AN IMMUNE STUDY OF NEWBORN INFANTS

WITH CONGENITAL SYPHILIS

Gregory R Samson

MB ChB, DCH (SA), FCP (SA)

Submitted for the Degree of Doctor of Medicine

University of Cape Town

1995

DECLARATION

I, Gregory R Samson, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed

.....
SIGNATURE

23/6/95

.....
DATE

The work reported in this thesis was performed in the Department of Paediatrics and Child Health, University of Cape Town, Cape Town

Dedicated to the memory of my beloved parents Henry and Daphne Samson whose unwavering support, loving attention and encouragement always served as the cornerstones of my life. And to my wife and best friend, Yvette, my children Karis and Tuzzio - a better family I could not have asked for.

ACKNOWLEDGMENTS

This thesis would not have been possible without the invaluable input and contributions made by several of my colleagues. It is therefore with gratitude and thanks that I extend my sincere appreciation to the following:

My fellow neonatologists, Professors Atties Malan and David Woods and Dr Clive van der Elst for the seeds that culminated in the germination of this project.

Jane Hughes, Di James and Margaret Cooper of the Immunology Laboratory, Red Cross Children's Hospital for their indefatigable technical assistance.

Di Blake, Dr Suzanne Mouton and Dr M C Cohen for their invaluable assistance in the work on the placenta.

Jane Hall, formerly of the Institute of Biostatistics at the Medical Research Council, who provided her expertise and patiently guided me through the complexities of statistics. Dr Dave Charlton for his assistance when I needed it most.

Professor Malcolm Bowie who critically and meticulously reviewed the manuscript, and whose constructive criticisms and suggestions led to its revision.

Professor ROC Kaschula who reviewed the chapters on the placental study and provided sound advice.

Professor Vincent Harrison, my esteemed colleague, for his considerable help with the preparation of the manuscript.

Professor David Beatty reviewed and critically analyzed a number of specialized chapters.

The staff of the Renal Laboratory, Groote Schuur Hospital who performed the immune complex assays, and, to those in the Haematology and Chemical Pathology Laboratories who performed the full blood counts and immunoglobulin assays.

Dr Michael Meyer for his constructive comments.

Professor H de V Heese for his tireless efforts of encouragement.

Mrs Vicky Melly who typed the manuscript, coped with endless revisions and gave editorial advice. I wish her the best for her new life in England. Our department is the poorer for her loss.

Finally and importantly, a special thanks to the Medical Research Council and the Cooper Lowveld Fund, University of Cape Town for their financial assistance and support for this project.

PUBLICATIONS

The following publications were derived from the Thesis.

1. Samson GR, Beatty DW, Malan AF. Immune studies in infants with congenital syphilis. Clin Exp Immunol 1990; 81:315-318.

2. Samson GR, Meyer MP, Blake DRB, Cohen MC, Mouton SCE. Syphilitic placentitis: an immunopathy. Placenta 1994; 15:67-77.

ABSTRACT

INTRODUCTION

Several studies of congenital syphilis have focused on the epidemiological, diagnostic, preventative, and therapeutic aspects of the disease. While adult syphilis has been extensively investigated, few studies have explored the potential immunological abnormalities in the fetus afflicted with the disease. Furthermore, little is known of the pathogenesis of the diseased placenta in syphilis.

The present study addresses the question of the immunological status of the newborn infant with congenital syphilis both prior to the commencement of (day 1) and on completion of antibiotic therapy (day 10). It further aims to test the hypothesis that the placentitis in congenital syphilis has an immune pathogenesis.

OBJECTIVE

(a) to examine the immune response in a cohort of newborn infants with congenital syphilis and

(b) to explore the hypothesis that the placentitis in fetal syphilis has an immune pathogenesis.

METHODS

Study design

A hospital based case - control study of newborn infants with congenital syphilis and a group of healthy matched controls. Only infants born in hospitals in the ambit of the Peninsula Maternity and Neonatal Services were enrolled in the study. The diagnosis of congenital syphilis was based on the physical and serological criteria of Kaufmann et al (1977). The controls were matched for gestational age, birth weight, sex and race.

Subjects

(a) Nineteen infants with congenital syphilis and 17 matched controls were enrolled.

(b) The placentas of 5 of the 19 infants were collected for study.

METHODS OF THE STUDY

Immune study

Standard immunological methods were employed in the study of the following immunological parameters on day 1 (pre-treatment) and day 10, the time of completion of treatment (post-treatment):

1. Humoral immunity.
   - 1.1 Peripheral B cells:
   - 1.2 Total serum Immunoglobulins. In addition to quantifying the total IgM levels, a correlation was sought between these levels and the extent of the disease.
2. Cell-mediated immunity.
   - 2.1 Lymphocyte transformation
   - 2.2 Total T (CD3) cells and T cell subsets (CD4 and CD8).
3. Circulating immune complexes. Correlation was also sought between the concentrations of the immune complexes and the extent of the disease process.
4. Total haemolytic complement (CH50) and C3.
5. Phagocytic function.
   - 5.1 Total white cell and differential counts
   - 5.2 Nitroblue tetrazolium test (NBT)

Placental study

Tissue sections were stained with haematoxylin and eosin (H&E) and examined histologically.Immune complexes were sought in the placentas by immunohistological staining using the streptavidin-biotin complex staining method.

STATISTICAL ANALYSIS

Immune study

The Wilcoxon signed rank test for matched pairs, a non parametric test, was employed in the comparative analysis of study subjects and controls. Spearmans rank correlation coefficient was used to determine concordance between two sets of variables.

Placental study

The Mann-Whitney two sample test was used to compare the placental -birth weight ratios between the case and control groups.

RESULTS

Immune study

Newborn infants with congenital syphilis demonstrated an heightened humoral immune response. Both the absolute numbers of B cells and the total serum IgM levels were significantly higher than that found in the controls ($p=0.006$ and $p=0.001$ respectively). No significant fall in B cell and IgM levels occurred at the time of completion of treatment ($p=0.85$ and $p=0.26$ respectively). There was a trend towards a positive correlation between the IgM levels and the extent of the disease but this did not reach statistical significance ($p=0.061$).

There was no difference in CD3, CD4 and CD8 counts between patients and controls ($p=0.236$, $p=0.887$ and $p=0.619$ respectively). However, PHA stimulated syphilitic

lymphocytes showed reduced blast transformation in autologous serum compared to the controls (40380 dpm vs 51583 dpm ; p=0.008). Culture of the syphilitic lymphocytes in AB serum showed a significant improvement in radioactive uptake (40380 dpm vs 45304 dpm ; p=0.011). Measurements on day 10 demonstrated an improvement in lymphocyte reactivity (40380 dpm vs 45295 dpm; p=0.08).

Circulating immune complex concentrations were markedly elevated in the study group (p=0.0007). On day 10 these levels had fallen significantly (p=0.0008).

Patients with congenital syphilis had significantly lower CH50 levels than the controls (0.014).

Total white cell counts and the outcome of the nitroblue tetrazolium tests were comparable to that found in the controls (p=0.201 and p=0.201 respectively).

Placental study

Placental-birth weight ratios for cases and controls were comparable (p=0.173). Histologically, large hypercellular villi, focal in distribution, and numerous macrophages were noted in the diseased placentas.

Immunohistochemical staining demonstrated significant reactivity against IgM and C3 on the endothelial surface of the fetal vessels in the stem, intermediate and terminal villi in the diseased placentas. Diffuse IgG reactivity was present in both the diseased and control placentas.

CONCLUSIONS

The results confirm that the fetus is capable of mounting a significant immune response to antigenic stimulation. Specifically, the Treponema pallidum antigen produced marked

stimulation of the humoral immune system with an outpouring of B cells and increased secretion of IgM, and the formation of high circulating levels of immune complexes.

Though the T cells were quantitatively normal, the reduced lymphocyte reactivity suggested an impaired cell-mediated immune response.This finding was attributed to the high levels of immune complexes.

The low CH50 concentrations suggested activation of the classical complement pathway by IgM and ICs, both potent activators of this pathway, and the incorporation of complement into immune complex formation.

Phagocytic function appeared unimpaired. This is in contrast to other bacterial infections where depressed NBT results are often encountered. This would suggest that the neutrophil is not a major role player in the killing of the T pallidum.

The histopathological findings were similar to those described in several studies. The finding of positive IgM and C3 reactivity and, to a lesser extent IgG, in similar distribution sites in the placenta suggested the deposition of immune complexes at these sites. Such immune complexes could set up a cascade of inflammatory events which would result in the histopathological findings often observed. It would appear that the placentitis in syphilis may have an immune pathogenesis.

TABLE OF CONTENTS

INTRODUCTION

During the 1980's the industrialised world experienced an unprecedented increase in sexually transmitted diseases. The incidence rates of primary and secondary syphilis reached levels that were last reported in the 1940's (Webster & Rolfs, 1993). The incidence of congenital syphilis has paralleled the increasing incidence of infectious syphilis (Handsfield and Lukehart, 1984). The number of cases reported to the Centres for Disease Control (CDC) (USA) in 1989 (CDC, 1989a) represented a 95% increase from 1987 and the highest yearly total since the introduction of penicillin (Ikeda and Jenson, 1990).

In 1991, the number of reported cases of primary and secondary syphilis in the United States declined for the first time since 1985 (Webster & Rolfs, 1993). In contrast infectious syphilis continues to be a major public health problem in Africa (Schulz et al 1987). In South Africa, the prevalence of reactive syphilis serology in pregnancy ranges from approximately 7.5% (Naicker & Moodley, 1983; Gonin, 1985; Dietrich et al, 1992) to 11% (Opai-Tetteh et al, 1993) and syphilis remains an important cause of perinatal morbidity and mortality. Ross et al (1978) at the King Edward VIII Hospital, Durban reported a perinatal mortality rate due to congenital syphilis of 3.2/1000 births. It is remarkable that the early neonatal mortality rate due to congenital syphilis has altered little in the past 40 years. A study in 1951 at the Philadelphia General Hospital, USA on the effect of untreated syphilis on the outcome of pregnancy reported that 22% of pregnancies would end in fetal wastage, that the chance of delivering an infected infant was 33%, and that the neonatal mortality rate was 12% (Schultz et al, 1987). Mascola et al (1985) reviewed 50 cases of congenital syphilis reported to the State of Texas in 1982. They reported a neonatal mortality rate of 8%, similar to the figure of 9.09% reported from the University Teaching Hospital in Lusaka, Zambia (Chawla, 1985). Schultz et al (1987) expounded on the seriousness and extent of the sexually transmitted diseases as major public health problems in Africa. They concluded that based on current studies 5 to 8% of

pregnancies surviving past 12 weeks would have an adverse outcome caused by syphilis assuming a seroreactivity prevalence of 10%.

These and other studies of congenital syphilis have focused primarily on the epidemiological, diagnostic, preventative and therapeutic aspects of the disease. Few studies have attempted to evaluate its immunological aspects. Moreover, no information is available on the status of the neonatal immune system at the time of completion of treatment. It is also well known that congenital syphilis is a multisystem disease. The placenta is one organ that is commonly affected. Its involvement in syphilis has been extensively studied and the histopathological changes have been well documented. However, there is a dearth of information on the potential pathogenesis.

The purpose of this study is an attempt to bridge this hiatus in our knowledge. The subject is introduced with a review of the published work in the literature on the immunological status of patients with syphilis. This is followed by a case-control study which explores the hypothesis that *Treponema pallidum* infection of the fetus produces gross quantitative immunological disturbances but that these disturbances are resolved and immunological normality restored at the time of completion of treatment. From the conclusions of the preceding work an additional hypothesis is formulated and tested, namely, that the placentitis in syphilis may have an immune pathogenesis. This part of the study is preceded by an overview of the development of the placenta with specific emphasis on the villous system, and a review of the literature in relation to the histopathology of the placenta in syphilis.

SECTION A

CHAPTER 1 - IMMUNE STUDIES IN SYPHILIS - A LITERATURE REVIEW

1.1 ANIMAL and HUMAN ADULT SYPHILIS

1.1.1 Introduction

By the mid-1970's the immune response of the human host to *T.pallidum* subspecies pallidum remained poorly understood. The underlying reason being that the study of syphilis remained outside the mainstream of modern immunologic research in the post World War 2 period. A number of factors accounted for this lack of research:

(a) the difficulty in culturing *T.pallidum* in vitro

(b) the difficulty in isolating the organisms from mammalian tissue precluding the determination of its antigenic characteristics

(c) the animal model used for studying *T.pallidum* viz., the rabbit was the least well characterized immunologically and the most expensive and difficult animal to work with

(d) the discovery and usage of penicillin in the treatment of syphilis led to a widely held assumption that the disease would soon be eradicated.

Methods for studying humoral immunity had been available for many years but it was only in the late 1960's and early 1970's that cell culture techniques became available allowing a variety of technical systems to be developed to study many different cellular mechanisms in vitro. Their development enabled further investigation into the relative contributions these mechanisms made to the pathogenesis and immunity to syphilis. The techniques most commonly employed were the lymphocyte transformation (LT) assay and the leucocyte migration inhibition assay.

In 1965 Cannefax reviewed the current knowledge on the immunity to syphilis. Certain immunological concepts had already evolved. At that time it was generally accepted that man had no natural immunity to the pathogenic treponemes and that resistance to syphilitic infection mainly involved the maintenance of a barrier of intact skin and mucous membrane. No effective humoral or tissue (cellular) resistance had been demonstrated.

This review (Cannefax, 1965) and a subsequent one (Cannefax, Norins and Gillespie, 1967) focused on what was known of acquired immunity to syphilis. The common theme that emerged in these reviews was that resistance to reinfection seemed to depend on the duration of the disease before its termination by treatment. If the primary disease was terminated within 3 months, reinfection was highly likely. If the disease process was allowed to proceed for longer than 3 months, subsequent re-inoculation did not result in symptomatic disease. This was not absolute as the introduction of a large enough inoculum could result in disease. The immunological mechanisms responsible for this acquired immunity had at that stage not been determined. While both humoral and cellular mechanisms were considered to play a role, the relative importance of each was unknown. Few studies pertaining to the role of cellular immunity had, up to that stage, been done. Cannefax refers to a study done in 1927 in which the investigators implanted tissue infected with virulent treponemes into previously infected but untreated rabbits and into normal control rabbits. The result was a rapid diffusion of the organisms in the control rabbits, whereas in the infected rabbits the organisms were fixed at the implantation site and then gradually disappeared. They concluded that certain, as yet unknown, tissue factors played an essential role in the containment of the organisms.

Wigfield (1965) focused on the tissue reaction to the spirochaete, stating that histologically there was a basic similarity between the chancre, the macular-papular skin rashes and the gummata - the difference being one of degree only. This reaction of the tissues to the spirochaete was considered to be a hypersensitivity reaction resulting in the clinical formation of the chancre and later condylomata. He proposed that the clinical response of

the patient could be related to grades of humoral resistance and cellular tissue sensitivity. In other words increasing degrees of sensitivity were associated with different stages of syphilis, ranging from the chancre of primary syphilis to the gumma of the tertiary stage. Moreover, increasing grades of humoral antibody response would be associated with either spontaneous cure or lead to the stages of early or late latency.

By 1967 two other important observations had been noted and they relate to the role of complement and the process of autoimmunity (Cannefax, Norins and Gillespie, 1967). Whether complement played an essential role or not was uncertain as studies had shown no clinical differences between two groups of mice challenged with *T.pallidum*, one deficient in complement, the other not. In addition, the discovery of the Donath-Landsteiner haemolysins, rheumatoid factors, and other autoantibodies raised the possibility that an autoimmune process may play a role in some of the clinical manifestations of the disease.

Cannefax, Norins and Gillespie (1967) ended their review thus "In short, because many of its intriguing immunological aspects remain unexplored, syphilis immunity can now be profitably examined using the concepts and tools developed recently in immunology".

1.1.2 The humoral immune response

The humoral response produces 2 types of antibodies, the one a nonspecific (anti-cardiolipin) antibody, and the other a specific antitreponemal antibody. Experimental studies designed to evaluate the humoral system were divided into 3 broad groups (Metzger, 1979). Those designed to:

a) detect treponemicidal activity in syphilitic serum

b) find a correlation between resistance and antibody activity,

c) demonstrate a passive transfer of immunity utilizing syphilitic serum

The outcome of these investigations were as follows:

i) There is an antibody response specifically directed against the *T.pallidum* (a finding which led to the development of the specific serological tests eg. the *Treponema pallidum* immobilization and the *Treponema pallidum* haemagglutination assays).

ii) no correlation was found between the presence and titre of the antibody and resistance to *T.pallidum* ie. disease progression occurs from primary to secondary stages despite high titres of antibody.

iii) partial immunity could be passively transferred using large quantities of hyper-immune serum. The immunity was considered partial as lesions appeared on challenge with *T.pallidum* but were delayed and their severity and duration were diminished.

Several authors have reviewed the immunologic mechanisms at play in infectious syphilis with particular emphasis on the relative contributory roles of humoral and cell-mediated immune (CMI) responses (Musher, Schell & Knox, 1976; Pavia, Folds & Baseman, 1978; Metzger, 1979). The apparent inconsistency of the role of the humoral immune system led investigators to turn their attention to the CMI system.

1.1.3 The cell-mediated immune response

The earliest experiments performed implicating the CMI system in the host defense against *T.pallidum* infection involved the demonstration of delayed hypersensitivity skin reactions (Noguchi, 1912; Marshack & Rothman, 1951). This allergic response was notably absent in the early stages of syphilis (primary and secondary) - humans and rabbits - whereas a significant percentage of tertiary syphilitics reacted positively to an intradermal injection of treponemal extract. Laird & Thorburn (1966) showed that 67% of their patients with tertiary syphilis reacted positively to treponemal extract.

Another model utilized to demonstrate activation of the CMI system in the face of syphilis was to show that infection by one organism confers resistance to infection by another

antigenically unrelated organism known to activate that particular host immune response. Listeria monocytogenes is an organism known to activate the CMI response. In these experiments rabbits were initially infected with virulent *T.pallidum* followed by an inoculum of Listeria monocytogenes. The subsequent killing of the Listeria provided further indirect evidence for activation of the CMI system by *T.pallidum* (Schell et al, 1975).

The tests currently and most commonly employed are the LT assay and the cell migration inhibition tests, the former being the more popular assay.

The LT assay is based on the premise that peripheral blood lymphocytes undergo blastogenesis when cultured with a mitogen (phytohaemaglutinin (PHA) or concanavalin A (ConA)) or specific test antigen. By incorporating a radioactively labelled DNA precursor into newly synthesized DNA and then measuring the degree of incorporation (and therefore, degree of DNA synthesis) on a scintillation counter, one obtains a measure of the degree of blastogenesis, and therefore a quantitative assessment of lymphocyte responsiveness and presumably functional capacity.

The early literature abounds with studies utilizing this assay as an indicator of the CMI response. Levene et al, (1969 & 1971) and Musher & co-workers,(1974 & 1975) were able to show that, not only is the LT assay suppressed in syphilis, but syphilitic serum can be shown to suppress the LT of normal cells. Levene et al (1969) suggested that the impaired LT in early syphilis may be related to the partial depletion of the lymphocytes in the lymph nodes and spleen. They further postulated that the suppression of normal lymphocyte activity by syphilitic serum was most likely due to the presence of a plasma factor, either an auto antibody or a product of the spirochaete. Friedmann & Turk (1975) in a landmark study showed that in human syphilis, a spectrum of lymphocyte responsiveness exists, and that this responsiveness depended to a large degree on the stage of the syphilitic process. During the phase of dissemination of the disease, the early

secondary stage, reactivity is lost. As the disease progressed towards the latent stage significant degrees of lymphocyte stimulation can be demonstrated. Similar degrees of reactivity were also demonstrated during therapy. Pavia et al (1976 & 1977) demonstrated suppression of the LT assay in infected rabbits in the first 4 weeks of the infection utilizing 3 different mitogens, PHA, CON A and pokeweed. After 4 weeks, normal levels of blastogenesis were observed. The conclusion drawn from these findings pointed to an interrelationship between the appearance of symptomatic infection and the in vitro response to the T cell mitogens viz., that during the weeks of infection and dissemination of the spirochaete there was poor lymphocyte reactivity, followed by a blastogenic response during the phase of healing and recovery. The conclusion reached from all these studies is that an effective CMI response develops during the late secondary stage and, therefore, may be influential in the clearing of the organism, while resolution of the lesions occurs as the latent stage is entered.

However, the results of subsequent studies refuted the concept of immunosuppression in early syphilis. The theory of cellular immunosuppresion was based on work on peripheral blood lymphocytes. Lukehart and coworkers (1980) investigated the reactivity of lymph node and splenic lymphocytes from syphilitic rabbits to T.pallidum antigens and CON A. Both splenic and lymph node cells showed an early marked responsiveness to the T.pallidum antigens. In the case of the splenic cells the response was 100-600 fold higher than that of the uninfected control rabbits. The response of these cells to CON A remained unaffected. The investigators concluded that the results were consistent with a role for T-cell-mediated specific immunity to treponemal antigens early after infection and, therefore, did not support the hypothesis of depressed cellular immunity during syphilitic infection. Maret et al (1980) corroborated these findings showing that lymphocytes from the popliteal lymph nodes of infected rabbits did not exhibit depressed responsiveness to both T.pallidum antigens and mitogens.

The outcome of these studies laid to rest the popularly held theory that T-cell activity was depressed in the early stages of syphilis.

1.1.4 Immune complexes in syphilis

By the mid 1970s pieces of the research puzzle were being put together and a clearer picture was being formed. The successful vaccination of rabbits with live attenuated *T.pallidum* by Miller in 1973 suggested that protection against *T.pallidum* depended on activation of the CMI system. However, what had been clearly demonstrated was that the CMI response was defective in the highly infectious stages of syphilis. This phenomenon of immunosuppression (though transient) was thought to be due to inhibitory factors present in the syphilitic serum which were blocking the effector function of the lymphocytes (Levene et al, 1969; Kantor, 1975 and Ryan et al, 1975). As mentioned previously, the inhibitory factor was considered to be either an autoantibody or a product of the spirochaete. It was postulated that the consequence of such immunosuppression could lead to spirochaetal proliferation and the production of soluble products which would react with circulating antibodies resulting in the formation of "immune complexes" which in turn could be the basis for the clinical manifestations of secondary syphilis " many of which resemble the state of chronic serum sickness" (Levene et al 1969). Confirmation of these inhibitory factors as circulating immune complexes (ICs) was provided by the work Baughn et al (1980) and Folds, Maret & Rauchbach (1982). Studies in both animal (Baughn et al, 1980) and human subjects (Folds, Maret, & Rauchbach, 1982) provided corrobatory evidence of the suppressive effect of ICs on lymphocyte responsiveness in vitro.

The novel and astute concept by Levene et al (1969) that the manifestations of syphilis could be the consequence of immune complex mediated tissue damage fell on fertile ground and was later to lead to a flourish of investigative reports on the subject. The presence of circulating ICs in syphilis was first suggested by the evidence of ICs in syphilitic nephropathy (Braunstein et al, 1970; Bhorade et al, 1971). Several further studies

confirmed the presence of circulating ICs in adult syphilis (Solling et al, 1978; Engel and Diezel, 1980; Baughn et al, 1986). The detection of antitreponemal antibodies and treponemal antigen within IC deposits in the kidney (Gamble and Reardon, 1975; Tourville et al, 1976) and in the skin (Jorizzo et al, 1986) was firm evidence of an immunopathogenic role in syphilis.

