hours, very few PMN cells had migrated into the inflammatory site while adequate numbers of monocytes were present after 24 hours.

These results suggest that monocytes from patients with measles are not significantly affected by infection and that the defect in cellular immunity probably resides in the lymphocytes.

C H A P T E R 10

Conclusions

CONCLUSIONS

Since von Pirquet's (1908) observation that measles inhibits the dermal tuberculin reaction, a number of *in vitro* tests of lymphocyte function have made it possible to re-examine under defined conditions the suppressive effect of measles on delayed hypersensitivity.

The use of nonspecific mitogens as a tool to investigate lymphocyte responsiveness *in vitro*, has led to conflicting results. For example, Kadowaki *et al*, (1970) and Finkel and Dent (1973) described depressed PHA activation of measles MN cells whereas Fireman *et al*, (1969) and Osunkoya *et al*, (1974a) have reported normal responses. In the present study, normal PHA-induced blastogenisis was found in children with measles, prompting further investigation using both allogeneic stimulation of measles MN cells as well as the measurement of lymphokine production, two parameters of cell-mediated immunity which have not previously been extensively studied. The depression of the latter two aspects of cellular immunity found in this study have posed many questions which at this stage, can perhaps only be answered in terms of what is already known in other disease states. In spite of these difficulties, it is possible to see some trend amongst the results obtained leaning towards either a selective inactivation of specific lymphocyte subpopulations (either by direct infection of the cells concerned or by some as yet unknown indirect effect) or a

redistribution of effector cells in the infected individual. Whatever the precise mechanism of altered cellular immunity in measles, future studies should aim at investigating defined cell populations such as helper and suppressor cells; their isolation and respective rôles in the cell-mediated immune response in this disease. As mentioned previously, the undertaking of such studies will undoubtedly necessite the use of micro-techniques to compensate for the generally young age and scarcity of leucocytes in these patients.

Although the results of this study together with results reported in other publications generally tend to regard the fact that lymphocytes, rather than monocytes are adversely affected by measles virus, more detailed investigations of monocyte function are imperative. It is becoming increasingly clear that monocyte factors or monokines can affect lymphocyte function by stimulating or inhibiting their *in vitro* immune response (Waksman and Namba, 1976).

A further complication in understanding the interaction of measles virus with the immune system arises from comparisons between the responses of *in vivo* and *in vitro* infection. Sullivan *et al*, (1975a) showed quite clearly that *in vitro* infection of lymphocytes with measles virus resulted in significant inhibition of PHA-induced ^{3}H-thymidine incorporation. Although most of the depressive action is probably due to the presence of live virus, other factors may also be of importance. In culture, lymphocytes may be exposed to

a greater concentration and variety of stimuli than individual lymphocytes *in vivo*, thus increasing the difficulties of direct comparison.

In the present study, use has been made of a nonspecific mitogen, PHA. Use of soluble antigens such as Candida or PPD would have perhaps given a better insight into the effects of measles infection on specific clones of cells, but definite proof of the immune status of the young children used in this study prior to infection, was impossible to ascertain. The problems involved in the use of measles-specific antigen preparations to determine immune function have been previously emphasized. Whole virus and even purified measles virus haemagglutinin will probably react with leucocytes non-immunologically and Valdimarrson *et al*, (1975) and Nordal *et al*, (1976) advised against interpreting results of lymphocyte stimulation by measles virus as wholly due to immunological causes.

This dissertation has shown that the effects of measles infection on the cellular immune response is complex. Further studies on the influence of the virus on the functioning of subpopulations of lymphocytes are necessary to unravel the immune mechanisms involved in this ancient disease.

APPENDIX

SOURCE OF MATERIALS AND EQUIPMENT

Materials

Agarose	B.D.H. Chemicals, Poole, England
Ammonium chloride (NH_4Cl)	Merck, Darmstadt, Germany
Amphotericin B	Squibb, Isando, Transvaal, South Africa
Casein (Nach Hammerstein)	Merck, Germany
Endotoxin, bacterial lipopolysaccharide (*E. coli* O127:B8)	Difco Laboratories, Detroit, Michigan U.S.A.
Fetal calf serum (FCS)	Wellcome Research Laboratories, Beckenham, England
Ficoll	Pharmacia, Uppsala, Sweden
Fluorescent sheep anti-rabbit serum	Miles Research Products, Goodwood, Cape Province, South Africa
L-Glutamine	Merck, Darmstadt, Germany
Hank's balanced salt solution	Polio Research Institute, Johannesburg South Africa
Heparin, preservative free	Evans Medical, Liverpool, England
Hepes, 2-(4-(2-hydroxyethyl) -1-piperazinyl) ethanesulfonic acid	Merck, Darmstadt, Germany
Horse Serum	Grand Island Biological Company, New York, U.S.A.
Hypaque	Winthrop Laboratories, Durban South Africa
Insta-Fluor	Packard Instrument Company, Downer Grove, Illinoise, U.S.A.
Levamisole, 1,2,3,5,6-tetrahydro-6-phenylimidaza (2,1-b) thiazole	Ethnor Laboratories, Johannesburg South Africa
Measles Complement Fixing Antigen	Wellcome Research Laboratories, England
Minimal essential medium (MEM)	Polio Research Institute, Johannesburg South Africa
Mitomycin C	Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Penicillin G, sodium benzylpenicillin	Glaxo-Allenbury's, Wadeville, Transvaal, South Africa
Phytohaemagglutinin (PHA) reagent grade	Wellcome Research Laboratories, Beckenham, England
Rabbit anti-hamster serum	Miles Laboratories, Goodwood, Cape Province, South Africa
Streptomycin sulphate	Glaxo-Allenbury's, Wadeville, Transvaal, South Africa
Sodium Bicarbonate, 4.4%	Wellcome Research Laboratories Beckenham, England
TC 199 medium	Wellcome Research Laboratories, Beckenham, England
Tritiated thymidine, (methyl-^{3}H) thymidine 17 Ci/m mol	Radio Chemical Centre, Amersham, England

EQUIPMENT

Capillary tubes, 50µl	Dade, Miami, Florida, U.S.A.
Electron Microscope	Philips, EM300, Holland
Fluorescent Microscope	Ortholux 2, Leitz GMBH, Wetzlar, Germany
Gel cutter and template	Hoechst Pharmaceuticals, Johannesburg South Africa
Millipore filters (8.0µ) cellulose, SCWP 013 00	Millipore Corporation, Bedford, Massachusetts, U.S.A.
Microtiter plates, round bottom (Cooke system)	Greiner, Nürtingen, Germany
Multiple automated sample harvester (MASH II)	Microbiological Associates, Bethesda, Maryland, U.S.A.
Nucleopore filters (5.0µ) N500 CPR013 00	Nucleopore Corporation, California, U.S.A.
Petri dishes, (Falcon), 60 x 15mm	Becton Dickinson & Co., California, U.S.A.
Planimeter, Coradi	Zurich, Switzerland
Projector, 'Prado Universal'	Leitz, Wetzlar, Germany
Scintillation vials (plastic)	Packard Instrument Company
Tri-carb liquid scintillation counter, model 3390	Packard Instrument Company
Tuberculin syringes, 1ml	AHSG S.A. (Pty) Ltd., Johannesburg South Africa
UHU glue	H.u.M. Fischer GmbH, West Germany

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