1.1.5 Complement activity in syphilis

The role of complement in the immune response to the *T.pallidum* is not entirely clear. In vitro studies strongly suggest that complement exerts a suppressive effect on treponemal motility. Results of the study by Rice and Fitzgerald (1985) showed that treponemal motility fell significantly in the presence of complement. In its absence treponemal mobility was unaffected. Similarly, the work of Radolf et al (1986) using syphilitic immune rabbit and human sera confirmed that the immobilization of the treponemes occurred only when complement was present, corroborating the findings of Rice and Fitzgerald (1985).

Measurement of complement components in sera in human secondary syphilis by Baughn and co-workers (1986) demonstrated significantly reduced total haemolytic complement (CH50) and C3 levels. In addition, a strong inverse correlation existed between these complement components and levels of the circulating ICs measured. This relationship lent further support to the idea that syphilis may have an immune complex-mediated pathogenesis.

The role of complement in the pathogenesis of the Jarisch-Herxheimer reaction was evaluated by Loveday and Bingham (1985) in 9 patients with secondary syphilis. Major complement components including CH50, C3 and to a lesser extent C4 were all significantly reduced and the degree of fall paralleled the intensity of the reaction. The findings suggested that complement activation played an important role in relation to the clinical features in the Jarisch-Herxhiemer reaction.

1.2 CONGENITAL SYPHILIS

While a phenomenal amount of information and knowledge based on both animal and human studies has accumulated over the years, uncertainty still exists concerning the immune response in congenital syphilis. The major limiting factor had been the volume of blood required to perform the standard tests but with refinements in techniques such studies have now become possible.

1.2.1 The humoral immune response

The landmark study of Silverstein in 1962 debunked the prevailing concept that the human fetus is immunologically disadvantaged. He showed that the human fetus does in fact respond immunologically to *T.pallidum* infection. Aiuti and co-workers(1966) in an elegant study further demonstrated a significant elevation in the total serum IgM level in an infant with congenital syphilis. A bone marrow aspirate from the same patient showed increased plasmablast and plasma cell activity. These studies represented the earliest indication of the activation of the humoral response in congenital syphilis. They confirmed that the fetus, when antigenically stimulated, is capable of mounting an appropriate immune response. Subsequently, heightened total serum IgM activity has been observed in other chronic intrauterine infections and came to represent a marker of a chronic fetal infection (Alford, 1971). Its nonspecificity for congenital syphilis led to the adaptation of several other techniques in an attempt to improve its specificity e.g. the Fluorescent Treponemal Antibody Absorbed (FTA-Abs) IgM test (Scotti & Logan, 1968) and Western blot analysis (Lewis et al, 1990; Meyer et al, 1994).

Current evaluation of the humoral response in congenital syphilis continues to be directed primarily at measurement of immunoglobulin levels, particularly IgM. There is, however, a paucity of data on the activity of the B cells in congenital syphilis. Abstracts from the

Polish literature on B cell activity in perinatally acquired infections by Gajewska (1982) and Stachowski et al (1991) made no reference to the specific infections studied.

1.2.2 The cell-mediated immune response

The earliest reports on the cell-mediated immune response in congenital syphilis involved the histological examination of autopsy lymph node and splenic tissue (Festenstein et al, 1967; Levene et al, 1971; Turner & Wright, 1973). These investigators reported a significant depletion of lymphocytes from the paracortical areas of the lymph nodes and around the central arteriole of the spleen. The plasma cells around the medullary cord remained unaffected. These changes were present in neonatal rabbits and infants who had died of congenital syphilis.

Friedmann (1977) studied the cell-mediated immune response in infants with congenital syphilis by the LT assay. The study comprised of 3 groups of mother -infant pairs. Of the 38 infants, 7 were symptomatic, 17 were asymptomatic but were considered at risk of disease because of the diagnosis of latent syphilis in the mothers, and the remainder were normal newborn babies. The results showed that lymphocytic reactivity in the diseased group were significantly higher than that given by cells in either the "at risk" or the control groups. In addition cells in both latter groups were unresponsive compared to their mothers. The findings suggested that some infants with congenital syphilis have heightened lymphocytic reactivity in response to *T.pallidum* antigenic stimulation while those considered "at risk" have poorly developed cellular reactivity consistent with that observed in adults in the early infectious stages of syphilis.

The work of Gamboa et al (1984) in neonatal rabbits with experimental syphilis showed that not only were the cellular immune mechanisms functional as measured by the LT assay, but that the lymphocyte responsiveness to *T.pallidum* antigens was greater than that of the same cells from adults. These results did not support the concept expressed by other investigators that depressed cellular responsiveness is induced by *T.pallidum* infection.

1.2.3 Immune complexes in congenital syphilis

As in adult syphilis, evidence for ICs in congenital syphilis was first suggested by their presence in renal tissue in infants with nephrotic syndrome and syphilitic nephropathy (Kaplan et al, 1972; Wiggelinkhuizen et al, 1973).

The work of Dobson and co-workers (1988a) confirmed that elevated circulating ICs, predominantly IgM complexes, are present in infants with congenital syphilis. Analysis of the ICs demonstrated the presence of an 83-kilodalton treponemal antigen in all of the complexes isolated from the sera of the infected infants.

1.2.4 Complement Activity

Studies evaluating complement activity in syphilis has been conducted predominantly in human adults and in animal models as mentioned previously. There is no data available on the status of complement in infants with congenital syphilis.

1.2.5 The total and differential white blood cell counts and the nitroblue tetrazolium test

Numerous studies have reported on the nature of the white blood cell response in congenital syphilis. Whitaker and co-workers (1965) investigated the haematological changes in 9 diseased infants. The leucocyte count covered a wide range, and the differential counts varied from a lymphocytosis to a myeloid leukemoid response. Saxoni et al (1967) provided no further information in their study of 18 infants, except to state that they observed an increase in the count of immature cells. Chawla and associates (1985) observed 12 cases (14.8%) of leucocytosis. The highest cell count was 48.0 x 10^9/L, with immature forms present in a few of the patients. Other investigators have remarked on the presence of a significant monocytosis in some infants (Karayalcin et al, 1977; Stevens et al, 1987).

In contrast to these reports on the quantitative leucocytic response, few studies have been performed to evaluate white blood cell functional activity in syphilis in general, and in congenital syphilis in particular. One such study by Musher et al (1983) looked at the interaction between *T.pallidum* and human polymorphonuclear leucocytes (PMNLs) in vitro. They observed the rapid phagocytic uptake of the spirochaete into vacuoles, followed by the process of degranulation and oxidative burst activity as revealed by the stimulation of the chemiluminescence assay. These data suggested that the response of PMNLs to *T.pallidum* infection was one of normal phagocytic activity. However, the data failed to explain the continued survival of these organisms in the face of such intense PMNL activity.

Several studies have evaluated the outcome of the nitroblue tetrazolium test (NBT) in neonatal sepsis (Cocchi et al, 1971; Anderson et al, 1974). Similar studies have not been conducted in infants with congenital syphilis.

Since the publication of the aforementioned reports no further in-depth immune studies on congenital syphilis have emerged. This dearth of information has left an hiatus in our knowledge creating an ill-defined picture of the fetal immune response to the invading spirochaete. This study attempts to address some of these issues and thereby hopes to provide further insight and definition of a complex problem.

CHAPTER 2 - AIMS, MATERIALS and METHODS

2.1 INTRODUCTION

This chapter describes (a) the aims of the study, (b) the selection criteria, demographic and clinical data of the cohort of patients enrolled in the study, and (c) the methods employed, all of which are standard immunological laboratory tests.

2.2 AIMS

The following aspects of the immune system were evaluated:

1) Humoral Immunity (B cells and serum Immunoglobulins)
2) Cell-mediated Immunity (Lymphocyte transformation and T cell subsets)
3) Circulating Immune Complexes
4) The Classical Complement pathway (Total haemolytic complement (CH50) and C3)
5) Phagocytic function (Full blood and differential white cell counts, and the Nitroblue tetrazolium test)

2.2.1 Humoral Immunity

2.2.1.1 B Cells

Evaluation of the humoral response in congenital syphilis has been directed primarily at measurement of immunoglobulin levels, particularly IgM. A similar evaluation of the B cell response has not been reported. The aim of this study is to explore further the humoral response in congenital syphilis by quantifying the B cell response in the peripheral blood of affected infants.

2.2.1.2 Serum Immunoglobulins

Cogniscant of its limitations clinicians continue to utilize the total serum IgM as a screening test for congenital syphilis. Quantifying the total serum IgM is an integral part of the workup in suspected congenital syphilis. However, there are few published reports on the serum immunoglobulin concentrations following treatment. Furthermore no attempt has been made to correlate the serum IgM concentrations with the extent of the disease. Thus, the purpose was twofold:

a) to quantitate the pre-treatment and post-treatment levels of serum immunoglobulins (IgG, IgM and IgA) and
b) to correlate the serum IgM levels with the extent of the disease.

2.2.2 Cell-mediated Immunity

While there are numerous reports on animal and adult human studies relating to syphilis, there is a notable lack of information on the cell-mediated immune response in infants with congenital syphilis. This investigation addresses the phenomenon of blast transformation and T cell responses in newborns with congenital syphilis.

2.2.3 Circulating Immune Complexes

Dobson et al (1988a) were the first to report on the presence of ICs in congenital syphilis. These investigators sought firstly, to demonstrate the presence of ICs in infants with congenital syphilis and, secondly to characterize and analyze the components of the complexes. There are however, few published reports verifying the presence of ICs in congenital syphilis. In addition, there is no data available on the relationship between ICs and (a) other immunological variables and (b) the extent of the disease. To answer these questions the study set out to:

a) establish a reference range of ICs in the cord blood of normal term infants in a local newborn population;

b) confirm the presence of and quantitate the concentration of serum immune complexes in newborn infants with congenital syphilis;

c) correlate IC levels with other immunological variables;

d) correlate IC levels with the extent of the disease process and

e) determine IC concentrations in response to therapy.

2.2.4 Complement

The complement system plays an important role in the opsonization and lysis of organisms, neutrophil chemotaxis and activation of vascular responses to infection. Over the past few years it has become increasingly apparent that complement activation occurs in a number of disease states and the products of complement activation may play a pathogenetic role.

During complement activation enzymatic cleavage of components leads to their increases utilization and, in many disease states, reductions in the circulating levels of particular components occur (Whaley, 1985).

Complement can be assessed either functionally or by estimation of the individual components as proteins by immunochemical assays. The advantage of the functional assays e.g total haemolytic complement (CH50), is that they measure solely the functional activity of the proteins. Thus inactive complement components or cleavage products are not detected, a limitation of the immunochemical assays.

To my knowledge no formal study of the activity of the complement system in congenital syphilis has been reported. This study was designed to investigate the classical complement pathway in a group of newborn infants with congenital syphilis by measuring the total haemolytic complement (CH50) and C3 levels. The results were compared with a population of healthy matched control infants. Correlation between CH50 and IgM and IC levels were also sought.

2.2.5 Phagocytic Function

Numerous studies have been performed designed to investigate phagocytic function in the well and ill term and preterm newborn. Most evaluations are centered on the study of neutrophils which are considerably easier to isolate than monocytes or macrophages. Phagocytosis by neutrophils can be depressed for 2 basic reasons: (a) a quantitative deficiency or (b) an intrinsic functional defect. Most studies have concentrated on the latter, providing insight into the migratory, chemotactic, phagocytic and killing capacities of the neonatal neutrophil.

Several studies in healthy term (Dosset et al, 1969; Xanthou et al, 1975; Harris et al, 1983) and preterm infants (Forman and Stiem, 1969; Gahr et al, 1985) have shown normal phagocytosis over a wide spectrum of pathogenic organisms. Furthermore, following microbial ingestion, neutrophils from a healthy newborn population have shown an increased capacity (relative to adults) to produce oxygen metabolites by oxidative burst as measured by the nitroblue tetrazolium dye test (NBT) (Humbert et al, 1970; Anderson et al, 1974; Chandler et al, 1978).

In contrast, the ill infant appears to have a depressed capacity to oxygen metabolite production. Both Cocchi and co-workers (1971) and Anderson et al (1974) reported decreased NBT responses in infected infants. Using the

chemiluminescence assay, Shigeoka and associates (1979) clearly documented an abnormality in oxidative metabolism in the ill infant.

While the effect of a wide variety of organisms on neonatal neutrophil function has been evaluated, there exists, however, a distinct lack of information on the effect that the *T.pallidum* has on the neutrophil in neonates with congenital syphilis.

The purpose of the study was therefore, firstly, to document the total white cell and differential count responses in this population of newborns with congenital syphilis and then to compare the results with that of previous studies; and, secondly, to evaluate the effect of the *T.pallidum* on neutrophil function by examining the phagocytosis-associated respiratory burst activity utilizing the endotoxin-stimulated NBT dye test.

2.3 STUDY POPULATION

Eligibility for enrollment in this case-control study was based on the following criteria: 1) the cohort had to be newborn infants of less than 5 days postnatal age, born within the region serviced by the Peninsula Maternity and Neonatal Services (PMNS), University of Cape Town, 2) they had to fulfill the diagnostic criteria for congenital syphilis as set out by Kaufman et al, (1977) and modified by Mascola et al, (1985) (Appendix A), and 3) no antibiotics or other forms of therapy which may have an influence on the function of the immune system should have been administered to the patient prior to the study. The postnatal age of < 5 days was chosen because in the majority of hospitalised patients the diagnosis would have been made during that period of time, and the risk of such an infant acquiring a nosocomial infection would be low. It was assumed that the risk of acquisition of such infections in babies born at home or referred in from outlying hospitals would be high.

Twenty-five infants with congenital syphilis were seen between the 1st July, 1985 and the 31st March, 1989. Nineteen infants met the selection criteria. Nine were black and 10 were of mixed race. There were 11 males and 8 females. The 6 patients excluded from the study were older (3 weeks-3 months), were born outside the PMNS and were referred in from outlying hospitals.The absence of white infants from the study population was not a selection bias, but were due to two major reasons: firstly, hospitals within the ambit of the PMNS serve the indigent population, the majority of whom are black, of the metropole of CAPE TOWN; and secondly, the white population, historically the socio-economically advantaged group, are cared for predominantly in the private medical sector.

The demographic data collected on each patient included gestational age, postnatal age, birth weight, sex and race. The gestational age of each infant was evaluated by the Ballard score as set out by Ballard, Novak and Driver, 1979. This method of evaluating gestational age was adopted because it was not possible in all cases to obtain this information historically from the mother's last day of her menstrual cycle. The prenatal care or booking status of the mother was also recorded.

Prior to entry into the study the infants underwent a full clinical examination. Radiographs of the long bones were obtained and the serological tests for syphilis viz, the Venereal Disease Research Laboratory (VDRL) and the Fluorescent Treponemal Antibody Absorbed - IgM (FTA - Abs IgM) tests were performed on peripheral blood.

Peripheral blood samples for the specific laboratory investigations were obtained by venipuncture at the time of clinical presentation prior to the commencement of a 10 day course of Penicillin therapy. This was designated as Day 1. The entire cohort was studied within 48 hours of delivery. The tests were then repeated on completion of the course of therapy, and this was designated Day 10. Thus each infant acted as his/her own control. Details of the laboratory investigations are included under "Methods" section.

The subsequent management of the infants was left to the discretion of the attending physician. However all aspects of management were detailed, particularly the need for blood transfusion.

2.4 COMPARISON GROUP FOR THE DAY 1 STUDY PERIOD

The control sample was selected from a healthy population of neonates born within the PMNS during the same period as the study group. The relevant maternal data recorded included the booking and serological (VDRL & TPHA) status, the presence or absence of maternal fever, clinical chorioamnionitis or fetal distress in the intrapartum period. Infants eligible for enrollment into the comparative population were delivered of mothers who sought early prenatal care, had a negative serological status and had no significant intrapartum complications. In addition, those infants who initially met these inclusion criteria but who later became symptomatic were excluded.

Seventeen healthy newborns, matched for gestational age, birth weight, sex, race, and postnatal age met the selection criteria. The laboratory investigations outlined in the "Methods" section were performed within 24 hours of delivery (designated as day 1), on peripheral blood samples obtained by venipuncture.

2.5 CONSENT

Informed consent was obtained from the mothers prior to patient entry. The research protocol was approved by the Ethics and Research Committee of the University of Cape Town.

2.6 METHODS

The following investigations were performed on the cohort and controls on Day 1 and repeated in the syphilitic infants on Day 10:

2.6.1 Humoral Immune Response

2.6.1.1 B cells

B cells were quantified by labelling surface membrane immunoglobulins with F(ab)2 rabbit antihuman IgG, IgM and IgA in a 1/10 dilution and then counted with fluorescein - conjugated goat anti rabbit IgG, IgM and IgA (1/20 dilution). At least 50 cells were counted. The results were expressed in absolute numbers x 10^9/L (Samson et al, 1990).

2.6.1.2 Serum Immunoglobulins

2.6.1.2.1 *Measurement of immunoglobulins*

1 ml of clotted blood was collected for the immunoglobulins M, G and A assay by the nephilometry method on a Multistat III Centrifugal Analyser in the Chemical Pathology Laboratory, Red Cross Children's Hospital, Cape Town. Results were expressed in g/L.

2.6.1.2.2 *Correlation of IgM levels with the extent of the disease*

The total serum IgM levels were compared with degree of systemic involvement. The following clinical features were identified: skin signs; hepatomegaly; splenomegaly; pneumonia on chest radiography; skeletal abnormalities on radiography; haematological changes viz., anaemia and thrombocyopaenia; the presence of hydrops; and renal disease as evidenced by proteinuria ($\geq$ 2+) on urinary dipstix. The extent of the disease was

then scored according to the number of signs that were present. The maximum score was eight. The score obtained was then correlated with the IgM concentration.

2.6.2 Cell-mediated Immune Response

2.6.2.1 The Lymphocyte Transformation Assay

The cells and serum used in the Lymphocyte transformation studies were obtained from the following sources:

a) Newborn infants with congenital syphilis (patient cells and serum)
b) uninfected, healthy matched newborn infants (control cells and serum)
c) healthy, adult medical colleagues (reference cells) and
d) Group AB Rh positive serum from a pool of healthy adult male donors obtained from the Western Province Blood Transfusion Service (reference serum).

In each individual lymphocyte transformation assay in which the responses of the patient or control cells were studied, reference cells were cultured in parallel to provide a "normal" reference for comparison.

The processing, culturing and assaying of the lymphocytes were performed as described by Beatty and Dowdle (1978 & 1979). Modifications to this method included the substitution of Eagles medium buffered with Tris-HCl for RPMI 1640 (Gibco, New York) supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml), and the substitution of round bottom microtitre plates (Cat.No M 220 - 24 AR) for stoppered 11 x 70 mm polystyrene tubes utilized in the culturing of lymphocytes. Phytohaemaglutinin (PHA) was the mitogen utilized.

Any significant alteration or change in lymphocyte transformation may be secondary to either an inherent cellular dysfunction or to a factor(s) present within the serum. In order to distinguish between this possible effect, twelve crossover experiments were performed in which the patient (or control) and reference lymphocytes were incubated in patient (or control) and reference AB serum. An example of the format is as follows:

Microtitre plates Well numbers

A.	**Volume**	**1 - 3**	**4 - 6**	**7 - 9**	**10 - 12**
	x y z	Pat.cells Pat.serum Medium	Pat.cells AB serum Medium	Pat.cells Pat.serum PHA	Pat.cells AB serum PHA
B.	**Volume**	**1 - 3**	**4 - 6**	**7 - 9**	**10 - 12**
	x y z	Ref.cells Pat.serum Medium	Ref.cells AB serum Medium	Ref.cells Pat.serum PHA	Ref.cells AB serum PHA

The results expressed in disintegration per minute (dpm) reflect the degree of radioactive uptake by the stimulated cells.

2.6.2.2 T cells and subsets

CD3 (T cells), CD4 (helper T cells) and CD8 (suppressor T cells) cells were quantified using monoclonal antibodies (OKT3, OKT4, OKT8) (Ortho mune Ortho Diagnostics) as described by Beatty et al (1984). Cells from a healthy adult was used as a positive control. A negative control was effected by omitting the primary monoclonal antibody. The results were expressed in absolute numbers $x10^9/L$.

2.6.3 Circulating Immune Complexes

2.6.3.1 Measurement of Circulating Immune Complexes

Normal reference values for serum immune complexes in our healthy newborn population were unknown. To obtain such values clotted cord blood from an additional 20 normal term male and 16 female infants delivered vaginally following uncomplicated pregnancies, were assayed for immune complexes. One ml of clotted blood obtained by venipuncture from the syphilitic patients and the controls were also assayed. The samples were prepared as described by Haeney (1981). The assay was performed by the Renal Laboratory, Groote Schuur Hospital using the C1q binding method as described by Zubler et al (1976). Assays were preformed in batches on a weekly basis. The results were expressed as a percentage ^{125}I-C1q binding activity (%).

2.6.3.2 Comparison of immune complex levels with extent of the disease

Levels of ICs were compared with the extent of systemic involvement. The method employed was that described previously for the IgM correlation (2.6.1.3).

2.6.4 Complement

2.6.4.1 Total haemolytic complement (CH50) assay

Total haemolytic complement (CH50) was used as a measure of activation of the classical complement pathway. One ml of clotted blood was assayed using the standard method described by Kabat and Mayer (1971). The results were expressed in units of CH50 activity. The assay was performed in the Immunology

Laboratory, Red Cross Children's Hospital. The established reference range for the laboratory was 28 ± 7.4 units.

2.6.4.2 C3 assay

C3 was measured by radial immunodiffusion (NOR-PARTIGEN, BEHRING) on the sera obtained for the CH50 assay. Accuracy was checked with the Control serum for NOR-PARTIGEN (Cat.No. OSMH07). The results expressed in iu/ml were read from a Table of Calibration values supplied with the commercial kit.

2.6.5 Phagocytic cell function

2.6.5.1 Full blood and differential white cell counts

A full blood count (FBC), including a total white cell and differential count, were obtained on EDTA blood and performed by the Haematology Department, Red Cross Children's Hospital using standard automated techniques (Coulter PLUS IV) and the differential count was determined manually by reading 100 cells.

2.6.5.2 Measurement of the nitroblue tetrazolium test

The Nitroblue Tetrazolium Test, a qualitative assay of phagocytic cell function as described by Park et al (1968), was performed by the Immunology Laboratory, Red Cross Children's Hospital. NBT was obtained from Sigma (Cat.No N6876). The percentage of stained polymorphs with intracellular blue/black formazan was counted after stimulating with endotoxin (E.coli 027.B6 LPS, sigma L3254) (Goddard et al, 1992).

2.7 STATISTICAL METHODS

Unless stated otherwise, the Wilcoxon signed ranked test, a non-parametric statistical method used to compare two groups in matched or paired samples, was employed for all the comparative data. Spearman's rank correlation coefficient was used to determine concordance between two sets of variables. Significance was set at a $p < 0.05$

CHAPTER 3 - RESULTS

3.1 ANTENATAL AND CLINICAL PROFILE OF MOTHERS AND THE COHORT

Table 3-1: Clinical details of the patients

	Congenital syphilis (n = 19)	Controls (n = 17)
Birthweight (g)	2028 ± 643*	2139 ± 600*
Gestational age (weeks)	35.4 ± 1.9*	35.5 ± 1.6*
Sex (M/F)	12/7	11/6
Clinical features:		
⁝⁝ Hepatomegaly and/or splenomegaly	16 (84.2%)	-
Skin lesions	11 (57.9%)	-
▒ Anaemia	5 (26.3%)	-
Thrombocytopaenia	7 (36.8%)	-
Metaphysitis	17 (89.5%)	-
▪Conjugated hyperbilirubinaemia	3 (15.8%)	-
Syphilitic pneumonia	5 (26.3%)	
*f*Proteinuria	2 (10.5%)	
Serology:		
VDRL Positive	19	
FTA-Abs IgM positive	12	

* mean +- sd, ⁝⁝ > 2cm, ▒ Hb < 13g %, ▪ Direct reacting bilirubin > 15% of total, *f* ≥ 2+ on labstix

The clinical details of the patients are depicted in Table 3-1. Of the 5 infants with anaemia (8.4 to 11.5 g %), 3 infants received a single packed red blood cell transfusion (10ml/kg) on the 2nd day of life. There were 7 infants with a thrombocytopaenia ($< 100 \times 10^9$/L) and two infants with a thrombocytosis (626×10^9 and 978×10^9/L respectively).

There were 3 deaths (fatality rate, 15.8%) each on the 2nd day of life. The cause of death in each case was multisystem failure.

Table 3-2: Booking and treatment profile of mothers

Mothers of infants with congenital syphilis

n = 19

Antenatal	n	Rx status (n)
Unbooked	6 (31.6%)	0
Booked	4 (21.1%)	0
Late Booker	7 (36.8%)	5 (single injection)
Booked	2 (10.5%)	2 (Erythromycin)

n = number

The booking and treatment profile of the mothers are shown in Table 3-2. Approximately 32% of the mothers had not sought any form of antenatal care. Significantly 21.1% of the mothers had booked early but had received no Rx, either at the antenatal clinic or anywhere else. In 7 of the 19 infants (36.8%) the mothers had booked too late to receive any form of antenatal care. Five of the mothers received a single dose of Benzathine Penicillin in the week prior to delivery. Of these, 3 infants were symptomatic with clinical evidence of congenital syphilis. Of the two asymptomatic infants, both were VDRL positive with markedly elevated total serum IgM levels, while one had a positive FTA - Abs IgM.

Two mothers who were serologically positive received Erythromycin as they were considered to be allergic to penicillin. Of the infants born to these mothers, one infant was symptomatic at birth, while the other was asymptomatic but had abnormal radiographic metaphyseal changes and abnormal serology with an elevated total IgM, positive VDRL and FTA - Abs IgM.

3.2 HUMORAL IMMUNITY

3.2.1 B cells

3.2.1.1 Patients vs controls

Table 3-3 Comparison of peripheral blood B cells in syphilitic and healthy control infants, and infants between pre-treatment (Day 1) and post-treatment (D 10) phases

B Cells ($X10^9$ /L)

	Cases n = 19	Controls n = 17	p val	D1 n = 19	D10 n = 14	p val
Median	1.06	0.27	0.006	1.06	1.12	0.85
(*IQ range)	(0.52-1.38)	(0.17-0.80)		(0.52-1.38)	(0.65-1.50)	
Mean(**SD)	1.13(0.97)	0.45(0.33)		1.13(0.97)	1.22(0.66)	

* IQ = 25th - 75th interquartile range

** SD = standard deviation

n = number of patients

A significant difference in B cell numbers was found between the syphilitic infants and the controls (p=0.006) (Table 3-3).

Correlations were sought with other variables. B cell numbers correlated with the total lymphocyte count (r=0.53), p=0.019). No significant correlation was found with the other variables, in particular, with IgM levels (r = 0.16; p = 0.518).

In the control groups, B cells correlated significantly with the total lymphocyte count (r-0.846,p=0.0001) total T cells (r=0.87, p=0.0001), helper (CD4) cells (r=0.612, p=0.009) Suppressor (CD8) cells (r=0.931, p=0.0001).

3.2.1.2 Post-treatment vs Pre-treatment levels

Of the 16 infants who survived till completion of therapy, 14 infants were available to study. The two remaining infants were discharged prior to completion of treatment in hospital. As depicted in Table 3-3, the absolute numbers of B cells at completion of therapy still remained persistently raised.

3.2.2 Serum Immunoglobulins

3.2.2.1 Patients vs Controls

The total serum IgM level was markedly elevated in the syphilitic group. The median (interquartile range) was 2.32 g/L (1.06 - 4.76), compared to a level of 0.11 g/L (0.08 - 0.21) in the control group (p = 0.001) (Table 3-4). Immunoglobulins G and A showed no differences between the cases and the controls (p = 0.448 and 0.766 respectively). With reference to the IgA levels, the results of only 9 of the control group were available for comparison. Insufficient sera was available to technically conclude the IgA study in the control group.

3.2.2.2 Correlation between IgM levels and the extent of the disease

The total IgM levels, and the extent of the clinical disease are depicted in Table 3-5. No statistical correlation was found between the total IgM levels and the extent of syphilitic disease. Spearman's rank correlation coefficient was 0.436 which was not significant at the 5% level ($p = 0.061$)

3.2.2.3 Post-treatment vs Pre-treatment levels

The total serum IgM levels fell on completion of treatment but was not significant statistically ($p = 0.26$) (Table 3-4). Immunoglobulins G and A remained unchanged.

TABLE 3-4: Comparison of total serum IgM in syphilitic and healthy control infants, and between pre-treatment (day 1) and post-treatment (day 10) phases in the syphilitic infants

Total serum IgM (g/L)

	Syphilis	**Control**	**p-value**	**Day 1**	**Day 10**	**p-value**
Numbers	19	17		19	14	
Median (interquartile range)	2.32 (1.06-4.76)	0.11 (0.08-0.21)	0.001	2.32 (1.06-4.76)	1.66 (1.09-4.08)	0.26
Mean (SD)	3.58 (3.51)	0.14 (0.08)		3.58 (3.51)	3.46 (4.09)	

TABLE 3-5 Relationship between total serum IgM levels and the extent of the disease

Clinical features

IgM Level g/l	CLINICAL FEATURES FTA-Abs IgM	Skin	Liver	Spleen	Pneumonia	Bones	Haem changes	Hydrops	Protein	Extent of disease
12.48	+	+	-	+	+	+	+	-	-	5
11.12	-	-	-	+	-	+	+	-	-	3
8.24	+	+	+	-	+	+	+	+	+	7
5.79	-	-	-	+	-	-	-	+	-	2
4.76	-	+	+	+	-	+	+	-	-	5
3.78	-	+	+	+	-	+	-	-	-	4
3.10	+	-	+	+	-	+	-	-	-	3
3.08	+	+	+	+	+	+	+	-	-	6
3.00	+	+	+	+	-	+	-	-	-	4
2.32	+	-	+	+	-	+	-	-	-	3
2.11	+	+	+	+	+	+	+	-	-	6
1.75	+	+	-	-	-	+	-	-	-	2
1.19	-	+	+	-	-	+	-	-	-	3
1.10	+	-	+	+	+	+	-	-	+	5
1.06	+	-	-	-	-	+	-	-	-	1
1.00	+	+	+	+	-	+	+	-	-	5
0.86	-	-	-	-	-	-	-	-	-	0
0.64	-	+	+	-	-	+	-	-	-	3
0.63	+	-	-	-	-	-	-	-	-	0

Pneum. = pneumonia
Protein. = proteinuria
haem. = haematological changes

3.3 CELL-MEDIATED IMMUNITY

3.3.1 Lymphocyte Transformation Assay

3.3.1.1 Patients vs controls

Incubation of the PHA stimulated patient and control lymphocytes in autologous serum showed a significant difference in radioactive uptake between the 2 sets of lymphocytes. The syphilitic lymphocytes had a radioactive uptake median of 40380 dpm compared to 51583 for the control lymphocytes ($p=0.008$) (Table 3-6).

When the syphilitic lymphocytes were incubated in AB serum, radioactive uptake increased significantly from 40380 to 45304 dpm ($p = 0.011$). There was no significant difference in uptake when the control lymphocytes were incubated in the AB serum (median 51583 to 52766 dpm, $p = 0.087$). However, though smaller, the difference between the syphilitic and control lymphocytes in their degree of radioactive uptake in the AB serum was still significant (median 45304 vs 52766 dpm, $p = 0.023$) (Table 3-6).

Incubation of the PHA stimulated reference lymphocytes in syphilitic and control serum produced no differences in the degree of radioactive uptake (median 22641 vs 23261 dpm, $p = 0.660$).

Table 3-6 Lymphocyte transformation: PHA stimulated lymphocytes of syphilitic infants compared to matched controls cultured in autologous and AB serum (dpm)

	CONGENITAL SYPHILIS n = 19			CONTROL n = 17			p value
	Median	IQ range	Mean (SD)	Median	IQ range	Mean (SD)	
Autologous serum	40380	20257-47582	35574 (14750)	51583	38683-58544	49938 (10609)	0.008
AB serum	45304	32213-51083	42109 (13196)	52766	43449-58761	52254 (9631)	0.023

IQ range = 25th - 75th interquartile range

SD = standard deviation

3.3.1.2 Post-treatment vs Pre-treatment levels

Of the sixteen survivors, 14 infants had lymphocyte transformation tests performed at the time of completion of treatment.

Lymphocyte reactivity in autologous serum had increased from a pre-treatment level of 40380 (range 20257 - 47582) dpm to 45295 (range 35030 - 53269) dpm on day 10. Though the degree of reactivity had shown an upward trend the difference was not statistically significant ($p = 0.08$).

Incubation of the lymphocytes in AB serum showed a further rise in radioactive uptake to a median of 48925 dpm (range 39524 - 53613). However, the difference was not significant ($p = 0.19$).

When lymphocyte reactivity in syphilitic serum and in AB serum on days 1 and 10 were compared, no statistically significant differences were found ($p = 0.53$ and 0.45 respectively).

Figure 3-1 graphically summarizes the lymphocyte transformation responses in autologous and AB serum in the infants with congenital syphilis on day 1 and day 10, and in the matched controls. The individual values with the medians, mean, interquartile ranges and standard deviations are shown in Appendix B.

3.3.2 T cells (CD3) and subsets (CD4 and CD8 cells)

3.3.2.1 Patients vs controls

An analysis of CD3, CD4 and CD8 counts in infants with congenital syphilis and matched controls, showed no difference in the absolute numbers of CD3, CD4 and CD8 cells between the two groups (Table 3-7).

Table 3-7 Lymphocyte subsets of syphilitic infants compared with matched controls showing absolute numbers X 10^9/L (median and IQ range)

Group	CD3	CD4	CD8	CD4:CD8 ratio
Congenital Syphilis	3.68 (3.16-4.98)	2.16 (1.25-2.79)	0.93 (0.68-1.76)	1.67 (1.19-3.33)
Controls	3.37 (2.0-4.05)	2.02 (1.14-2.62)	1.10 0.66-1.45)	2.0 (1.34-2.79)
p-value	0.236	0.887	0.619	0.776

No significant difference found between congenital syphilis and controls.

LYMPHOCYTE STIMULATION IN THE PRESENCE OF PHYTOHAEMAGLUTININ

Results expressed in disintegration per minute (dpm).

Lymphocytes were cultured in autologous or pooled human serum

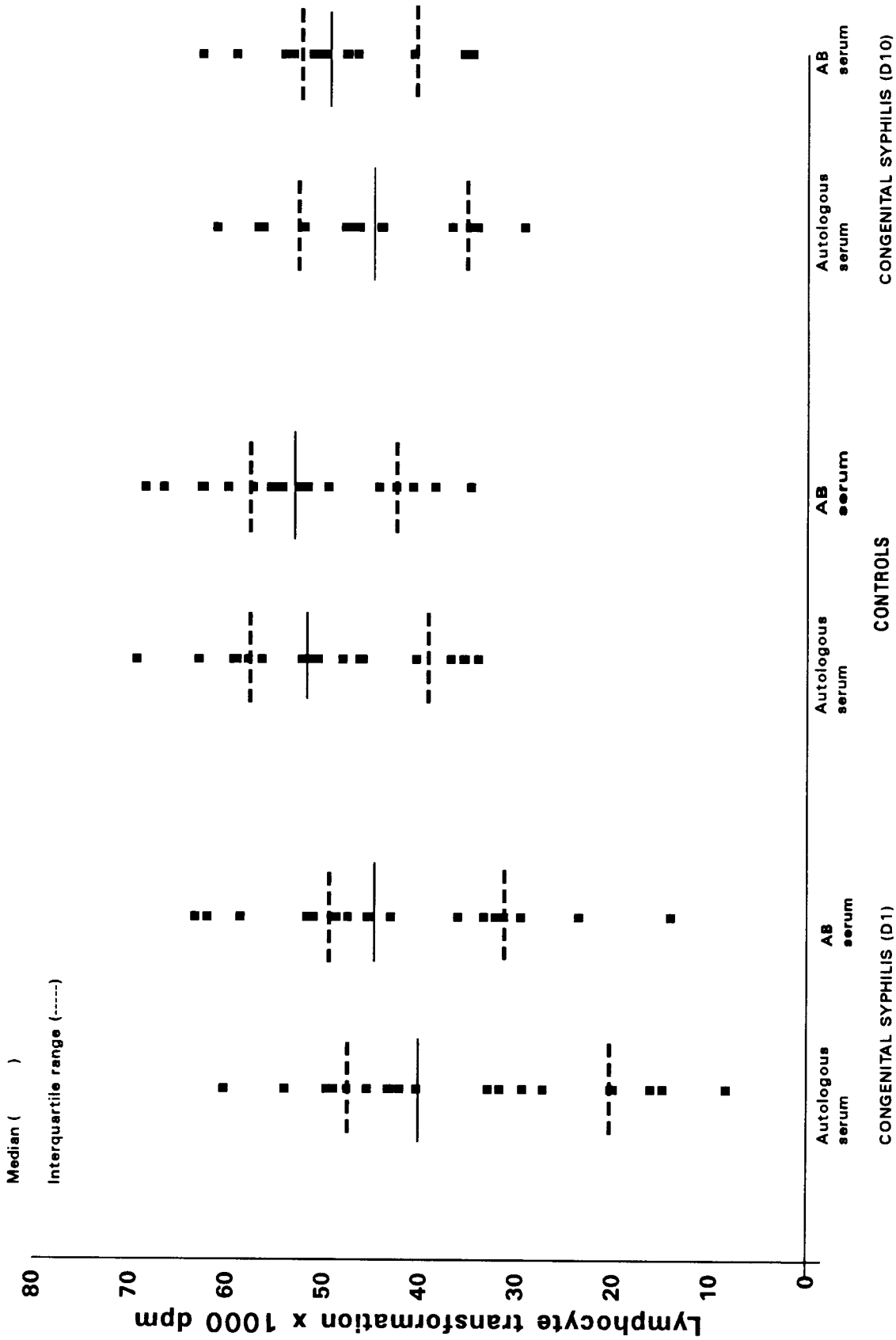


Fig. 3-1

3.3.2.2 Post-treatment and pre-treatment comparison of CD3, CD4 and CD8 concentrations

Further evaluation of the lymphocyte subsets after completion of therapy demonstrated no changes in the CD3, CD4 and CD8 counts (Table 3-8)

Table 3-8 Lymphocyte subsets of syphilitic infants and day 1 and day 10 at completion of penicillin therapy (median and IQ range) (x 10^9/L)

Period	CD3	CD4	CD8	CD4:CD8 Ratio
Day 1	3.68 (3.16-4.98)	2.16 (1.25-2.79)	0.93 (0.68-1.76)	1.67 (1.19-3.33)
Day 10	3.90 (3.22-5.51)	2.45 (1.74-4.47)	1.13 0.85-1.56)	2.2 (1.34-2.79)
p-value	0.71	0.38	0.67	0.80

3.4 CIRCULATING IMMUNE COMPLEXES

3.4.1 Immune complexes in cord blood, congenital syphilis and controls

Figure 3-2 depicts the IC results in cord blood, and in the syphilitic and control infants.

The range of serum IC concentrations in cord blood was similar for both the sexes, 0 - 4.3% in females and 0 - 4.2% in males. The medians were 3.35% and 3.2% respectively. These values served as the reference range.

Sixteen infants with congenital syphilis were studied. All had significantly elevated levels of ICs. The lowest value was 5.4% and the highest 62% with a median (IQ range) of 13.4% (8.6-24.5). In contrast the controls had levels similar to that detected in cord blood (median 3.2%). The difference was highly significant, $p = 0.0007$.

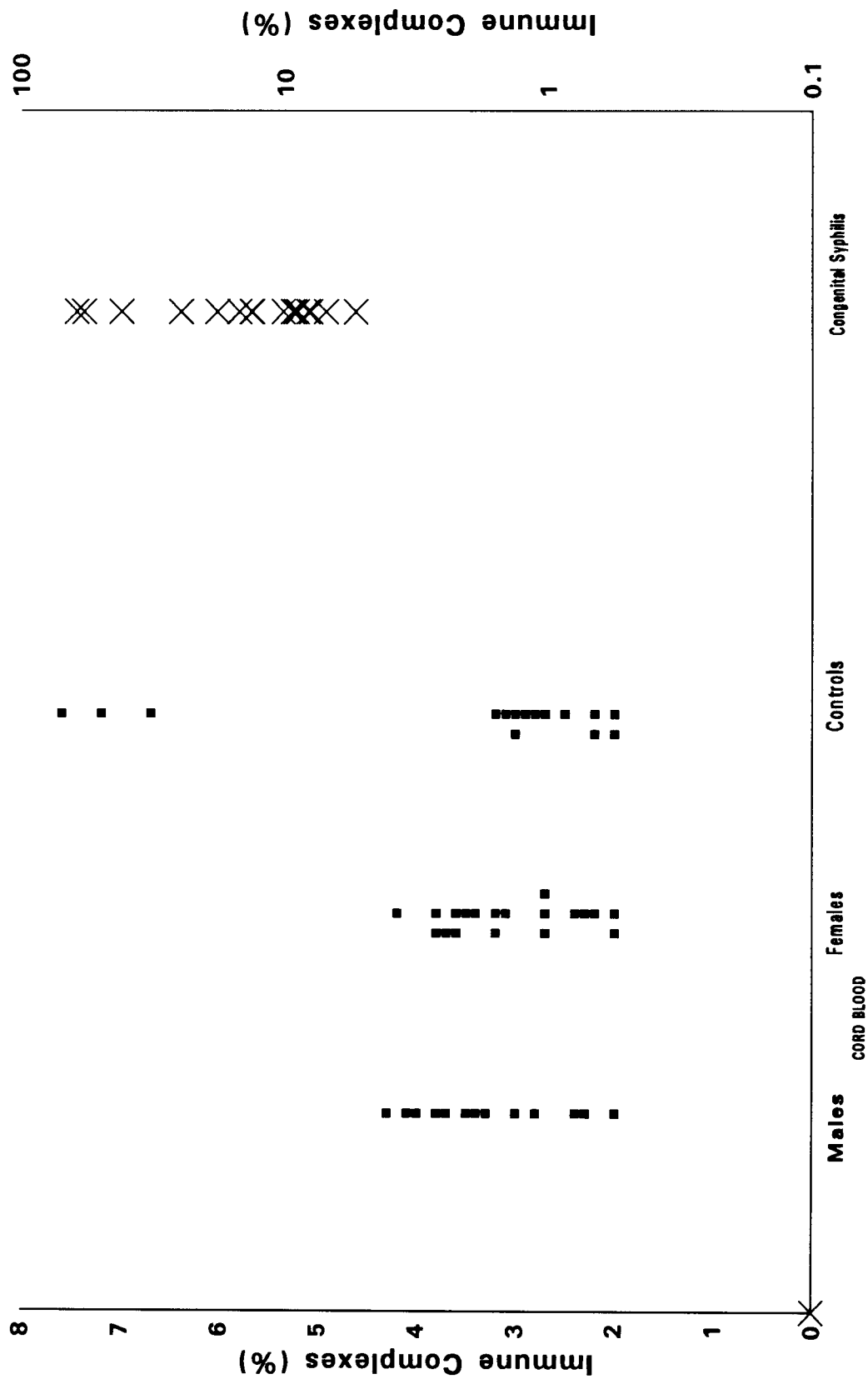
3.4.2 Correlation between immune complexes and other variables

There was a significant positive correlation between total IgM and IC concentrations in the syphilitic infants ($r = 0.765$, $p = 0.0003$). This is depicted graphically on the scattergram (Fig 3-3). The correlation between ICs and the other variables was not significant. More specifically, there was no significant correlation between IC and CH50 levels ($r = - 0.365$, $p = 0.179$).

In the control group, no correlation was found between IC levels and the other immunological variables.

Fig. 3-2

CIRCULATING IMMUNE COMPLEXES IN CORD BLOOD, IN CONTROLS AND IN CONGENITAL SYPHILIS



RELATIONSHIP OF IMMUNE COMPLEXES AND TOTAL SERUM IgM LEVELS

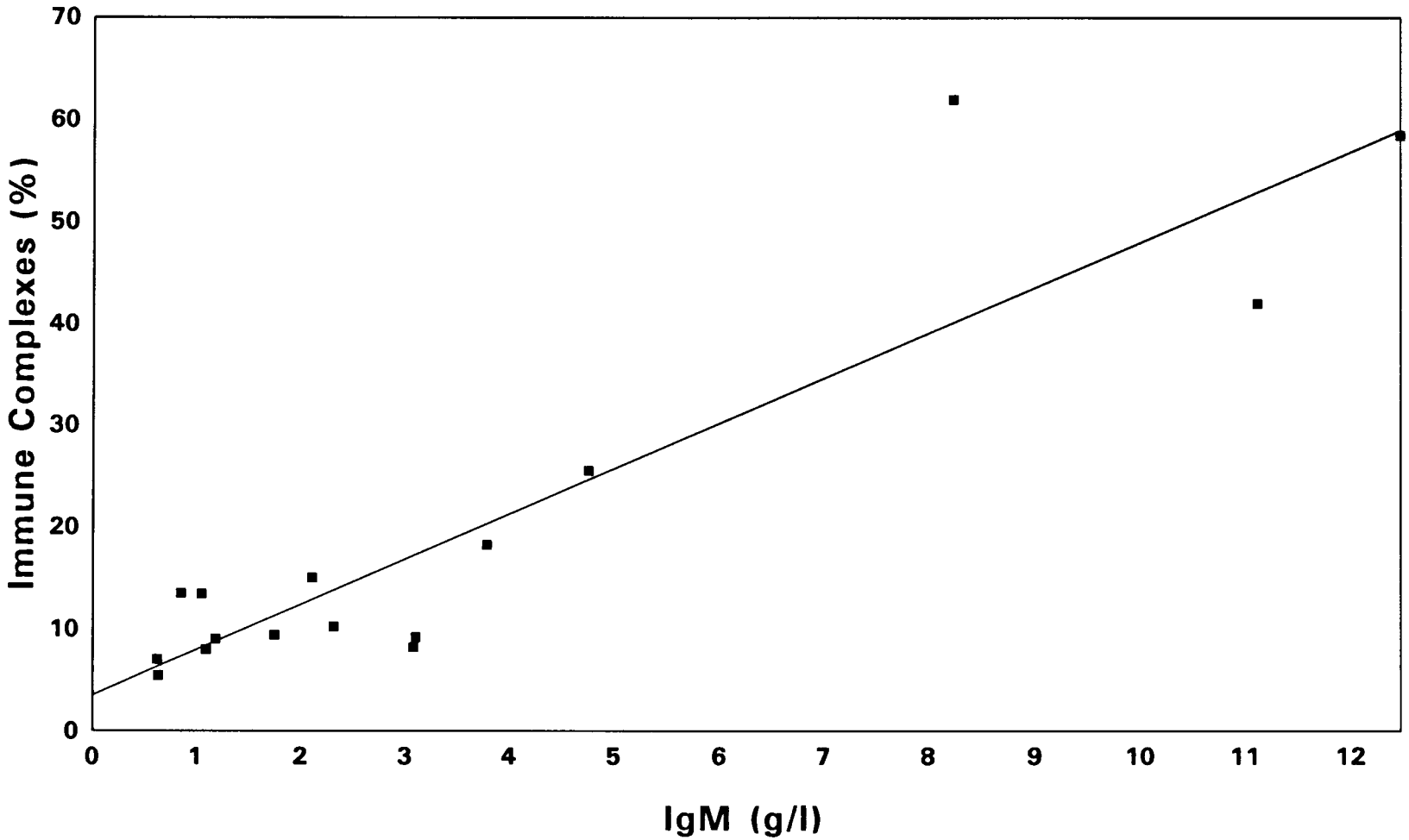


Fig. 3-3

3.4.3 Relationship between immune complex levels and the extent of the disease

Immune complex concentrations and the extent of the disease for each patient is shown in Table 3-9. In addition, Figure 3-4 graphically depicts the relationship between IC levels and the score of the extent of the disease.

Though the plot of the data shows that there is a tendency for IC concentrations to increase with the severity of the disease or vice versa, the correlation between the two variables was not significant ($r = 0.361$, $p = 0.169$).

3.4.4 Effect of treatment

Follow up of determinations of ICs was possible on 12 syphilitic infants. Overall, there was a significant reduction in IC levels at completion of therapy (median 6.95%, $p = 0.0008$). This reduction was observed particularly in those patients with IC concentrations of $\leq 18\%$. There were four infants (case numbers 15, 6, 13 and 1) who had markedly elevated levels prior to therapy (Table 3-9) and this persisted post therapy, with levels of 56.2%, 56.0%, 35% and 24.4% respectively.

The clinical course of all the patients were similar irrespective of the initial IC level. At the time of discharge the infants were thriving and on physical examination, the organomegaly had significantly receded in size and the skin lesions had healed. However, the persistence of the high IC levels in four infants was reason for concern, thus, a repeat IC assay and clinical evaluation one month after discharge was arranged. Unfortunately, the patients did not return for follow up.

Table 3-9 Relationship between IC levels and the extent of the disease

CaseNo	IC	CLINICAL FEATURES								Extent
		Skin	Liver	Spleen	Pneum	Pruria	Bones	Haem	Hydrops	
15	62	+	+	-	+	+	+	+	+	7
6	58.5	+	-	+	+	-	+	+	-	5
13	42	-	-	+	-	-	+	+	-	3
1	25	+	+	+	-	-	+	+	-	5
12	18.2	+	+	+	-	-	+	-	-	4
17	15	+	+	+	+	-	+	+	-	6
5	13.5	-	-	-	-	-	-	-	-	0
10	13.4	-	-	-	-	-	+	-	-	1
14	10.2	-	+	+	-	-	+	-	-	3
9	9.4	+	-	-	-	-	+	-	-	2
4	9.2	-	+	+	-	-	+	-	-	3
2	9.0	+	+	-	-	-	+	-	-	3
16	8.2	+	+	+	+	-	+	+	-	6
11	8.0	-	+	+	+	+	+	-	-	5
18	7.0	-	-	-	-	-	-	-	-	0
3	5.4	+	+	-	-	-	+	-	-	3

Pneum. = pneumonia

Pruria. = proteinuria

haem. = haematological changes

PLOT OF IC AGAINST EXTENT OF DISEASE

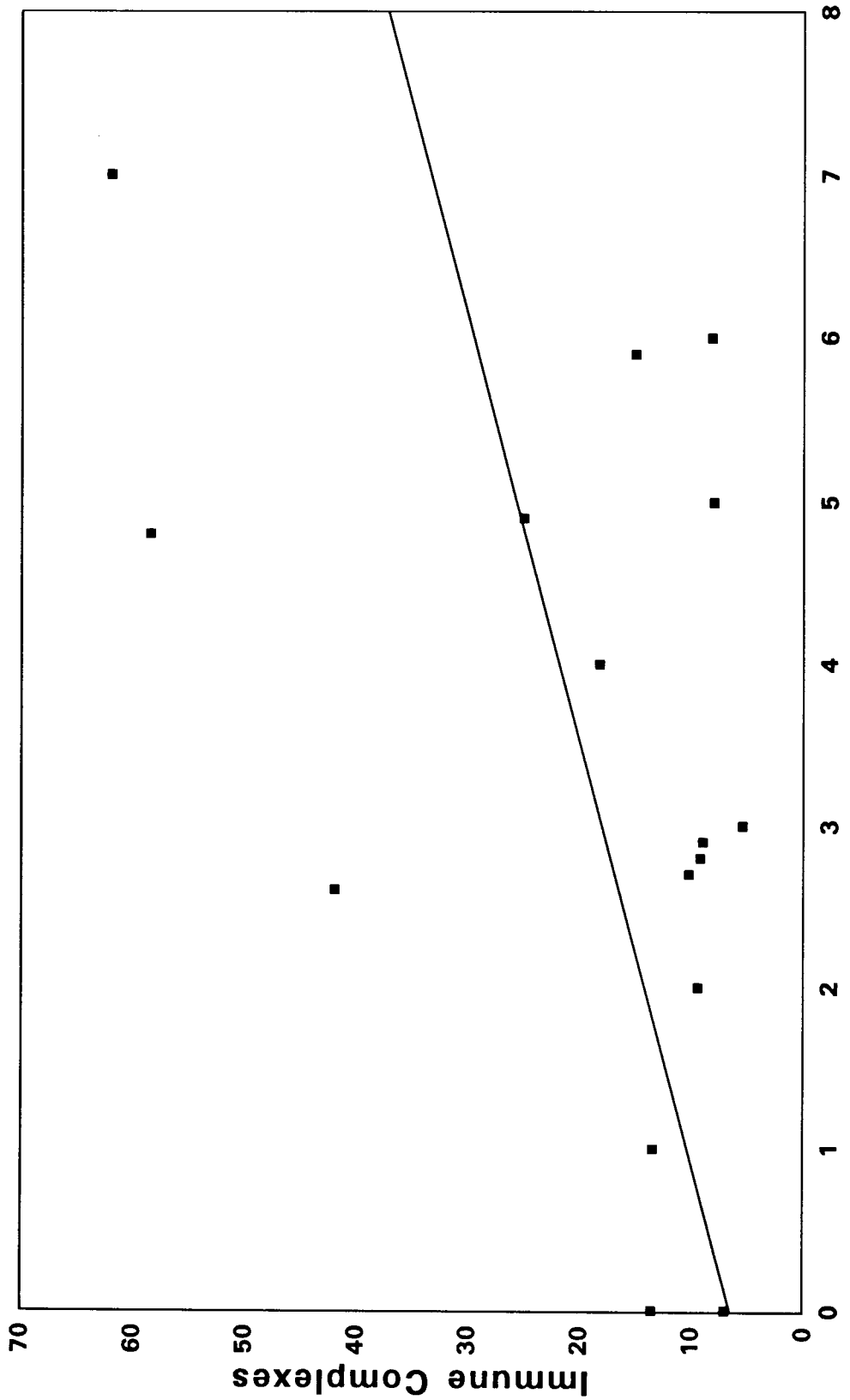


Fig. 3

3.5 COMPLEMENT

3.5.1 Patients vs controls

Table 3-10: Total haemolytic activity (CH50) (units/ml) in syphilitic newborn infants compared with matched controls

	n	Mean	SD	Median	IQ range	p value
Congenital Syphilis	11	16.3	8.0	12.5	10-24.4	0.014
Controls	11	23.4	6.7	21.4	17.2-27.0	

n = number of patients or controls

The CH50 assay was performed on 16 infants with congenital syphilis and 14 controls. The signed rank test for paired data was performed on the 11 matched samples. The results are depicted in Table 3-10. The data set for the 2 groups is set out in Appendix C.

The CH50 levels were significantly lower in those infants with syphilis compared to their healthy matched controls ($p = 0.014$).

The Spearmans correlation coefficient demonstrated a negative linear correlation between the CH50 and serum IgM levels in the syphilitic patients ($r = - 0.512$; $p = 0.042$) The scatterplot illustrates the relationship graphically (Fig 3-5). There was no correlation in the control group.

The relationship between CH50 and IC levels was not significant at the 5% level with a correlation coefficient of $r= -0.365$ and a p value of 0.179.

RELATIONSHIP BETWEEN CH50 AND IgM LEVELS IN NEWBORNS WITH CONGENITAL SYPHILIS

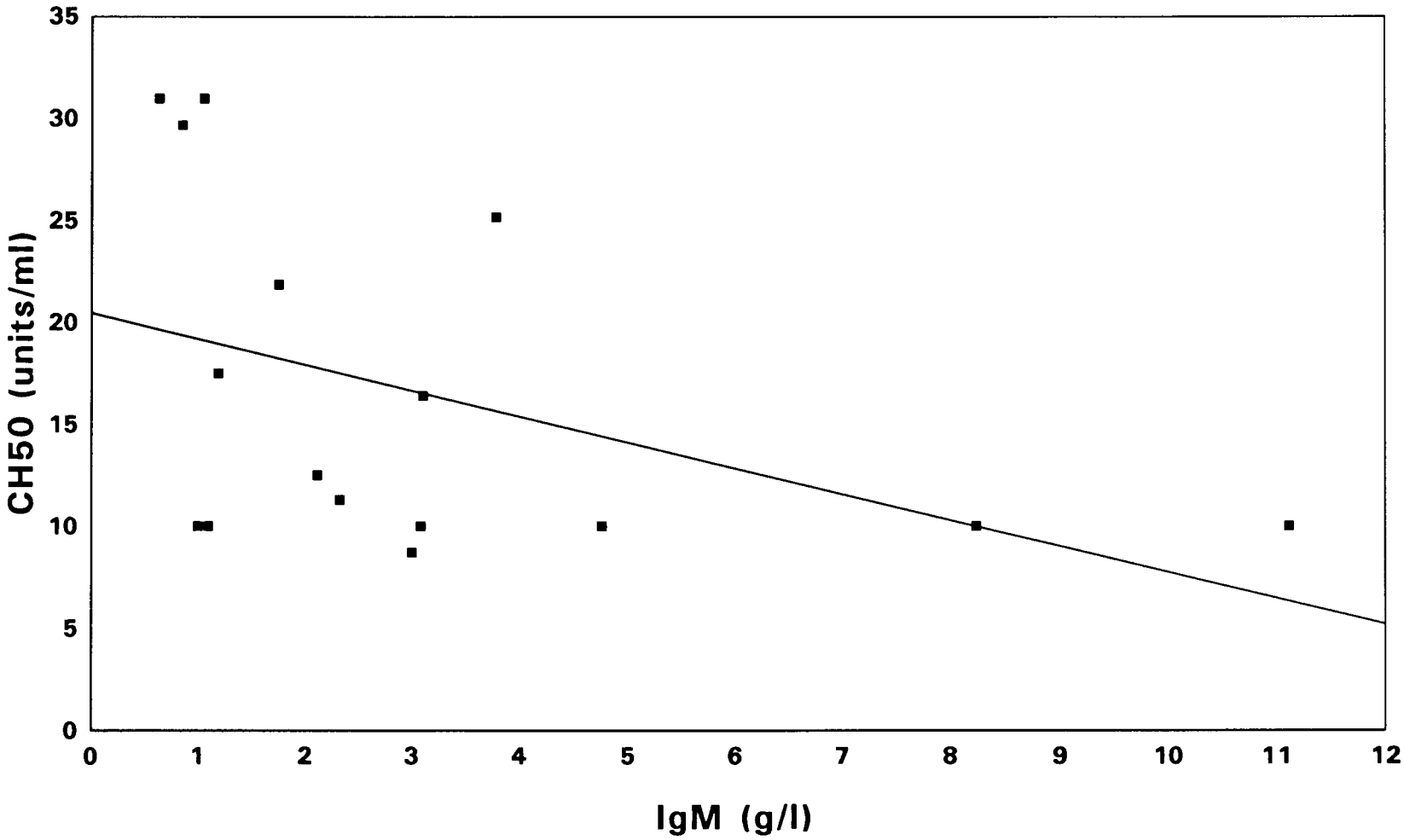


Fig. 3-5

Sera sufficient for the C3 assay was available for only 7 of the study infants and 6 controls. The data set is set out in Appendix D. Because the Wilcoxon signed rank test was not suitable for this data set the Mann-Whitney U test for unpaired data was used to compare the C3 results. No difference between the 2 groups was found (Table 3-11).

Table 3-11 C3 levels (iu\ml) in infants with congenital syphilis and healthy matched controls.

	n	mean	sd	median	p-value
Congenital syphilis	7	85.84	26.23	76.20	
controls	6	88.83	24.56	85.40	0.66

3.5.2 Post-treatment CH50 levels

At completion of therapy sera was obtained from 12 of the 16 survivors. There was no significant difference in CH50 levels on day 10 when compared with the pre-treatment levels (Table 3-12).

Table 3-12 CH50 levels on day1 and day 10 in infants with congenital syphilis on completion of treatment

day	n	mean	sd	median	IQ range	p-value
1	12	18.56	8.85	16.95	17.45	
10	12	20.34	8.93	20.40	20.45	0.27

3.6 PHAGOCYTIC FUNCTION

3.6.1 Patients vs controls

3.6.1.1 Total white cell and differential counts

Table 3-13 The white blood cell (WCC) (10^9/L) and % differential counts in infants with congenital syphilis

Patient	Leucocytes	Neutrophils	Lymphocytes	Monocytes	Eosinophils
1	6.7	56	28	2	6
2	7.6	35	53	9	3
3	12.7	42	33	17	8
4	12.0	35	58	7	-
5	8.4	36	57	3	4
6	15.6	49	41	9	1
7	34.6	71	18	11	-
8	41.0	54	37	1	-
9	15.5	37	49	10	3
10	12.4	25	64	8	3
11	57.3	75	14	5	1
12	16.2	55	40	5	-
13	11.9	25	73	1	1
14	7.8	39	55	6	-
15	14.1	80	18	-	2
16	25.3	64	14	7	-
17	10.2	45	10	5	5
18	10.8	31	64	4	1
19	14.3	57	37	6	-

White cell and differential counts were performed on all 19 infants with congenital syphilis. The results are shown in Table 3-13.

The leucocyte counts ranged from 6.7 to 57.3 x 10^9/L with wide variations in differential counts. Three infants (15.8%) had a leucocytosis (patients 7, 8, 11) with one infant (patient 11) displaying a leukaemoid response. In 2 of the patients (7 and 11) the leukocytosis was associated with a marked neutrophilia. Eight of the 19 infants (42.1%) had a lymphocyte predominance. Three infants (patients 3, 7 and 9) presented with absolute monocyte counts greater than the upper limit of normal (Weinberg, 1985).

3.6.1.2 Nitroblue tetrazolium test (NBT)

The stimulated NBT test was performed in 18 patients and 17 controls. Infants with congenital syphilis had a median NBT test result of 70% (range 35% - 77%). In comparison, the median value in the control population was 74% (62 - 84%). The differences were not significant (p = 0.20) Table 3-14.

Table 3-14 Comparison of Nitroblue tetrazolium test (NBT) in congenital syphilis and matched controls

	NBT (%)		
	Congenital syphilis n = 18	**Controls n = 17**	**p value**
Median	70	74	0.20
Interquartile range	35-77	62-84	
Mean (SD)	57.7 (27.4)	71.9 (15.7)	

3.6.2 Post-treatment vs Pre-treatment levels

There were no differences in white cell counts or NBT levels between day 1 and day 10. The p values were 0.32 and 0.68 respectively.

CHAPTER 4 - DISCUSSION

4.1 Antenatal and clinical profile of mother and cohort

The management of the "unbooked" mother continues to be a major problem for both the obstetrician and the neonatologist alike.

In the metropolitan area of Cape Town, midwife obstetric units (MOU) have been set up to provide antenatal, natal and postnatal care for mothers residing in the peri-`urban' areas of greater Cape Town (Van Coeverden de Groot et al 1978). The residents here make up the indigent populace of the city and thus provide the major public health problems.

Specifically, the overall prevalence of unbooked mothers in the MOU's is 6.8% (D Greenfield - personal communication). The perinatal mortality rate (PMR) in this group of mothers for 1992 was 120,3/1000 total births, in contrast to the overall PNM rate of 24.8. The unbooked mother as a high risk category is further highlighted in relation to syphilis. In a recent study by Mlisana et al (1992), of 114 unbooked pregnant mothers who delivered at King Edward VIII Hospital in Durban, 30.7% had reactive syphilis serology, of whom 11.5% delivered infants with clinical evidence of congenital syphilis. A similar prevalence of 32.3% was documented by Swingler & Van Coeverden de Groot (1993) in their study in an MOU in Khayelitsha, Cape Town. This was in contrast to the overall prevalence rate of 12.7% of reactive syphilis serology for mothers delivering in the midwife obstetric units.

Other authors have previously highlighted the problem that mothers who do not present for antenatal care present the major problem in the antenatal prevention of syphilis (Stray-Pedersen, 1983; Kaufman et al, 1977).

In the present study 31.6% of the infants with congenital syphilis were born to mothers who had not presented for routine antenatal care. Furthermore, $\pm$ 37% of the mothers had

sought antenatal care too late to be adequately treated and in so doing placed the fetuses at risk. It is accepted that a single injection of penicillin early in pregnancy is regarded as adequate treatment (CDC, 1985). Thus any mother who is treated less than 30 days prior to delivery should be regarded as being inadequately treated and the infant should be regarded as having congenital syphilis (Ikeda & Jenson, 1990). As can be seen from this study, 3 of the five infants whose mothers had been treated late in pregnancy were symptomatic at birth. In addition, of even greater concern were 4 infants (21.1%) whose mothers had booked but had received no treatment.

The mother who is regarded as being allergic to penicillin presents a special management problem. Two of the mothers in the study were considered to have penicillin allergy and were consequently treated with a course of erythromycin. Both infants had evidence of early stage congenital syphilis. South et al (1964) were the first to recognise and record the failure of erythromycin to adequately treat the fetus. Subsequently a number of other authors have highlighted this problem (Fenton & Light, 1976; Hashiasaki et al, 1983). Consequently, the broad guidelines strongly discourage the use of erythromycin because of the high risk of failure to cure infection in the fetus. It is suggested that in cases of suspected hypersensitivity to penicillin in maternal syphilis, the history of allergy should initially, be closely scrutinized. If necessary they should be skin tested and either treated with penicillin or referred for desensitization. However, if mothers have been treated with erythromycin, the infants require close surveillance and follow-up, and if this is not possible, they should be treated (CDC, 1989b).

The occurrence of haematological disturbances in congenital syphilis is not uncommon. The frequency of anaemia has been observed to be as low as 9% (Mascola et al, 1985) or as high as 75% to 95% (Chawla et al 1985, Sartin et al 1965). The differences probably reflect the different ages at which the patients were initially seen. In the retrospective review of Mascola et al (1985) the patients were all newborn infants seen within the 1st

week of life, while in the study of Chawla et al (1985) the patients represented a wide range of postnatal ages, the eldest infant being 6 months of age.

Of particular relevance to this study was the treatment of the anaemia with packed cell transfusions and the possible impact this would have in the interpretation of the results. Blumberg and Heal (1994) recently reviewed the published work on the effects of transfusion on immune function. Current opinion, based on animal and clinical studies, suggests that allogeneic transfusions are associated with immune dysregulation. Recent data from adult human studies have shown that a reduction in CD4 helper and natural killer cells and an increase in CD8 suppressor and B cells, are associated with allogeneic transfusions. Leucocytes have long been thought to be the main mediators of such transfusion induced immuno-modulation. This effect has been shown to be minimized by the use of red blood cell concentrates or leucocyte-depleted blood products.

Pahwa et al (1985) investigated the effect that age and blood transfusions would have on the T lymphocyte sub-populations in a group of high risk infants. The postnatal ages of their infants ranged from 1 week to 7 months and their gestational ages from 27 to 43 weeks. A significant number of the infants had more than one blood transfusion. They observed that, normally, infants <3 months of age had increased T4/T8 ratios when compared with normal adult values. This increase in the T4/T8 ratio was due to an increase in the proportion of helper cells and a concomitant decrease in the suppressor cells. This increase ratio decreased with increasing postnatal age to reach adult values between 3 and 7 months of age.

To determine whether the frequency of blood transfusions bore any relationship to the T cell subset distribution, the investigators plotted T4/T8 ratios against the number of blood transfusions. The time interval between the last transfusion and the study varied between 1 and 3 weeks.

There was an inverse relationship between the number of blood transfusions and the T4/T8 ratios particularly in the infants aged 2 to 4 weeks. Unfortunately the influence of blood transfusions on T cell subsets in infants less than 2 weeks could not be evaluated because the sample number in this category was too small. The explanation advanced by the investigators to explain their findings is based on the existence of the phenomenon of transfusion-associated immunosuppression. Decreased T4/T8 ratios in multitransfused patients have been correlated with elevated serum ferritin levels. This iron-related immuno-suppression has been observed in other multi-transfusion status (De Sousa 1983).

Three of the five anaemic infants (Hb $<13g\%$) (Oski and Naiman 1972) in the present study received a single packed cell transfusion at 10ml/kg on the second day of their illness. As the initial blood samples for study was taken prior to therapy being instituted, the blood transfusion would not have clouded the interpretation of these results. However,while there may have been concern in the interpretation of the results on the 10th day of study, the work of Pahwa et al (1985) showed that a single blood transfusion did not have a significant influence on the T4/T8 ratios. Furthermore, the use of red cell concentrates would have minimized the effect of transfusion induced immuno-modulation.

4.2 HUMORAL IMMUNITY

4.2.1 B Cells

This study has demonstrated that the fetus is capable of mounting a significant B cell response to the *T.pallidum* antigen. Associated with this is an outpouring of immunoglobulin M but not IgG and A antibodies.

These observations are in accordance with that which is currently known about the functional maturity of fetal B cells, namely (a) that the fetal lymphocyte population is capable of recognizing and responding to virtually any antigenic determinant (Lawton, 1984), and (b) that the humoral response comprises initially of an IgM-producing B cell

clone which can give rise subsequently to IgG, IgA and IgE (Dudley and Weidmeier, 1991), a process which is dependent on the functional competency of the T cells.

The current literature is sparse with respect to studies of B cell responsiveness in neonatal infections. Gajewska (1982) studied 90 full term and preterm infants with perinatally acquired infections. The percentage B cells were increased, as was the levels of IgM and IgA. Stachowski et al (1991) reported an increased production of IgM and reduced synthesis of IgG and IgA antibodies in newborns stressed with perinatal infections. The present findings corroborate that of the previous two authors with the exception of the finding of elevated IgA levels reported by Gajewska (1982) which was not present in either this study or that described by Stachowski et al (1991).

The correlation between the B and total lymphocyte counts is expected, considering that B cells are an integral component of peripheral blood lymphocytes. The lack of correlation between the B cell and IgM levels cannot be fully explained but it suggests that, notwithstanding both variables being significantly raised, their rate of increase is probably disproportionate.

The evaluation of B cell activity on the 10th day demonstrated that these cells remain persistently elevated at completion of therapy. There are 2 possible reasons for this finding: firstly, viable organisms may persist following treatment as reported by Tramont, (1976) and Engel and Diezel (1980) resulting in continued antigenic stimulation. Though individual treatment failures have been reported with Benzathine Penicillin (Beck-Sague and Alexander, 1987), there is sufficient evidence to attest to the overall clinical efficacy of the procaine penicillin regimen recommended by the Centres for Disease Control (CDC, 1988) making this a less likely explanation. A more plausible consideration is that the continued elevation in B cell levels is an expression of a normally reactive and functional humoral immune system whose natural history following an antigenic stimulus is one of an exponential increase in activity till a steady state is reached, followed by an exponential

decrease over a period of time following elimination of the antigen and the down regulation of B cell activity (Virella, 1993a).

4.2.2 Immunoglobuins

Elevations in serum IgM observed in earlier studies have been corrobrated by a number of later studies (Alford, 1967; Borobio et al, 1980; Meyer & Malan, 1989) including the present one. In this study newborns with congenital syphilis had IgM levels $\pm$ 20 times greater than that found in the controls. Such a profound humoral response has been observed in chronic intrauterine infections other than congenital syphilis. However, symptomatic infection by the rubella virus and the Treponema pallidum organism have been noted to produce IgM levels considerably higher than those detected in symptomatic cytomegalovirus infections (Alford, 1971). This may, in part, be due to the variable antigenic potency of the different pathogens, or it may be related to the duration of the primary or secondary infection in the mother and to the degree of clinical involvement in the infant (Alford, 1971), an observation further alluded to by McCracken in relation to congenital rubella (McCracken, 1969).

The present study did not show a statistically significant relationship between IgM levels and the extent of the disease. Meyer and Beatty (1991) in their study of rheumatoid factor (IgM RF) levels in congenital syphilis established a positive correlation between these levels and the extent of the disease. The major difference between the present study and that of Meyer and Beatty (1991) lies in the characteristics of the study population. In the latter study the cohort was larger ($n = 41$) of which 27 were newborn and the variation in age was greater (0 - 4 months). It is therefore possible that a greater sample size in the present study would have reduced a possible type II error producing a similar result.

Immunoglobulins G and A were not raised and levels were comparable to that observed in the controls. Measurement of IgE antibody was not performed in this study, thus precluding comment on its status in congenital syphilis. To the best of my knowledge,

serum IgE level have not been studied in congenital syphilis, though Bos et al (1980a) have demonstrated raised levels in adult patients with early syphilis.

A possible explanation for the dominant IgM response lies in the ontogeny of B cell development in the fetus. Immature B cells express only the IgM isotype on their cell membranes (Wilson, 1985; Goldman et al, 1985: Dudley and Weidmeier, 1991). Maturity heralds the expression of both surface IgM and IgD. The activation of mature B cells by antigen initiates the secretion of IgM specific antibody and in the process produces a clone of IgM - producing cells.

The diminished capacity to produce IgG and IgA in response to antigenic stimulation has been attributed to a state of functional immaturity on the part of B cells. Though some of the immaturity may be secondary to an increase in suppressor cell activity (Durandy et al, 1979; Pedersen et al, 1983; Punnonen, 1989;) there is mounting evidence for an intrinsic delay in B cell development. As early as 1977, Hayward and Lawton proposed that the relative deficiency of IgG and IgA responses to antigenic stimulation observed in their study, and documented in congenital neonatal infections, was due to a maturational delay of the B lymphocytes. Miyawaki et al (1988) corroborated these findings by demonstrating the reduced ability of the B cells to produce IgG and IgA in vitro in response to a T cell independent stimulator (Ebstein-Barr virus). In contrast IgM production was unimpaired.

More recent work lends support to the view of the previous investigator. Punnonen (1989) reported on the role of interleukin 2 (IL-2) in relation to B cell proliferation and IgM production. The results suggested that there may be both an increase in suppressor cell function induced by IL-2, and, an intrinsic B cell deficiency. Similarly, Watson et al (1991) showed that IgM synthesis by cord blood B cells can be enhanced by interleukin-6 (IL-6) and decreased by IL-2. However, these cells produced no IgG or IgA irrespective of the cytokine preparation employed, further underlining the intrinsic limitation to respond to immunostimulatory factors.

The evaluation of the IgM levels at the end of therapy showed a definite downward trend, though the median of 1.66g/L was still 8 times the upper limit of the normal range. The serum levels were not monitored beyond completion of therapy and therefore, the time period over which the IgM decreased to normal serum concentrations is not known. Interestingly, in this respect, Borobio and co-workers (1980) monitored 9 syphilitic infants serologically over a period of months, and showed a gradual fall in total serum concentration over a 3 to 6 month period. As expected as long as the total IgM remained raised, so the FTA-ABS IgM remained positive.

There are two possible reasons for the continued elevation in IgM levels. The first relates to the rate of metabolism of IgM in the body. Studies have shown that IgM has one of the lowest rates of catabolism compared to the other immunoglobulin classes. Its rate of catabolism (2.2 mg/k/ day) is 12 to 15 times lower than that described for IgA and IgG. It would also appear that for the most part the rate of IgM catabolism is unaffected by its serum concentration (Mariani and Strober, 1990). Secondly, as alluded to previously, with the down regulation in B cell function, the IgM secreting B cells follow an exponential decrease over time producing a parallel fall-off in IgM levels.

4.2.3 Summary

This study has shown that newborn infants with congenital syphilis have an augmented humoral response as demonstrated by significant elevations in peripheral B cell and serum IgM levels. The nature of this reponse is accordance with present knowledge on the ontogeny of the humoral immune response. Though lower than the pre-treatment levels, this heightened B cell and IgM activity still persists at completion of therapy. A possible explanation for this finding relates to the slow catabolism of the IgM molecule and the exponential fall-off of B cell levels over time. From a clinical standpoint this infers that the total serum IgM and the FTA-ABS IgM tests may remain positive for a few months in an infant who is fully treated.

4.3 CELL-MEDIATED IMMUNITY

4.3.1 Lymphocyte Transformation

Lymphocyte reactivity to PHA stimulation in infants with congenital syphilis was suppressed when cultured in autologous serum. Subsequent culture in AB serum produced an enhanced responsiveness. In addition, lymphocyte responsiveness had improved at the time of completion of penicillin therapy. These observations suggest that the CMI response, as measured by the lymphocyte transformation assay, is suppressed in early neonatal syphilis. Furthermore the cause of the immunosuppression appears to reside in the syphilitic serum as there was a significant improvement in lymphocyte reactivity to PHA stimulation when the lymphocytes were cultured in AB serum. Treatment of the disease was also associated with a reduction in the degree of immunosuppression.

The depressed lymphocyte transformation in this investigation corroborates the report by Friedmann (1977) in which the paired lymphocyte reactivity of infants with congenital syphilis and their mothers' utilizing *T.pallidum* antigen was studied. The results, expressed as stimulation ratios, demonstrated that lymphocytes of neonates with syphilis were unresponsive to stimulation with *T.pallidum*.

Evidence that CMI was impaired in congenital syphilis and in early venereal syphilis was based on the clinicopathological observations of Festenstein et al (1967) and Levene et al (1971), and the experimental in-vitro studies of numerous investigators (Fulford and Brostoff, 1972; Wicher and Wicher 1975; Pavia et al, 1976; Bey et al, 1979; Maret et al, 1980; Baker-Zander et al, 1982). Despite the many investigations, the nature of the inhibitory factors and the mechanism of its inhibition remains obscure.

Bey et al (1979) reported inhibition of Con A responses by sera from intratesticularly infected rabbits and found it to be associated with the presence of a mucopolysaccharide material; the inhibition was abolished with hyaluronidase. This finding suggested that the

mucopolysaccharide capsular material is the immunosuppressive component of *T.pallidum*. In contrast, Baughn et al (1980) demonstrated the presence of immune complexes in the sera of rabbits infected with *T.pallidum*. They proposed that these complexes played a significant immunoregulatory role and that the complexes, rather than mucopolysaccharide, were responsible for immunosuppression. Further supportive evidence came from reports that had implicated soluble complexes in antibody excess to have an inhibitory effect on lymphocyte responsiveness to specific antigens (Oppenheim, 1972; Theofilopoulous and Dixon, 1980). Folds et al (1982) studied the effect of sera from patients with secondary syphilis on lymphocyte transformation. The autologous syphilitic sera had a significant suppressive effect on blast transformation, an effect that was attributed to the presence of circulating immune complexes, and which subsequently improved pari passu the fall in serum complexes following penicillin therapy. This fall in immune complex levels with treatment is consistent with the work of Engel & Diezel (1980), and Solling et al (1978).

Results from the present study have shown that neonates with congenital syphilis have markedly elevated levels of serum ICs which decline significantly with treatment. The improvement in lymphocyte reactivity in AB serum and following treatment would strongly suggest that the presence of ICs may be immunosuppressive as they are absent in the AB serum and diminished after penicillin therapy. This finding would be consistent with that described by Folds et al (1982). This association suggests that serum immune complexes might possibly be the factors responsible for the suppressed lymphocyte transformation in these syphilitic infants.

However, contrary to the findings of Folds et al (1982) and Thompson et al (1980) where the sera of adult syphilitic patients suppressed the reactivity of lymphocytes from normal individuals, the sera of the congenital syphilitic infants in this study had no such effect on the normal reference lymphocytes. This contradictory finding cannot be fully explained. Suffice it to say that in all these studies, healthy adults were the source of the reference lymphocytes. With what is known about the differences between adult and neonatal

lymphocytes with respect to cell surface expression of different receptors (Wilson 1985), it is plausible that if cord blood was used as a source of reference lymphocytes, the reactivity of cord blood cells may well differ from adult cells when cultured in the sera from infants with congenital syphilis.

An additional finding in the study of Thompson et al (1980) which is at odds with this study, is their report that the sera from congenital syphilitic infants were immuno-stimulatory and not immunosuppressive as observed in this investigation. They proposed the lack of humoral regulation of the CMI response due to a possible diminution in suppressor cells as a possible explanation for their finding. This explanation is not borne out by the observations of the present study in which an evaluation of the total T cells and the T cell subsets demonstrated no difference in the absolute numbers of suppressor cells between the cases and the controls. It is likely that the difference in the sample size between the two studies (19 infants in the present study vs 6) is the reason for these contradictory findings.

Of prime concern is the question as to whether these in-vitro findings have any relevance to in-vivo biological and clinical events, a question that Baker-Zander and co-workers (1982) have previously alluded to. Immunosuppression has been put forward as an hypothesis by a number of workers (Levene et al, 1969; Pavia et al, 1978; Bey et al, 1979; Thompson et al, 1980) to explain the progression of adult syphilis through its various stages. There is the anecdotal evidence of clinicians implying that infants with congenital syphilis are at increased risk of secondary bacterial infections, an implication not borne out by any known clinical studies.

The concept of immunosuppression in syphilis is not universally accepted, as a number of reports have claimed that immune cells operate effectively, and immunosuppression does not occur in syphilis (Hardy et al, 1979; Lukehart et al, 1980; Baker-Zander et al, 1982). More importantly, the Oslo study (Gjestland, 1955) involving $\pm$ 2000 untreated syphilitic

patients that were followed for ± 20 years (1891-1910) showed that 75% of untreated patients did not progress beyond the primary stage of syphilis, implying a certain degree of immunity and thus no immunosuppression in the majority of patients. There is no equivalent data for infants with congenital syphilis.

Therefore, though this study has shown that the sera of neonates with congenital syphilis is immunosuppressive in-vitro, the clinical relevance thereof is uncertain.

4.3.2 T cells and subsets

The evaluation of cell-mediated immunity with respect to lymphocyte subsets has demonstrated no quantitative alteration in the absolute numbers of CD3, CD4 and CD8 counts in newborns with congenital syphilis when compared with matched controls. In addition, post-therapy cell counts were comparable to the controls. This study has demonstrated that quantitatively, certainly in terms of peripheral blood T-lymphocyte counts, there is no suppression of T-cell mobilization in newborns with congenital syphilis. A review of the literature has shown a dearth of data with which to compare these findings.

Bos et al (1980b) in their quantitative study of T-lymphocytes in 10 adults with primary syphilis utilizing the rosette technique, demonstrated a significant decrease in the number of T-lymphoid cells in the affected adults relative to the controls. In a subsequent study, these workers further demonstrated that this diminished levels of T-cells was accompanied by a significant increase in IgG-bearing lymphoid cells (Bos et al, 1980c).

Jensen and From (1982) studied T-cell changes in a population of 34 adult patients with primary and secondary syphilis before and after treatment. Their study revealed that the total number of T-cells was significantly reduced during both primary and secondary syphilis. Re-evaluation 1 and 2 months after treatment demonstrated a significant improvement in cell counts, similar to that present in the controls. Their study further

revealed that in primary syphilis the percentage of helper cells was significantly reduced, while in secondary syphilis the percentage of suppressor cells was diminished.

This peripheral T-lymphopenia was not observed in the present study. However, 2 of the 3 infants who died soon after birth had a marked peripheral T-lymphopenia (6% of total lymphocyte count). The phenomenon of T-cell depletion in the paracortical areas of lymph nodes and periarteriolar area of the spleen has been described in children with fatal congenital syphilis (Levene et al, 1971; Wright and Grimble, 1974). None of the survivors in the present study demonstrated a T-lymphopenia.

There are no obvious reasons to explain these seemingly contradictory findings between adults and newborns with early syphilis. However, there are a number of possible explanations that could be considered. Firstly, the techniques used to quantify the T cells were different. Both Bos et al (1980) and Jensen and From (1982) utilized the E-rosetting technique as opposed to the use of monoclonal antisera in the present study. The rosette technique is labour intensive and subject to many variables. Thus while this technique retains historic interest, T-cells are more readily and accurately measured with monoclonal antibodies (Moscicki et al, 1985). Secondly, and more importantly, several investigators have demonstrated a number of differences in lymphocyte subsets between newborn and adults. They differ not only with respect to the cell surface expression of certain antigens (Wilson, 1985), but, in addition the absolute numbers of lymphocytes (T and B cells) are significantly increased in newborns compared to adults (Fleisher et al, 1975; Thomas and Linch, 1983). These investigators further emphasized that the increase in absolute numbers of lymphocyte subsets in the newborn may easily be missed using techniques expressing results as a percentage of mononuclear or lymphocyte preparations.

The presence of comparable levels of absolute numbers of T cells and T cell subsets in the study population and the control could mean that in the face of an infection the newborn is capable of considerable mobilization of lymphocytes from the lymphoid tissues such that

peripheral blood concentrations are maintained within normal limits. Moreover, the lymphopenia observed in adult syphilis may be a phenomenon of the premorbid state as observed in the two moribund infants who died soon after birth. Support for this hypothesis is found in the observation of T-cell depletion in the lymph nodes and spleen in infants with fatal congenital syphilis (Levene et al, 1971; Wright & Grimble, 1974). What can be called into question is the quality of the T-lymphocyte response. Several investigators have shown that while newborns do have adequate numbers of phenotypically mature T and B cells, the cells do demonstrate functional immaturity relative to adult cells (Bonforte et al, 1972; Campbell et al, 1974; Chandra, 1975).

The present study suggests that syphilis further compromises the functional capabilities of the lymphocytes compared to the disease-free control population, as measured by the in-vitro lymphocyte transformation assay.

4.3.3 Summary

The outcome of this study suggests that in infants with congenital syphilis, cell-mediated immunity may be compromised with respect to lymphocyte responsiveness to antigen. This finding is consistent with that demonstrated in animal and adult human syphilis. It would seem that the presence of circulating immune complexes may be partly responsible for the compromise.

However, contrary to the observation in adults, congenital syphilis did not produce a peripheral T-cell lymphopenia except in the state of pre-morbidity. This may be related to quantitative differences in T-cells between infants and adults and secondly, to methodological differences in T-cell evaluation.

4.4 CIRCULATING IMMUNE COMPLEXES

4.4.1 Immune complexes in cord blood, syphilitic and control newborn infants

This study confirms the presence of elevated levels of ICs in infants with congenital syphilis. In the majority of infants, IC levels were 2 to 5 times greater than that detected in cord blood and in the sera of the control group.

This demonstration of ICs in infants with syphilis is in accord with earlier adult (Solling et al 1978; Engel and Diezel, 1980; Folds, Maret and Rauchbach, 1982; Baughn et al, 1986) and experimental animal (Baughn and Musher, 1983; Baughn, Adams and Musher, 1983) studies. Furthermore, the study corroborates the findings of Dobson et al (1988). As in the present study, Dobson et al (1988) also showed a wide spread of IC levels relative to the controls, notwithstanding the small sample of six patients in their study. The authors further showed that the ICs contained predominantly IgM antibody, and confirmed the presence of *T.pallidum* antigens within the ICs thus establishing its specificity. In addition, from a clinical perspective, 2 of 16 asymptomatic infants in an "at risk" group also exhibited elevated IgM immune complexes. A similar finding was observed in the present study. Two asymptomatic infants born to mothers with untreated positive VDRL titres, had IC levels 2 to 3 times that found in the control group (cases 5 and 18, Table 3-9).

However, the presence of ICs should be considered a nonspecific finding. Immune complexes have been demonstrated in several other chronic intrauterine infections. Shahin and co-workers (1974) reported the presence of IgM antibody, complement and toxoplasma antigen on renal biopsy in an infant with congenital toxoplasmosis suggesting the presence of an immune complex nephrosis. Stagno et al (1977) confirmed the presence of ICs in infants with congenital CMV infection, while Tardien et al (1980) investigated and demonstrated ICs in four infants with the rubella syndrome. Finally, Maszkiewicz (1983) was able to show a group neonates with various forms of sepsis to have circulating ICs,

while more than half of a group with suspected sepsis and who were later proved to be symptomatic, had elevated levels of ICs.

Though this humoral response should be considered nonspecific in the context of perinatal infections, there may possibly be a role for its clinical use in the screening of the newborn suspected of having or considered to be "at risk"" of developing congenital syphilis.

4.4.2 Correlation between immune complexes and other variables

A positive correlation was shown to exist between the total serum IgM and IC levels. This finding is consistent with what is known of the fetal humoral response. Following an antigenic stimulus, the predominant antibody produced by the fetus is of the IgM class (Stiehm, 1975; Goldman et al, 1985). Subsequent interaction between the Ig and the *T.pallidum* antigen would result in IC formation. Thus the level of ICs would be directly proportional to the quantity of antigen and antibody. Furthermore, corroboratory evidence is provided by the study of Dobson et al (1988) who showed that ICs in congenital syphilis have IgM as the dominant antibody.

What is more difficult to explain is the absence of a statistical correlation between IC and CH50 levels. IgM-immune complexes are known to be potent activators of the classical complement pathway. Such activation will ultimately lead to a fall in CH50, C3 and C4 levels. Measured levels of CH50 in this study has been shown to be significantly decreased. However, this did not translate into an inversely proportional relationship with the ICs as one would expect, and which has been shown in other ICDs such as systemic lupus erythematosis and rheumatoid arthritis (Blandford, 1979). It may be that the sample of the cohort in the present study was too small or that the rise in IC levels and the fall in CH50 levels are disproportionate.

4.4.3 Relationship between immune complex levels and the extent of the disease

Congenital syphilis has the hallmarks of an immune complex(IC)-mediated disorder. It is a multisystem disease with frequent involvement of the skin and bones, associated with high levels of circulating immune complexes containing IgM and treponemal antigen (Dobson et al, 1988) and low levels of total haemolytic complement (CH50). Moreover, there is a strong association between the nephritis of congenital syphilis and glomerular membrane deposition of ICs (Blandford, 1979). All these findings are highly suggestive of an IC-mediated event. However, except for the renal lesions, there is no data relating tissue complexes to other organ systems in congenital syphilis. Jorizzo and co-workers (1986) reported on the biopsy findings of Ag-containing ICs in the skin lesions of adults with secondary syphilis. If one accepts that congenital syphilis is analogous to adult secondary syphilis, both the result of haematogenous spread at different periods in time, then by inference the skin, and possibly the metaphyseal changes, may be due to the deposition of ICs.

Assuming that ICs play an important pathogenetic role in congenital syphilis, then the level of ICs would significantly influence the extent of the disease. This relationship has been conclusively demonstrated in known ICDs such as systemic lupus erythematosis and rheumatoid arthritis (Blandford, 1979). Such a relationship was not clearly established with respect to congenital syphilis in the present study. In contrast Meyer & Beatty, 1991 were able to demonstrate a positive correlation between the extent of the disease involvement and IgM RF levels, and between IgM RF and IC concentrations. The authors went on further to suggest (i) that the association between IgM RF and IC levels exists because the former is an integral component of the latter, and (ii) that IgM RF contributes to IC formation, complement activation and tissue damage and disease severity.

The principal difference between the study of Meyer and Beatty (1991) and the present one lies in the size of the sample of patients. Meyer and Beatty (1991) reported on the observations in 41 infants with congenital syphilis, 27 of whom were newborns. This,

compared to the 16 neonates in the present study. This differential in sample size more than likely accounted for the contrasting statistical outcome between the two studies.

4.4.4 The effect of treatment on serum immune complex levels

At completion of therapy, IC levels were demonstrated to be significantly diminished. This would suggest either (a) a reduced production of ICs following the killing of the organisms by penicillin and/or (b) removal of the ICs by the mononuclear phagocytic system and the liver (Virella, 1993b). The combined effect of these two mechanisms would be the effective and systematic elimination of the ICs.

In four infants the IC concentrations remained persistently elevated at the time of completion of the treatment. The clinical course of these infants during treatment did not differ from the rest of the cohort, and at the time of discharge they were thriving and well. Engel & Diezel (1980) expressed concern at the presence of persistently elevated levels of ICs at the end of therapy. They were of the opinion that this finding represented ongoing antigenic stimulation and, thus, the presence of live *T.pallidum* organisms. These investigators therefore suggested that penicillin treatment be continued until such time that there is a noticeable and significant fall in IC levels. If this postulate is correct, it implies that patients who continue to have raised levels of ICs and are not treated further, are at risk of relapse or of developing a latent form of syphilis with its attendant complications. Dobson et al (1988), however, performed serial IC levels on an infant who had the highest levels out of a group of six infants with congenital syphilis. By the 16th day of life, the serum still contained elevated concentrations of ICs, though the levels had diminished. By the 44th day, ICs were no longer detectable. Unfortunately the authors did not comment on the infant's clinical outcome. This may suggest that the outcome was no different from the rest of the group.

An alternative explanation for the persistence of the ICs may be related to an impairment in its clearance. It is well known that the reticuloendothelial system (RES) plays an essential

role in the clearance of ICs (Virella, 1993b). Furthermore, ICs are capable of modulating the immune response ie. either augmenting or suppressing it. It is therefore possible that very high levels of ICs could suppress and thus impair its clearance by the mononuclear phagocytic system. There would thus be a delay in its elimination, and consequently, remain persistently elevated in the serum.

While both explanations are feasible, from a clinical standpoint there is no epidemiological or clinical data to suggest that therapy should be continued longer than the recommended 10-14 days.

4.4.5 Summary

Newborn infants with congenital syphilis have quantitatively markedly elevated levels of ICs. Parallels are drawn between congenital syphilis and known immune complex-mediated disorders (ICD). It is suggested that the measurement of ICs may have a role as an adjunctive investigation of the "at risk" infant.

4.5 COMPLEMENT

The complement proteins have one of the highest turnover rates of any plasma constituent (Boackle, 1993). At any given time the level of a complement component is a direct function of its synthetic and catabolic rates. Complement levels alter in different disease situations. Raised levels, particularly of C3 and C4, can reflect an acute phase response as occurs in most inflammatory and infective disease states (Thompson, 1978). In contrast, subnormal levels occur in disorders that either adversely affect its hepatic synthesis or potentiate or augment its activation and consumption (Johnston, 1993).

The functioning of the classical pathway and the terminal components can be determined by the CH50 assay (Phimister & Whaley, 1990). The results of the present study demonstrate that newborns with congenital syphilis have significantly low levels of total

haemolytic complement. The possible reasons are twofold. Firstly, syphilitic disease of the liver can adversely influence the production of the complement components. While hepatomegaly is a common manifestation of congenital syphilis, hepatitis is an uncommon association. Wright et al (1974) in a study of 59 infants dying of untreated congenital syphilis in the first year of life, 50 of the infants had normal liver histology on the hemotoxylin and eosin stain. Clinical studies confirm this finding (Mascola et al, 1985; Chawla et al, 1985). Chronic hepatic disease with liver dysfunction is an even rarer finding. Of 13 infants with syphilitic hepatitis reported by Motala and co-workers (1990) one infant developed cirrhosis with portal hypertension. In the present study there were 16 infants with hepatosplenomegaly, 3 of whom had a conjugated hyperbilirubinaemia. This would suggest that liver dysfunction is unlikely to be responsible for the reduced complement activity demonstrated in this study.

Of greater significance is the presence of elevated levels of serum immune complexes. There are several reasons to suggest that it is their presence that is responsible for the reduced levels of total haemolytic complement. These molecules are known potent activators of the classical complement pathway. Their detection by the C1q binding assay is indicative of their complement -fixing ability. Dobson et al (1988) showed that immune complexes in congenital syphilis are predominantly IgM-complexes, and IgM is a potent complement binding antibody. This relationship is expressed in the inverse correlation between IgM and the CH50 demonstrated in this study. Several studies have, in addition, demonstrated the presence of complement components and cleavage products within immune complexes in experimental and human syphilis (Baughn et al, 1982; Baughn & Musher, 1983; Baughn et al, 1986). However, notably, there was no statistical correlation between CH50 and IC levels. This finding is difficult to explain in light of the previous argument. It may mean that either complement is not an integral feature of ICs in congenital syphilis - the argument presented favours such a relationship - or, more likely, the size of the study sample is too small to demonstrate a statistical correlation. The cumulative evidence is weighted in support of ICs as a major role player in the activation

and subsequent consumption of complement leading to reduced CH50 levels. Concomitant depletion of C3, C4 and the presence of the cleavage product, C3D would have strengthened this assertion. Unfortunately the data on C3 is grossly inadequate to draw any meaningful conclusions, and the question of C4 and C3D determinations was not addressed in this study.

That the CH50 level at completion of therapy remained unchanged is in keeping with the metabolism of the complement proteins. The synthetic rates of complement are controlled by a number of ill-defined mechanisms including the levels of complement activators, the class and subclass of immunoglobulin present in immune complexes and a host of other variables (Boackle, 1993). This accounts for the varying synthetic rates in disease states and during the course of a given disease. Measurement of ICs and IgM on day 10 of treatment has shown these still to be raised. Both potent activators of the complement system, their continued presence implies a greater consumption rate versus synthesis and therefore, continued low levels of CH50.

4.5.1 Summary

Newborns with congenital syphilis have reduced levels of CH50 suggesting activation of the classical complement cascade. This observation coincides with the presence of significantly elevated levels of ICs, potent activators of this complement pathway. The cumulative evidence suggests that activation and subsequent consumption of complement components accounts for the reduced CH50 activity.

4.6 PHAGOCYTIC FUNCTION

4.6.1 White cell and differential counts

The findings in the present study were similar to those previously reported (Whitaker et al, 1965; Chawla et al, 1985). Leucocytosis and wide variations in the differential counts, were common features with a neutrophilia in some patients and lymphocytosis in others. Absolute monocyte counts were considered to be elevated in three infants. Neither

Whitaker et al (1965) nor Chawla et al (1985) reported on the presence of a significant peripheral monocytosis. Whitaker and co-workers (1965) did, however, observe a marked monocytosis on bone marrow examination, and reported a rise in peripheral monocyte counts on repeated leukocyte measurements a week later. Stevens and colleagues (1987), in a case report, observed such a marked monocytic differentiation on a marrow aspirate that, among others, a diagnosis of acute monocytic leukemia was entertained. Karayalcin et al (1977) described a monocytosis in 8 out of the 10 infants they studied, one of whom had a monocytic leukemoid response. The authors further referred to the presence of this immune response in active syphilis in rabbits and went on to underline and emphasize the importance of monocytes in the cellular reaction to the Treponema pallidum. This opinion was given greater impetus by the work of Baker-Zander and Lukehart (1992) who accentuated the paramount role that macrophages play in the killing of the spirochaete.

4.6.2 Nitroblue tetrazolium test

The results of the NBT test has demonstrated that the respiratory burst activity of neutrophils in infants with congenital syphilis was equivalent to a healthy control population. This would suggest that the bactericidal function of syphilitic neutrophils is intact though a formal bactericidal assay was not performed. This assessment of phagocyte function contrasts with that of other workers who found that neutrophils of ill, stressed neonates have impaired bactericidal activity (Wright et al 1975; Shigeoka et al 1979). Wright and co-workers (1975) determined the effect of stress or illness on newborn leucocyte function by measuring the phagocyte and bactericidal activity of leucocytes from 40 sick newborns with a wide range of gestational ages and birth weights and compared them with a healthy newborn and adult population. Approximately 63% of stressed newborns had depressed in vitro activity compared with 17% in the well infants and 17% in the adult controls. Shigeoka et al (1979) included 8 infants with group B streptococcal (GBS) sepsis in their study group of stressed infants. Utilizing the chemiluminescence technique, they reported a depressed neutrophil metabolic activity in all the ill infants.

The reason for the discrepancy in neutrophil functional activity between the present study and those of other workers is not immediately obvious. The explanation may lie, partly, in the nature of the invading organism. Recent work has shown that it is the macrophage that plays a pivotal role in the elimination of the *T.pallidum* (Baker-Zander and Lukehart 1992). Thus the spirochaete may not regard the neutrophil defence as a "threat". In contrast, neutrophils exercise a key role in the killing of the GBS, S.aureus and E.coli microbes. Therefore, it is to the advantage of these organisms to employ mechanisms to depress and impair neutrophil activity.

One other possible explanation for the differences in outcome may relate to the method employed to measure the oxidative burst of the neutrophils. Shigeoka et al (1979) utilized the chemiluminescent technique which is perhaps the most sensitive and directly quantitative assay for the oxidative burst (Virella 1993c). Anderson and colleagues (1974) employed the NBT test in their evaluation of phagocytic function. In their report the unstimulated NBT test was depressed in the stressed infants, while stimulation of the cells in vitro resulted in a normal NBT dye reduction. This result is similar to that found in the present study where the results of the endotoxin stimulated assay were comparable to that found in the healthy control population.

Thus the outcome of the present study suggests that the neutrophils of infants with congenital syphilis are capable of producing toxic oxygen metabolites essential for microbicidal activity. However, what is questionable is the ability of such neutrophils to be treponemicidal. Musher et al (1983) have shown that, in vitro, human PMNs have the capacity to phagocytose, degranulate and exert an oxidative burst in response to the presence of *T.pallidum*. However, the authors significantly drew attention to the fact that the data from the animal experiments failed to explain why the *T.pallidum*, despite its apparent ingestion by neutrophils, fails to be eradicated.

In summary, the leukocyte and differential counts in infants with congenital syphilis vary widely, and no specific, peripheral white cell response characterizes the disease process. However, a leukemoid reaction and an elevated monocytic response should raise the suspicion of the attending physician and place congenital syphilis high in the differential diagnosis. The NBT assay has demonstrated results comparable to a healthy matched population. This would suggest that if neutrophil function is depressed in congenital syphilis it is more likely to involve one of the other steps in the phagocytic pathway and not the one concerned with the production of toxic oxygen metabolites.

4.7 CONCLUDING REMARKS

From a clinical perspective the enhanced humoral response, in particular the IgM response, finds application in the screening of an at risk population of infants. Alford (1971) recognized the usefulness of measuring serum IgM levels as a means of detecting subclinical congenital infections in the newborn. However its limitations have been firmly established. While IgM levels may be elevated in symptomatic congenital syphilis, they are often low or normal in asymptomatic or delayed onset disease (Kaufman et al, 1974). In addition, in the immature preterm infant with low immunoglobulin levels IgM may be absent (Ackerman, 1969).

In an attempt to overcome these limitations, Scotti and Logan (1968) developed a fluorescent treponemal antibody absorption test (FTA-ABS) that was specific for IgM antibodies to syphilis. However, experience with this test has shown that the false positive rate may be as high as 10% (Rosen et al, 1975) and the false negative rate may exceed 35%. (Kaufman et al 1974). Recognizing the limitations of the present syphilis-specific diagnostic tests, several new innovative methods have emerged in an attempt to improve the diagnostic capabilities of these tests. This is particularly relevant to the problem of identifying the asymptomatic possibly infected infant who is, in fact, uninfected. In a recent study Stoll and coworkers (1993) evaluated three diagnostic tests which could assist in the diagnosis of congenital syphilis: the FTA-ABS 19S IgM test, an IgM capture

ELISA for T.pallidum - both specific treponemal IgM antibody tests - and the reverse enzyme linked immunospot (RELISPOT) assay which detects immunoglobulin-secreting cells and is a non-specific indicator of infection. The FTA-ABS 19S IgM test differs from the original FTA-ABS IgM in that the serum is fractionated to eliminate the interference from rheumatoid factor (increasing its specificity) and the competitive inhibition of IgM by maternal IgG (increasing its sensitivity). In the symptomatic patient the three tests had greater sensitivity (73%, 88% and 78% for the FTA-ABS 19S IgM, IgM ELISA and RELISPOT respectively) and specificity (97-100%). However, no single diagnostic test was sufficiently sensitive to clarify the issue of the asymptomatic but possibly infected infant.

Meyer and Malan (1989) further explored the problem of the asymptomatic high risk infant by evaluating the role of the IgM rheumatoid factor (IgM RF) as a test for congenital syphilis. Their findings (a) confirmed the low sensitivity of the FTA-ABS IgM (26.6%) and (b) suggested, that despite the low sensitivity (46.7%) of the IgM RF, it's specificity and positive predictive value of 100%, makes a positive test in a high risk infant highly suspicious of congenital syphilis.

Finally, further efforts to improve identification of the asymptomatic infant led workers to explore and develop more sophisticated assays. While some investigators studied the IgM response to treponemal antigens by enzyme-linked immunosorbant assays (Farshy et al, 1984; Muller et al, 1987), others adapted protein immunoblot (Western blot) techniques to identify T pallidum antigens recognised by IgG and IgM (Baker-Zander et al, 1985; Dobson et al, 1988b; Lewis et al, 1990). The indirect immunofluorescent antibody (IFA) test for the detection of T.pallidum antigen (Bromberg et al, 1993) and the polymerase chain reaction (PCR) assay for the detection of T.pallidum DNA (Sanchez et al, 1993) have generated the most interest and excitement for the improved diagnosis of congenital syphilis. Both investigators have demonstrated the value of these tests in detecting the presence of T.pallidum in the asymptomatic infected infant who has early syphilis but who

has not yet mounted an immune response. The sensitivity and the specificity of the PCR assay has been shown to be comparable to the rabbit infectivity test (RIT) which is the current reference standard for the identification of T.pallidum in clinical specimens (Grimpel et al, 1991; Sanchez et al, 1993).

In the present study all the symptomatic infants had significantly elevated serum IgM levels. There were 2 asymptomatic infants who were detected on IgM screening. One of these two infants had a positive FTA-ABS IgM. A third infant had no clinical signs but the total serum IgM was raised, the FTA-ABS IgM was positive and radiographic metaphyseal changes were present. In the seventeen infants in whom the FTA-ABS IgM test was done twelve (70.6%) were positive. These findings were comparable to other reports (Kaufman et al 1974; Borobio et al, 1980). It is essential to emphasise that knowledge of the metabolism of the total IgM is paramount to an understanding of the prolonged period of positivity of the FTA-ABS IgM test even in the treated patient. In the study of Borobio et al (1980) this period ranged from 3 to 6 months. However, and importantly, Mlisana et al (1992) re-emphasized the marked limitations of these tests when they reported on the false negative results of both the total IgM and FTA-ABS IgM in 4 symptomatic infants.

Therefore the current diagnosis of congenital syphilis should still be based on a combination of epidemiological, clinical, serological and radiographic findings. Correct interpretation of the serological tests demands a sound knowledge of its limitations. The PCR assay and the IFA test hold out the best prospects for future improvements in diagnostic capability.

The results of the LT assay in the present study suggest that the CMI response in congenital syphilis may be functionally impaired. However, the clinical relevance of this finding is unclear. Overall these infants have shown no impairment in clinical response to treatment and on follow-up no major sequelae have been found. However, there are

several reports of the persistence of *T.pallidum* organisms following penicillin therapy (Ingall & Musher, 1983). It is speculative whether impairment of the CMI reponse may be responsible for the lack of complete eradication of this organism. However, as Ingall & Musher (1983) have pointed out the extent to which treponemes persist after treatment and the long term implications, is unknown.

The inference drawn from the present study is that congenital syphilis has the hallmarks of an ICD. In this respect, the role of steroids in treatment deserves consideration. Steroids play a pivotal role in the treatment of ICDs and are largely responsible for modifying the disease process. Their role in symptomatic congenital syphilis has not been fully addressed. In a study of 30 infants by Venter et al (1991), 15 received penicillin and prednisone (2 mg per kg per day for 5 days) and 15 received penicillin only. The addition of steroids to the treatment regimen did not significantly modify the clinical course or the biochemical abnormalities. Of the 4 infants who died in the first week of life, 3 were in the penicillin only group and 1 in the penicillin and steroid group. Certainly a much larger study is required to assess the efficacy of steroids on the mortality of congenital syphilis.

Several studies have addressed the role of complement activation in syphilis and though there is at present no evidence to suggest a direct spirochetocidal effect, observations from these studies place complement at the forefront of immobilization and inactivation of the *T.pallidum*. Rice and Fitzgerald (1985) reported on the loss of treponemal motility in the presence of complement. Their study showed that a 50% fall off in treponemal motility correlated with the consumption of 72% of the available complement. They suggested that both early and late components of the complement cascade are necessary, and that the reaction proceeds via the classical complement pathway.

Radolf et al (1986) corroborated the findings of Rice and Fitzgerald (1985). These investigators showed that *T.pallidum*-specific antibody renders the organism nonmotile and avirulent only in the presence of complement.

In the absence of complement, no or little antibody was found on the treponemal surface. However the addition of complement to syphilitic immune rabbit and human serum resulted in immobilisation and the deposition of antibody on the entire surface of the immobilized organisms.

The larger cleavage product of C3, C3b, has been established as a potent opsonin when fixed to the surface of a particle, whether it be a red blood cell or a micro-organism (Griffen, 1977). In the presence of activated macrophages, C3b serves to aid both in the attachment of the organisms to macrophages via the C3b receptor, and in its ingestion. Baker-Zander and Lukehart (1992), in their elegant study demonstrated decisively the indispensability and effectiveness of macrophages in the phagocytosis and killing of *T.pallidum*.

From the aforementioned observations there appears to be sufficient indirect evidence to suggest that complement plays a paramount and decisive role in the immobilization and killing of the *Treponema pallidum*.

The outcome of the present study of the newborn with congenital syphilis has provided a broader insight into the immune response of the fetus exposed to the invading *T.pallidum*. Corroborating the findings of previous studies, the fetus has demonstrated a profound immune response incorporating and mobilizing the major immunological role players.

SECTION B

INTRODUCTION

Congenital syphilis is a multisystem disease with multiple organ involvement, including the placenta. Several studies have reported on the histopathological findings in the syphilitic placenta. Essential to an understanding and appreciation of these observations is a sound knowledge of the maturational events that occur in the development of the placenta. This section serves to describe the essential elements in the development and structure of the placental villous system, followed by a literature review of the histopathological features encountered, and finally, the question of the pathogenesis is addressed.

CHAPTER 5 DEVELOPMENT AND STRUCTURE OF THE PLACENTAL VILLOUS SYSTEM

5.1. EARLY DEVELOPMENT OF THE VILLOUS TREE

(Wynn, 1975; Kaufmann and Scheffen, 1992)

Placental development commences around day 6-7 postconception at the time of implantation of the blastocyst into the endometrium. The blastocyst consists of an outer cellular layer, the trophoblast, and an inner cell mass, the embryoblast. The formation of the villous tree stems from invasion and proliferation of the trophoblast.

At day 7-8 the single layered trophoblast divides into 2 distinct layers, the outer syncytiotrophoblast, and the inner cytotrophoblast. Concomitant with proliferation of the syncyntiotrophoblast at the implantation pole, a system of intrasyncytial, confluent lacunae appear which will eventually form the intervillous space. The pillars of syncytiotrophoblast separating the lacunae are called trabeculae. The roof of this system of trabeculae and lacunae formed by syncytio- and cytotrophoblast is called the primary chorionic plate. The floor, a layer of syncytiotrophoblast, is called the trophoblastic shell.

From the chorionic plate cytotrophoblast invades and proliferates inside the trabeculae, resulting in longitudinal growth and branching. The branches that end blindly and protrude into the lacunae are called primary villi (day 12-15 postconception). At this time trophoblast from the trophoblastic shell erodes into maternal endometrial vessels. Between days 15 to 20, mesenchymal cells derived from the extraembryonic mesenchyme invade the primary villi transforming them into secondary villi. Around the 20th day postconception fetal vascularization from the mesenchymal cells commences, transforming the secondary into tertiary villi. Throughout the subsequent periods of development, the tertiary villi undergo a complex process differentiation that results in various villous types that differ from each other with regard to structure and function.

5.2. STRUCTURE OF THE VILLOUS TREE

The classification of the villous system as set out by Fox (1978a) was based on conventional paraffin fixed embedded tissue stained with haematoxylin and eosin. Anatomically the placental villi follow a well defined branching pattern. Primary stem villi running parallel to the chorionic plate divide into a number of secondary stem villi just below the plate. The latter in turn branch and divide into a series of tertiary stem villi which sweep down into the intervillous space towards the decidual plate into which they insert. These tertiary stem villi then turn back upon themselves to re-enter the intervillous space where they branch and form the terminal villous network.

The outcome of several studies concluded in the re-evaluation and modification of this conventional classification (Kaufmann et al, 1979; Sen et al, 1979; Castellucci and Kaufmann, 1982; Kaufmann, 1982). Five villous types are now recognized:

5.2.1 Mesenchymal Villi

These are the most primitive villi which prevail during the early stages of pregnancy, and are the forerunners of the immature intermediate villi. They correspond to the tertiary villi previously described in the early development of the villous system.

5.2.2 Immature Intermediate Villi

Characteristically these are bulbous villi that are continuations of the stem villi, and are prevalent in immature placentas. They represent the forerunnners of the stem villi and are, in addition, the principal sites of maternofetal exchange during the first two trimesters.

5.2.3 Stem Villi

These are characterized by a condensed fibrous stroma containing arteries and veins. In addition to the main stem which connects the villous tree to the chorionic plate, and the anchoring villi which connect to the basal plate, there are a number of generations of branchings which extend to the periphery of villous tree. Functionally, stem villi serve as mechanically supporting structures of the villous trees.

5.2.4 Mature Intermediate Villi

These are long slender villi which are functionally responsible for the production of the terminal villi. It has been suggested that they may play a significant role in placental hormone production (Kaufmann et al, 1979)

5.2.5 Terminal Villi

They are grape-like outgrowths originating from the mature intermediate villi. They are characterized by a high degree of capillarization and the presence of dilated sinusoids, making them the main sites of fetoplacental exchange.

5.3 DEVELOPMENTAL AND MORPHOLOGICAL CHANGES DURING PREGNANCY

The placenta undergoes a series of developmental and morphological changes with advancing pregnancy as viewed through the light and scanning electron microscope.

During the first trimester mesenchymal and immature intermediate villi predominate (Castellucci and Kaufmann, 1982). Between the 14th and 20th gestational weeks they comprise most of the villous cross sections. At term they may be completely absent.

These villi are poorly vascularised structures, few in number but large in size, and covered with easily recognizable syncytio and cytotrophoblastic epithelial layers. The syncytiotrophoblast forms a layer of uniform thickness around the periphery of the villi with no identifiable cell boundaries visible between the nuclei. In contrast the cytotrophoblast forms a complete layer of cells with well marked cell borders and pale staining nuclei. In general the architecture of the villous stroma is made up of connective tissue cells, fibres and fetal vessels (Kaufmann et al, 1977). This stage of pregnancy is characterized by a reticular villous stroma which is typified by numerous channels containing Hofbauer cells. Characteristically these cells have a coarsely vacuolated cytoplasm and eccentrically placed nuclei. Most authors consider the Hofbauer cell to be of chorionic mesenchymal origin (Wynn, 1967; Fox, 1967; Kaufmann et al, 1977) However, recent data suggest that these cells are, at least, in part, a self replicating population (Castellucci et al, 1987; Frauli and Ludwig, 1987).

Light and transmission electron microcsopy have shown that the most striking aspect of the Hofbauer cell is their vacuolated appearance (Castellucci et al, 1985). In 1970, Enders and King reported that the vacuolated type of cell is seen predominately in early pregnancy and that with advancing gestation these cells assume a more granulated appearance. They further observed that the cells displayed evidence of pinocytic activity suggesting the uptake of large volumes of fluid. Consequently it was postulated that Hofbauer cells played a pivotal role in water balance in the early placenta. This concept was strengthened by the identification of the great majority of the cells in stromal channels (Castellucci et al, 1980) suggesting ease of movement along the villous core and thus promoting their task of regulating water balance. Hofbauer cells thus function as a substitute for the lymphatic system which is notably absence from the placenta. Fox's contention that there is a direct relationship between the number of Hofbauer cells and the degree of villous edema at term (Fox, 1967), lends support to the speculative role of these cells in fluid balance in the chorionic villi.

The potential role of Hofbauer cells in placental host defence was underscored by the work of several investigators who observed the similarity between these cells and tissue macrophages (McKay et al, 1958; Bourne, 1962; Benirschke and Driscoll, 1967). Further evidence for their macrophagic character came from morphological (Wynn, 1967; Boyd and Hamilton, 1970; Enders and King, 1970), cytochemical (Fox and Kharkonger, 1969) and immunological (Moskalewski et al, 1975) studies. The 3-dimensional visualization of these cells by Castellucci and co-workers (1980) revealed morphological features which strongly suggested that Hofbauer cells are motile in the villous core thus allowing them to exert their role in maintaining host defence. Moreover, data collated from further studies have corroborated the immunological function of this cell population. This relates to the presence of Fc receptors for IgG (Moskalewski et al, 1975), C3b receptor activity (Wood, 1980), elimination of antigen-antibody complexes (Wood and King, 1982), and expression of class I and II major histocompatibility determinants (Bulmer and Johnson, 1984; Bulmer et al, 1988).

In the second half of pregnancy the villi are smaller, narrower but increased in number. The mature intermediate villi are now prominent, branching from the immature intermediate villi (Castellucci & Kaufmann, 1982). The trophoblastic layers have undergone a number of changes. The cytotrophoblast is less prominent while the syncytial layer is thinner, irregular with focal clustering of the the nuclei. Moreover, a distinct and well defined trophoblastic membrane is now visible, separating the trophoblast from the stroma. Capillaries are prominent, situated towards the centre of the villi and lined by mature endothelial cells. The Hofbauer cells are still easily discernible, interspersed within the stroma. Functionally the mature intermediate villi produce the terminal villi. These are the final ramifications of the villous tree during the last trimester.

The mature villous is also characterized by the presence of multinucleated syncytial protrusions known as syncytial knots. They are particularly prominent in prolonged post-term pregnancies. The significance of syncytial knots is not precisely known. Fox (1965)

and Jones (1977) have advanced the theory that they represent senescent syncytial nuclei which have no further functional activity. However, Kaufmann et al (1987) in their elegant study on the three-dimensional structure of the human placental villi showed that significant differences in interpretation exist between cross section and three-dimensional impressions of the same tissue section brought about by the tangential sectioning of tissue specimens. Trophoblastic tangential sectioning produces artificial impressions of syncytial sprouts and bridges. In the immature placenta with its wide calibre villi tangential sectioning is a rare event. Thus the correlation between scanning electron microscopy (SEM) and light microscopy (LM) is good. When viewing mature term placentas on SEM they demonstrate the presence of long and slender villi with grape-like terminal branches. Such viewing on LM will show the presence of sprouts and bridges which are regarded as typical signs of villous maturation. The relative absence of "sprouts" on SEM is due to the fact that there is less chance of tangential sectioning with the semi-thin sections (0,5-1.0 μm) employed in SEM than with the thicker (5-10.0 μm) paraffin sections used in LM. Therefore, semi-thin sections of mature placentas may be misinterpreted as immature placental tissue because of the absence of syncytial bridges and sprouts. In addition, the increased knots and sprouts seen in prolonged pregnancy and in pre-eclampsia, i.e. the features of hypermaturity (Salvatore 1968) is nothing more than tangential sectioning of long branched and twisted terminal villi. On SEM no excess true syncytial sprouting was noted or detected. Whatever the physical characteristics that explain how syncytial knots are seen to occur on LM, they are a recognizable phenomenon with interpretative value.

At this stage the syncytiotrophoblast itself is much thinner and the cytotrophoblast is much less prominent and flattened between the syncytial cells and the trophoblastic membrane. Although histologically less prominent, the cytotrophoblastic cells retain their ability to proliferate when stimulated. Probably the most striking feature of the villi of the mature placentas is their high degree of vascularization. The fetal vessels are sinusoidally dilated and situated towards the periphery of the villi in close proximity to the trophoblast. The reticular stroma which dominated early pregnancy is now replaced by a more fibrous type

of stroma resulting in partial obliteration of the stromal channels. The sinusoidal type of stroma is well suited to passive and facilitated transport. The Hofbauer cells are still present to significant degree but they are less easily recognizable as they are compressed by fetal vessels and collagen fibres.

This ordered stage of morphological development results in a reduction of the mean maternofetal diffusion distance from 50-100 μm in the 2nd month of gestation to between 4-5 μm at term. In other words, the high degree of vascularization and a significantly reduced maternofetal distance makes this villous type functionally the most appropriate for diffusional exchange (Sen et al, 1979).

In summary this synopsis of villous histological maturation during the different phases of pregnancy is designed to provide the basis and foundation essential to an understanding of the varied pathological changes observed in a placenta afflicted with syphilis, and, consequently, an appreciation of the findings of the present study which explores the pathogenesis of the syphilitic placentitis.

CHAPTER 6 PLACENTAL HISTOPATHOLOGY IN SYPHILIS - A REVIEW OF THE LITERATURE

Hormann's review in 1954 of placental syphilis provided the first in depth histological assessment of earlier studies of the diseased syphilitic placenta (Fox, 1978b). Corroboration of these findings only appeared twenty years later when Russel and Altshuler (1974) described their findings in a study of three placentas from infants with congenital syphilis. The placentas were described as large and bulky with a triad of villous immaturity, focal villitis and an obliterative endarteritis. Villous immaturity was deduced from the large size of the villi and the presence of increased cellularity relative to gestational age . These villous changes could be either focal or diffuse. In addition the fetal vessels showed various degrees of obliteration due to a proliferative endarteritis.

In a recent study of six cases, Walter, Blot and Ivanoff (1982) reported that hypercellularity of villi characterised by a loose poorly vascularized stroma with numerous mesenchymal and Hofbauer cells, was the outstanding histological feature. In addition numerous monocytes were present in the villous vascular lumina. Associated lesions included a peri-and/or intra-villous polymorphonuclear infiltration with or without a cytotrophoblastic or stromal necrosis.

Other reported series (Russell and Altshuler, 1974; Benirschke and Driscoll, 1967) found that vasculitis and villous lympho- plasmocytic infiltration were uncommon features. In a recent study Qureshi et al (1993) underscored the importance of the histopathological changes in the placenta in congenital syphilis. They observed that the presence of a chronic villitis, relative villous immaturity and proliferative vascular changes represented a well-defined constellation of histopathological changes.

Gross examination and placental-birth weight ratios were the subjects of study by Malan and co-workers (1990). They reported on the relative placental weights of 74 infants with

congenital syphilis. The majority of the placentas were heavier than normal; 38 were above the 90th centile on the placenta-birth weight chart while only 15 were below the 50th centile. The placentas themselves were noted to be pale and friable. Qureshi et al (1993) observed that 11 of the placentas studied were above the 50th centile and 2 were above 90th centile. Macroscopically the placentas appeared normal. The clinical significance of these large placentas was underscored by Walter, Blot and Ivanoff, (1982). They commented that in previously reported series such placental enlargement appeared to be associated with perinatal death in 50% of cases. As Malan et al (1990) focused their study on relative placental weights, there was no comment on the clinical outcome of the infants in this study.

This disparate and varied histological findings led Walter, Blott and Ivanoff (1982) to suggest that the placenta is involved in a spectrum of histological changes influenced by successive stages of the fetal immune response. The first phase, an inductive phase, in which there is absence of the host immune response would be characterized by the absence of inflammatory changes in both the infected placenta and fetus. This is followed by a reactive phase manifested by a villous inflammatory cell infiltrate. Corroboratory evidence for the concept is found in the work of Harter and Benirschke (1976) who demonstrated the presence of Treponema pallidum using silver and immunofluorescence stains in two first trimester (9 and 10 weeks) conceptuses and found no evidence of any inflammatory response. This discovery led to the revision of the long held theory that up to the 4-5th month of gestation the fetus is protected from the invading spirochaetes by a placental barrier residing in the trophoblastic Langhans cell layer. It seems certain now that the spectrum of pathological changes present in congenital syphilis is the result of the inflammatory response which becomes evident in the fetus at about the 5th month of gestation. This concept evolved from the pioneering work of Silverstein and Lukes (1962) on the fetal antigenic response. They concluded that the histological features of congenital syphilis were dependent on the development of fetal immune competence. This was all put

into perspective by Benirschke (1974) in an editorial comment titled "Syphilis - the placenta and the fetus".

While the aforementioned patterns of placental lesions are common, they are not specific for congenital syphilis. A chronic villitis is associated with cytomegalovirus (CMV), rubella, and toxoplasma intrauterine infections. The histological hallmarks of CMV placentitis include a chronic lympho-plasmacytic villitis, thrombosis of villous capillaries often with adjacent hemosiderin deposits, necrosis of villous tissue and trophoblast, fibrosis of villous stroma and inclusion-bearing cytomegalic cells (Benirschke and Kaufmann, 1990). Garcia et al (1989) observed that the majority of the placentas were grossly abnormal. There was a tendency to an increased placental weight relative to gestational age. Benirschke and co-workers (1974) reported no characteristic gross placental changes. Schwartz et al (1992) characterized the cellular infiltrate in CMV placentitis. Hofbauer cells and T lymphocytes predominated with a significant proportion of IgM and IgG bearing plasma cells. B lymphocytic reactivity was absent, suggesting that the B lymphocyte is apparently not a significant component of the inflammatory reaction in CMV placentitis.

Garcia and associates (1985) concurred with Blanc (1978) that placental hypoplasia is characteristic of intrauterine rubella infection. They reported placental weights well below the normal values for gestational age. Macroscopically, they observed abnormalities of the Whartons jelly and placental membranes. However, the microscopic changes, particularly the chronic villitis, though more intense in the last trimester, were nonspecific.

Though the placentas of pregnant women infected with the Human Immunodeficiency Virus (HIV) do not show any features of a villitis, they do demonstrate an increase in placental-fetal weight ratios with villous hypercellularity and hypervascularity (Jauniax et al, 1988). Similarly, in their study of term placentas from 43 HIV sero-positive mothers, Chandwani et al (1991) found that while 60% of the sero-positive placentas displayed a

chorionitis and 14% a funisitis there was no evidence of a villitis. As chorionitis and funisitis were present in 27% and 6, 1% respectively of the sero-negative placentas the authors concluded that there were no histopathological features specific to HIV infected placentas.

The entity of villitis of unknown origin (VUE) as described by Altshuler (1973) and Russell (1980) and reviewed by Benirschke and Kaufmann (1990) have histological features similar to that of the well documented infective villitides. Currently no infectious cause has been delineated for VUE. However, the severity and destructive nature of VUE is associated with fetal compromise and morbidity.

Consequently, whereas the infective villitides have features which allow their histological identification, it does not allow a definitive diagnosis to be made based on such a varied histological profile. Therefore, in the case of congenital syphilis epidemiological, historical, physical, and serological factors have to be incorporated in the diagnostic workup.

CHAPTER 7 SYPHILITIC PLACENTITIS: AN IMMUNOPATHY?

7.1 INTRODUCTION

The macroscopic and microscopic changes in the placentas of infants with congenital syphilis are well described and have been reviewed in the previous section. To date, all the factors involved in the pathogenesis and contributing to these placental changes have not been elucidated.

The membranous nephropathy of congenital syphilis and its clinical expression as the nephrotic syndrome has been shown by several investigators (Kaplan et al, 1972; Hill et al, 1972; Wiggelinkhuizen et al, 1973; Gamble and Reardon, 1975) to have an immune pathogenesis. Immunofluorescent and electron microscopic studies have demonstrated deposits of immune complexes on the epithelial aspect of the renal glomerular basement membrane. In all the immunofluorescent studies the presence of IgG has been consistently demonstrated in the immune deposits (Kaplan et al, 1972; Hill et al 1972; Wiggelinkhuizen et al, 1973; Gamble and Reardon, 1975). In addition, except for one study (Kaplan et al, 1972), IgM and to a lesser extent the IgA were also present. In two of the studies (Wiggelinkhuizen et al, 1973; Gamble and Reardon, 1975) complement was actively sought for and found.

Two recent studies on the immune status of infants with congenital syphilis have demonstrated the presence of elevated levels of circulating ICs (Samson et al, 1990) and IgM rheumatoid factor (IgMRF) (Meyer and Beatty, 1991) in such infants.

These products of the immune response to an antigenic stimulus, which in the main have a protective role, have, under conditions of intense and continued antigenic stimulation the potential for entrapment and deposition in various bodily organs. High levels of circulatory ICs have been well described in adult syphilis (Engel and Diezel, 1980; Folds,

Maret and Rauchbach, 1982) and implicated in the pathogenesis of the skin lesions in secondary syphilis (Jorizzo et al, 1986). Theofilopoulos and Dixon (1980) in their extensive review of immune complexes in human diseases describe a number of autoimmune and infectious diseases where ICs are thought to play an important immunopathological role. Similarly, several investigators have provided experimental evidence for an exacerbation of tissue damage by ICs in a variety of organs (McCormick et al, 1969; De Horatius and Williams, 1972). Specifically, IgM RF has been shown to bind to ICs deposited in glomeruli and to trap further ICs from the circulation and in this way may promote chronic disease and tissue damage (Ford, 1983). Baum et al (1964) and Floyd and Tesar (1979) have produced evidence for an aggravating role of IgM RF in IC induced vasculitis in rats.

The cumulative evidence from both animal and human studies suggest that ICs singularly or in combination with IgM RF play an important role in inducing and promoting tissue damage. Based on this evidence it is reasonable to assume and hypothesize that the placenta, a fetal organ, may be vulnerable to IC-IgM RF induced tissue damage in congenital syphilis especially when there are high levels of ICs (Samson et al, 1990) and IgM RF (Meyer and Beatty, 1991) The presence of IgM RF has previously been identified in syphilitic placental tissue (Meyer, 1990). but there are no reports on the presence of ICs in placental villous tissue. Baughn and Musher (1983) and Dobson et al (1988a) analysed ICs in syphilitic rabbits and in infants with congenital syphilis respectively, and demonstrated the presence of IgG, IgM, and C3 in immune complexes. The IgM isotype was shown to be the dominant isotype in congenital syphilis (Dobson et al, 1988a).

In order to test this hypothesis immune complexes in the placenta of infants with congenital syphilis were examined immunohistochemically for the presence of IgG, IgM, andC3.

7.2 MATERIALS AND METHODS

7.2.1 Gross examination

Five placentas were randomly selected from a cohort of nineteen neonates with congenital syphilis who were recruited for the immunological study (Chapter 2). Five placentas from a similar number of noninfected well infants matched for weight and gestational age were examined as controls. Placentas were considered noninfected when there was no evidence of overt chorioamnionitis, both clinically in the mother or on macroscopic examination of the fetal membranes and when clinical examination of the infants was normal. In addition, the mothers had negative serological tests for syphilis.

Both the infected and normal placentas were prepared as follows: All placentas were prepared within 24 hours of delivery. The placentas were drained and then weighed with the umbilical cord and fetal membranes intact on a Salter Thermoscale (No. 40A) calibrated in 20g markings. This was followed by a macroscopic examination with particular reference to texture, presence or absence of pallor, calcification and areas of infarction. A placental-birth weight ratio was calculated for each patient and control.

The fetal membranes were then stripped to the margins of the placenta. Full thickness sections were taken from the periphery and centre of the organ as set out by Fox (1978c) (Appendix E).

The tissue blocks were fixed in 10% neutral buffered formalin. For both the histological examination and the immunohistochemical staining 5μm thick blocks were prepared and paraffin embedded.

7.2.2 Histological examination

The blocks were stained with standard haematoxylin - eosin (H & E) stain for light microscopical examination.

7.2.3 Immunohistochemical stain

The immunohistochemical staining method employed to identify IgG, IgM and C3 in tissues was based on the Streptavidin - biotin methodology (Zymed LAB-SA System, Histostain-Bulk Kits, Zymed Laboratories Inc., Ca, USA), a tool widely used in immunology and diagnostic medicine. The system is based on the strong binding between streptavidin, a protein isolated from Streptomyces avidinii, and biotin, a water soluble vitamin, which in turn binds strongly to a variety of proteins and nucleic acids. This system is considered to have an advantage over the previous peroxidase anti-peroxidase (PAP) system in that only a single universal labelling agent is employed, namely, horse-radish peroxidase-streptavidin conjugate, instead of several different PAP complexes for each animal system.

The staining procedure was carried out according to the manufacturer's instructions accompanying the kit (Appendix F) where most of the reagents supplied were ready-to-use. Optimum dilutions for the primary antibody was established in the Histopathology Laboratory, Red Cross Childrens Hospital by a chequer board dilution method for the primary antibody. The paraffin embedded tissue sections were deparaffinated with xylene and then rehydrated with ethanol. They were then washed in a PBS bath (10mM phosphate-buffered saline, Ph 7.5) for 10 minutes. Primary antibodies (Behring, Germany) were directed at IgM, IgG and C3 using rabbit antihuman IgG, IgM and C3 in dilutions of 1:1500, 1:1500 and 1:400 respectively. They were incubated overnight at 4°C.

Biotinylated antirabbit serum was applied for ten minutes followed by the streptavidin - peroxidase complex for five minutes. A substrate-chromogen solution containing 3-amino-9-ethylcarbazole stained the complexes for 20 minutes and haematoxylin was used as a counterstain. Negative controls on each block were prepared by omitting the primary antibody. This step was essential to reduce the problem of nonspecific binding.

7.3 STATISTICAL METHODS

The Mann-Whitney two sample statistical method was used to compare the placental-birth weight ratios between the case and control groups. A $p < 0.05$ was regarded as significant.

7.4 RESULTS

7.4.1 Gross examination and placental-birth weight ratio

A feature consistently observed during the gross examination of all the syphilitic placentas was its textural friability. In only three cases (1, 4 & 5) were the placentas were pale. Of the five, these placentas were also the heaviest (Table 7-1) with weights on or above the 90th centile when plotted on the placental-birth weight chart (Malan et al, 1990). However, when a placental-birth weight ratio was calculated for each case and control and then compared, the mean ratio of the cases was not significantly increased. ($p=0.173$) (Tables 7-1 and 7-2)

Table 7-1 Placental and birth weights of infants with congenital syphilis

Case	G.A. (wks)	Plac wt (gm)	B. W. (gm)	Plac-B.W.
1	36	660	2020	0.33
2	33	380	1450	0.26
3	36	450	1400	0.32
4	33	600	1460	0.41
5	37	640	1980	0.32

Plac-B.W. Mean $\pm$ sd = 0.33 $\pm$ 0.05

G.A. = gestational age in weeks.

B.W. = birth weight in grams

Plac. wt = placental weight

Plac-BW = placental-birth weight ratio

Table 7-2 Placental and birth weights of healthy control population

Control	G.A. (wks)	Plac wt (gm)	B. W. (gm)	Plac-B.W.
1	37	400	2300	0.17
2	34	420	1400	0.30
3	34	540	1500	0.36
4	34	420	1360	0.31
5	36	390	2200	0.18

Plac-B.W. Mean $\pm$ SD = 0.26 $\pm$ 0.08

G.A. = gestational age in weeks.

B.W. = birth weight in grams

Plac. wt = placental weight

Plac-BW = placental-birth weight ratio

In none of the syphilitic placentas was any significant calcification or areas of infarction observed. In one control placenta (no. 1) patchy areas of calcification on the maternal surface was noted. The remainder of the gross examination of the control placentas was normal.

7.4.2 Histological examination

Examination of the tissue sections from central and peripheral placental sites revealed no significant difference using both H & E and the immunohistochemical stains. Thus the observations reported apply equally to both central and peripheral tissue sections.

Histologically (H & E stain) the outstanding feature of the placentas with congenital syphilis was the presence of large hypercellular villi with a loose, poorly vascularised stroma. Numerous macrophages (Hofbauer cells) were present in the villous stroma in all these placentas. In only one case was an obliterative endarteritis easily recognised. In all the cases, the syncytiotrophoblast was diffusely flattened and in 3 cases a villous plasma cell infiltrate was observed. All these histopatholgical observations were focal in distribution with normal villi abutting those with marked histological changes.

In the control placentas the villous development was considered appropriate for the gestational age of the placenta as indirectly evaluated from the gestational age of the infant, and no villitis or vessel wall changes were observed.

7.4.3 Immunohistochemical stain

The distribution of the immune complexes as determined by the immunoreactivity pattern of the IgM, C3 and IgG are depicted in Tables 7-3, 7-4, 7-5.

Table 7-3 Immunoreactivity pattern of Syphilitic and Control placentas for IgM

Villous distribution

No		Stem	Intermediate	Terminal
1.	Disease	+	+	*+
	Control	-	-	-
2.	Disease	+ S	+	*+
	Control	-	-	-
3.	Disease	±	+	*+
	Control	-	-	-
4.	Disease	+S	+S	*+
	Control	-/ patchy	-/ patchy	-
5.	Disease	+S	+S	*+
	Control	-/ patchy	-/ patchy	-

+ = diffuse intense endothelial positivity

± = focal intense endothelial positivity

*+ = very few terminal villi present, but those present are positive

+S = includes positivity of serum

+T = includes positivity of the trophoblast

Table 7-4 Immunoreactivity pattern of Syphilitic and Control Placentas for C3

Villous distribution

No		Stem	Intermediate	Terminal
1.	Disease	+	+	*+
	Control	/patchy	/patchy	/patchy
2.	Disease	+S	+	*±
	Control	-	-	-
3.	Disease	±	±	±
	Control	-	patchy	patchy
4.	Disease	+S	+S	*+S
	Control	-	-	-
5.	Disease	±	± focal	focal
	Control	patchy	patchy	patchy

+ = diffuse intense endothelial positivity

± = focal intense endothelial positivity

*+ = very few terminal villi present, but those present are positive

+S = includes positivity of serum

+T = includes positivity of the trophoblast

Table 7-5 Immunoreactivity pattern of Syphilitic and Control placentas for IgG

Villous distribution

No		Stem	Intermediate	Terminal
1.	Disease	+T/S	+	*+
	Control	+T	+T	+T
2.	Disease	+T	+T	*+
	Control	+T	+	+
3.	Disease	±	±	±
	Control	+	+	+
4.	Disease	+T	+T	*+
	Control	+	+	+
5.	Disease	+T	+	*+
	Control	+T	+	+

+ = diffuse intense endothelial positivity

± = focal intense endothelial positivity

*+ = very few terminal villi present, but those present are positive

+S = includes positivity of serum

+T = includes positivity of the trophoblast

IgM reactivity (Table 7-3)

In the syphilitic placentas the pattern of IgM immunoreactivity had its greatest intensity in the vascular endothelium of the stem and intermediate villi (Figure 7-1). Though the terminal villi were few in number those that were present demonstrated positive endothelial reactivity (Figure 7-2). In two of the tissue sections (no's 2 & 4) some degree of reactivity appeared within the serum present in the lumen of the fetal vessels. In contrast, neither the villous stroma nor the villous trophoblast showed any degree of reactivity. The control placentas showed no IgM immunoreactivity.

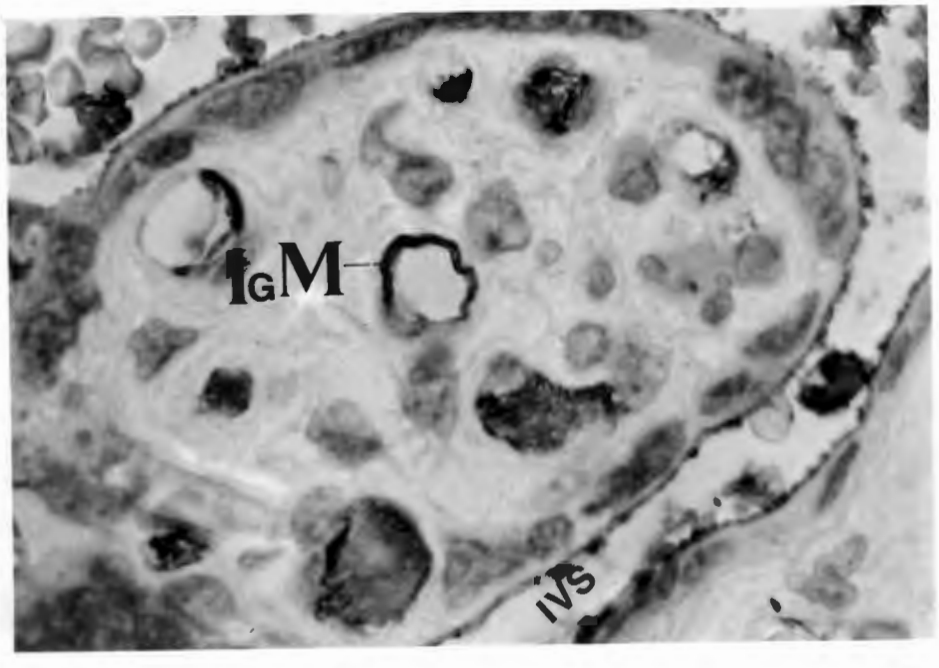


Fig 7-1
Endothelial IgM reactivity in tertiary villous (Streptavidin-Biotin immunohisto-chemical stain x 630; IVS, intervillous space)

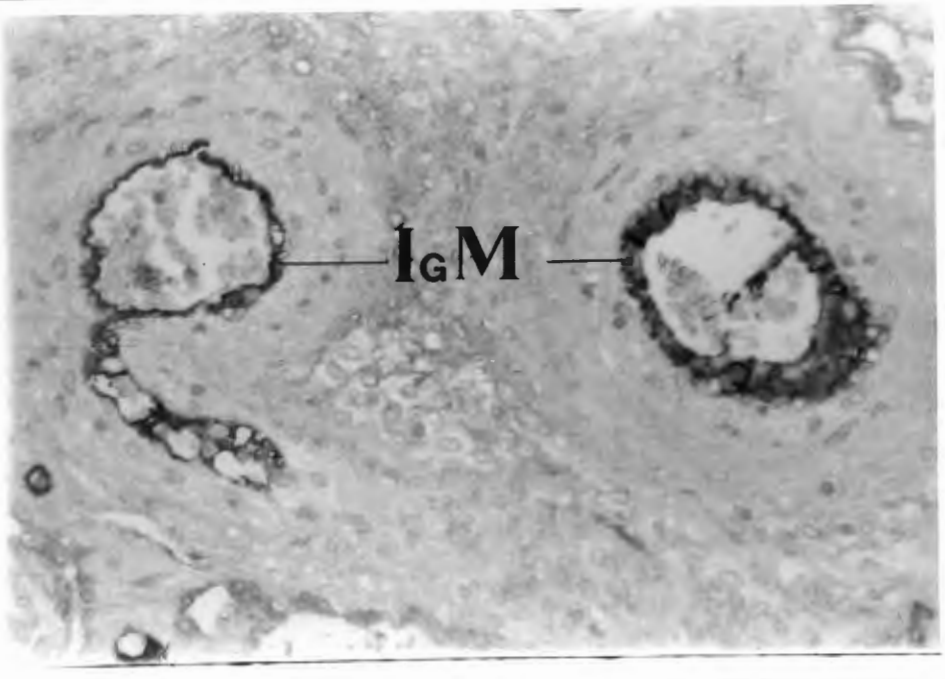


Fig 7-2
Endothelial IgM in a stem villous (Streptavidin-Biotin immunohistochemical stain x 252)

C3 reactivity (Table 7-4)

The positive reactivity for C3 in the syphilis placentas (Figure 7-3) was not as distinct and well defined as that for either IgM or IgG. In three out of five placentas diffuse positive staining was demonstrated in the vascular endothelium of the stem and intermediate villi and in those few terminal villi that were present. In the remaining diseased placentas the reactivity was more focal in distribution. In none of the placentas was reactivity observed within the stroma or in the trophoblast layer.

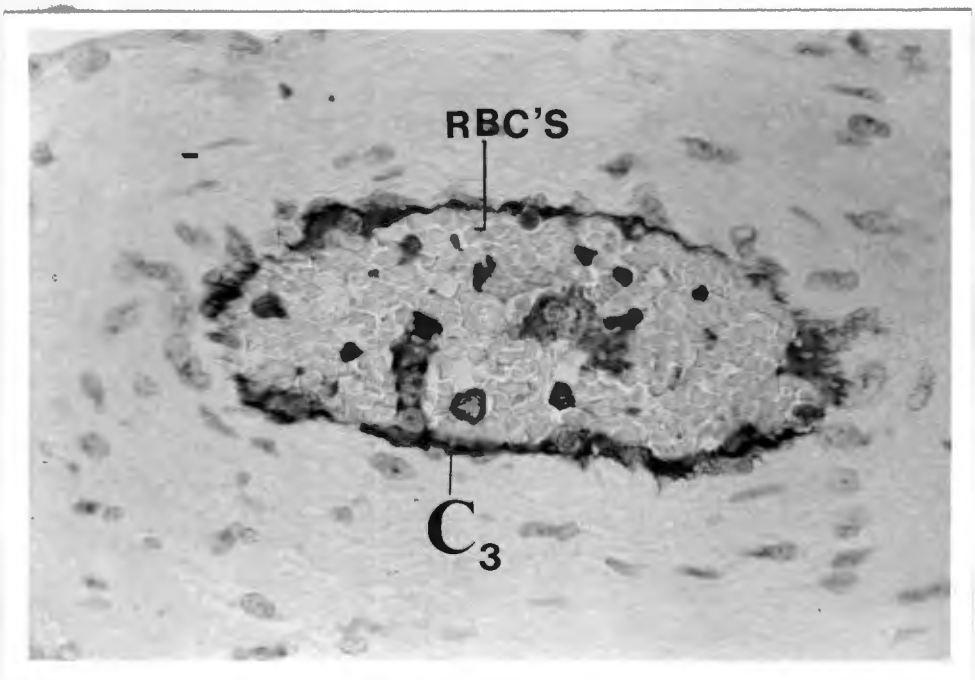


Fig 7-3

C3 reactivity in endothelium of stem villous (Streptavidin-Biotin immunohistochemical stain x 160)

The normal control placentas showed very patchy reactivity in three placentas in all the villi that were looked at.

Tissue sections, stained immunohistochemically for IgM, C3 and IgG, without prior exposure to the primary antibody, all showed a negative reaction (Figure 7-4).

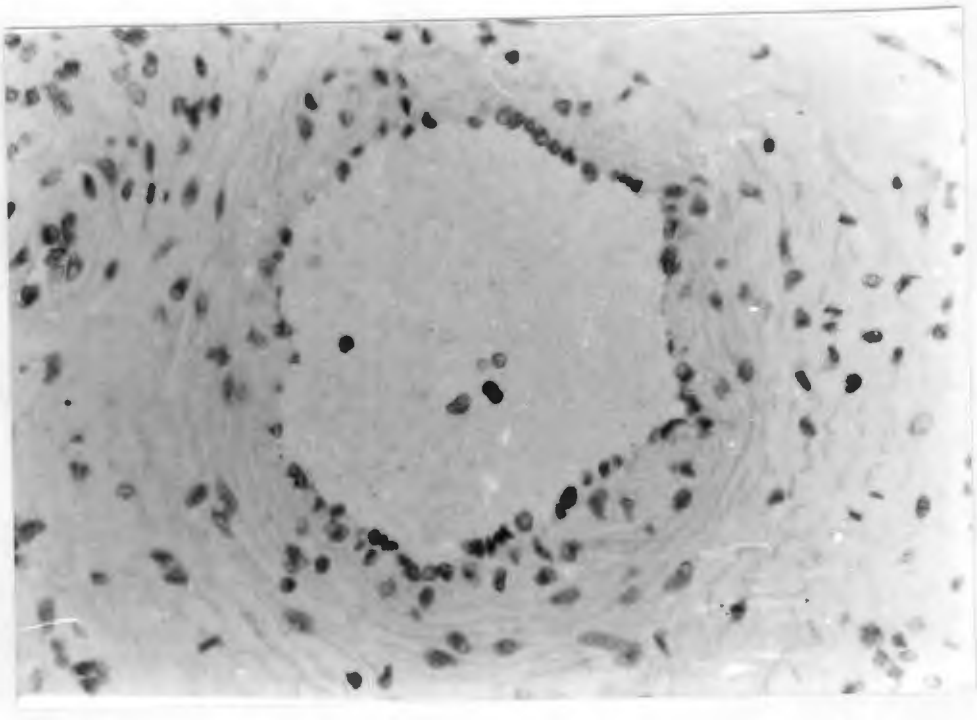


Fig 7-4

Negative control showing absence of reactivity in stem villous x 160

IgG reactivity (Table 7-5)

The terminal villi were quantitatively reduced in number in the diseased placentas, but in those that were present endothelial IgG was demonstrated. In the majority of the placentas positive trophoblastic associated IgG was well defined particularly in the stem villi.

In the control placentas all branches of the villous tree were positive for IgG staining including the trophoblast layer. In some of the sections the stroma showed weak IgG positivity but in the majority no stromal reactivity was seen. This observation was also seen in the diseased placentas.

7.5 DISCUSSION

The gross anatomy and the varied histological patterns described in this study are well known and have been extensively studied and described by a number of investigators in the 1930's and more recently in the 1970's (McCord, 1934; Dorman and Sahyun, 1937; Russel and Altshuler, 1974; Harter and Benirschke, 1976; Walter et al, 1982). The major histopathological changes of a focal villitis, villous immaturity and macrophage infiltration of the villous stroma were observed in this study and are not at variance to that which has previously been described.

The aim of the study was to test the hypothesis that the placentitis in intrauterine syphilis is mediated by products of the fetal immune response, in particular, by the formation of immune complexes. Immune complexes were sought for by staining for it's components namely, IgM, C3 and IgG which on the basis of previous studies (Baughn and Musher, 1983; Dobson et al, 1988) are assumed to be constituents of these complexes.

The following evaluation and analysis of the patterns of reactivity were formed:

IgM reactivity had a distinct and defined pattern, being located and distributed in the endothelium of the stem, intermediate and terminal villous vessels and notably absent from the stroma and trophoblastic layers and in the normal control placentas. The IgM immunoglubulin, being a macromolecular pentametric molecule, does not allow for maternal-fetal transfer (Gitlin et al, 1964; Hay et al, 1971). Thus its presence within the fetal vessels implies that the origin of this isotype is from the

fetus itself. The presence of the IgM isotype within chorionic villous vessels may in itself not be an abnormal finding as IgM is produced by the fetus, from ± 10 - 11 weeks of gestation (Stiehm, 1975). The presence of IgM on the vascular endothelia of the syphilitic placentas was, however, markedly increased when compared to normal control placentas, and the absence of reactivity in the negative controls indicate that these are not nonspecific findings. Johnson et al (1975) were unable to demonstrate IgM on the endothelial cells of normal placentas using FITC-antihuman IgM, though in a subsequent study by Johnson and co-workers (1977), weak staining for IgM was observed in some of the chorionic villi. Further evidence lending support to the fact that the positive IgM reactivity is a distinctly abnormal finding is the presence of significantly elevated levels of serum IgM in infected newborns as demonstrated in the present (Chapter 3) and other studies (Alford, 1971). An assimilation of all these observations is supportive evidence for the deposition of IgM on the endothelial surface of fetal vessels within the chorionic villi of syphilitic placentas.

In 1980, Faulk and his fellow workers in their study of complement in normal mature and immature human placentas, noted the absence of receptors to unaltered or native C3. However, specific components of complement were present at characteristic locations in the placentas. CIq was found within vessel walls, endothelium and certain stromal cells, while C3d, a product of activated C3, and C9 were present in the trophoblastic basement membrane. C6 was seen as clustered cytoplasmic granules within the endothelium of fetal stem vessels suggesting that C6 may be stored and/or synthesized in these cells.

The findings in this study of absent or weak C3 staining in some villi in the normal placentas supports the findings of Faulk et al, (1980). In the majority of the syphilitic placentas the reactivity was intensely positive but less than that observed for IgM. No stromal or trophoblastic reactivity was observed. These findings

suggest that C3 is activated and altered during the immune response to syphilis permitting its endothelial deposition in sites similar to that found for IgM, and was therefore probably a component of an immune complex .

The staining pattern for IgG was entirely different. Much of what is known of the presence of IgG in the placenta has been summarised in two recent reviews (Faulk and Johnson, 1980; Johnson and Brown, 1981). The Fcy receptor activity is located in the placenta at the principal site of contact and molecular exchange between mother and fetus viz., the syncytiotrophoblastic microvillous plasma membrane. Such receptor activity is far more evident in mature than immature placentas. That maternal-fetal IgG transfer does occur has been proven by the fact that the bulk of the IgG in cord blood is of the maternal phenotype based on the Gm allotype studies, though a percentage of the IgG does express the phenotypic markers of the fetal origin (Martensson and Fudenberg, 1965). In an extension of this study, Johnson and co-workers (1977) in attempting to define and characterize the Ig classes and subclasses in the human placenta using an immunofluorescent technique, observed that the distribution of IgG was confined to the endothelium, the perivascular tissues and in areas of fibrinoid necrosis. The majority of this IgG proved to be of maternal origin with a small amount of fetal IgG in fibrinoid deposits and in the cytoplasm of some stromal cells. These investigators did not observe IgG within the syncytiotrophoblast. Subsequent studies by Martre, (1977) and Johnson and Martre, (1979) using a different detection system, (erythrocyte indicator system), have consistently shown the presence of IgG on trophoblastic tissue.

Further studies have demonstrated Fcy receptors on cells other than villous syncytiotrophoblast, namely endothelial and Hofbauer cells (Johnson and Brown, 1981). However, in contrast to trophoblastic tissue, the specificity of those receptors is more pronounced for complexed or aggregated IgG rather than native

IgG. From this has evolved the concept that these Fcy receptors have an immunobiological function that is distinct from those receptors situated on syncytiotrophoblastic cells. It would appear that these receptors have been assigned the responsibility of ensuring the sequestration of soluble immune complexes formed in situ by maternal IgG antibody and paternally derived allo-antigens (Johnson and Brown, 1981). Conceptually this trapping of immune complexes can be viewed as a type of physiological sink into which immune complexes are trapped, preventing their entry into the fetal circulation and in so doing, help to protect the fetus from immune complex mediated tissue damage (Faulk and Johnson, 1980).

It could be argued that the positive IgM and C3 staining represents nonspecific binding of the primary and secondary antibodies (IgG) to the Fc receptors in placental tissue. However, two factors mitigate against this contention. Firstly, fixing of placental tissue in formalin has been shown to destroy the Fc receptors (Matre and Johnson, 1977; Matre and Haugen, 1978) and, secondly, the lack of significant reactivity within the normal control placentas and in the negative controls, indicate that the IgM and C3 staining observed are specific.

It is far more difficult to draw conclusions from the pattern of reactivity of IgG. The majority of the fetuses at the time of study were at a stage of gestational maturity when placental IgG transfer is optimal. Thus, the intensity of reactivity for IgG in both the normal and diseased placentas is not an unusual phenomenon. It also implies that despite extensive placental disease, maternal-fetal IgG transfer is not adversely affected. It further implies, that because of the absence of reactivity in the negative control, that the positive IgG staining is specific.

These findings beg the question of the significance of the IgG in the diseased placentas. Is it part of the normal physiological process of placental IgG transfer,

or does the IgG represent part of an immune complex molecule with IgM and C3? In the absence of fetal/maternal identification of the IgG molecules in the syphilitic placentas, it is difficult to prove that the IgG reactivity observed is the result of trapping of fetal IgG following the immune response to a chronic syphilitic infection. However, there is some indirect evidence to support the involvement of IgG in the pathological immune response to syphilis. Meyer (1990) has demonstrated the presence of IgM rheumatoid factor in fetal vessels placentas of infants with congenital syphilis in a distribution similar to that noted for IgG. Samson et al, (1990) showed that infants with congenital syphilis have elevated levels of circulating immune complexes (IC's). Analysis and characterization of these IC's by Dobson and co-workers (1988a) showed that though they contain predominantly IgM, there were, in addition, IgG immune complexes.

The patterns of reactivity for IgM, C3 and possibly IgG, lends strong support to the postulate that they represent the presence of immune complexes attached to the endothelium of the fetal vessels throughout the villous tree, formed in response to the invading spirochaete. These IC's are distinct and separate from those normally formed and trapped in the placental sink as part of an immunobiological response to protect the fetus from immunological injury as the latter contain neither IgM nor C3 (Faulk and Johnson, 1980; Johnson and Brown, 1981). Rheumatoid factor, as Meyer (1990) has pointed out, probably exacerbates the problem by binding the IC's and being multivalent, further trap complexes from the cirulation as Ford (1983) has demonstrated in a number of experiments using a rat model.

It is therefore postulated that IC's alone or in concert with RF play an important role in the pathogenesis of the syphilitic placental disease. The immunopathological role of trapped IC's have been recognized in a number of pathological disorders (Theofilopolous and Dixon, 1980). Its role in syphilitic glomerulonephritis has been well described (Kaplan. et al, 1972; Hill et al, 1972; Wiggelinkhuizen et al, 1973; Gamble and Reardon, 1975)

Furthermore, the role of RF as an aggravating factor in IC-mediated vasculitis has also been recognized (Baum et al, 1964; Floyd and Tesar, 1979).

A consequence of the deposition of IC's may lead to a type III immunological reaction associated with the accumulation of inflammatory cells and the exudation of edema fluid. It is possible that such a reaction could account for the macroscopic features of a large, pale, friable placenta with a histological focal villitis, vasculitis and stromal edema.

CHAPTER 8 EPILOGUE

Intrauterine syphilis, like secondary syphilis, is characterized by haematogenous dissemination. Up until the 20th week of gestation, the fetus is incapable of mounting an appropriate immune response. Thereafter, recruitment of the major immunological role players takes place. The overall aim of this study was to document this immune response and to test the hypothesis that the placental histopathology may have an immune pathogenesis.

The humoral response was characterized by active mobilization of the B cells associated with a substantial increase in the peripheral cell count. In accordance with the ontogeny of the B cell, there was an outpouring of IgM antibodies, a feature which had been well described previously and which still is of particular relevance to clinical practice.

Quantitatively the CD3, CD4 and CD8 counts were similar to that measured in the healthy control population. However, the inference drawn from the lymphocyte transformation assay was that there appeared to be a qualitative defect in the T cell response. The immediate cause was unknown. Published reports implicated the immuno-modulatory influence of circulating immune complexes. Though high levels of immune complexes were demonstrated in the study group, this theory was not tested. It is uncertain how this impacts on the infants clinical course. Practically, the clinical response of these patients to penicillin therapy has been appropriate and uncomplicated.

Markedly elevated levels of immune complexes were measured in the neonates with congenital syphilis. Concordance was sought between these levels and the extent of the systemic disease. Though there was a trend, a positive correlation was not demonstrated. A positive correlation did exist between the IgM and immune complex concentrations. This was attributed to the formation of IgM-immune complexes, a feature previously observed in other studies.

Measurement of serum total haemolytic complement (CH50) demonstrated significantly low levels. This finding was attributed to the activation of the classical complement pathway by two of its most potent activators, namely the IgM molecule and circulating immune complexes.

Variations in total while cell and differential counts observed in the present study were similar to that reported previously. However, no studies have evaluated the oxidative burst during phagocytosis in congenital syphilis. Studies in ill stressed newborns have shown a depression in the oxidative burst pattern as measured by the nitroblue tetrazolium test. This pattern was not observed in this study. The results of the NBT test was equivalent to that seen in the control population. This finding is in accordance with other reports which has shown the macrophage to be the prime line of defence against *T.pallidum* infection.

At the time of completion of treatment, no major changes in the immune status had occurred. B cell and IgM concentrations were still elevated. The persistently high level of IgM has clinical and practical implications in the IgM-incorporated serological tests eg. the FTA-ABS IgM, remains positive well beyond completion of treatment. Improvement in blast transformation occurred but did not reach normal levels. Immune complex concentrations diminished significantly and precipitously, but did not disappear altogether. Several investigators have raised concern about the presence of persistent immune complexes, inferring the persistence of *T.pallidum*. This study did not address this concern; however, until proved otherwise, cognizance of such a finding should be made and appropriate follow-up of the patients arranged.

The histopathological findings in the diseased placentas corroborated the observations of previous reports. Its pathogenesis had not been investigated previously. The hypothesis of an immune pathogenesis was tested. Components of immune complexes, namely, IgM, C3 and IgM were sought for and found in the endothelia of the fetal vessels.

Immune-complex-mediated tissue damage could be the likely consequence with resultant inflammatory cell infiltration and villous edema. Immune complex deposits had been observed previously in the renal lesions of congenital syphilis. By inference other organ systems, particularly bone, may show similar involvement.

The combination of multisystem tissue damage plus the aforementioned immunological findings, in particular that of the high IC, and low CH50 levels, confers on congenital syphilis the hallmarks of an immune-complex-mediated disorder. The role of steroids as an adjunct to penicillin therapy had been addressed as part of a much broader study. While the addition of steroids made no impact on the clinical course, a larger study designed specifically to address this question, is warranted.

In conclusion, the fetus has been afforded the capability of mounting an appropriate immune response to the *T.pallidum* antigen. However the outcome of such a response is not always beneficial to the fetus. Diffuse tissue and organ damage are the consequences. Fortunately with adequate therapy the sequelae are minimal to nil.

APPENDIX A

Criteria for the Diagnosis of Neonatal and Early Congenital Syphilis*

Diagnostic Criteria

Clinical

Absolute

1. Specimen from lesions showing presence of *Treponema pallidum* by dark-field examination, or by histologic examination

Major

2. Condyloma lata
3. Osteochondritis, periostitis
4. Snuffles or hemorrhagic rhinitis

Minor

5. Fissures of lips
6. Cutaneous lesions
7. Mucous patches
8. Hepatomegaly, splenomegaly
9. Generalized lymphadenopathy
10. Central nervous system signs
11. Hemolytic anemia
12. Elevated No. of cells or protein level in cerebrospinal fluid

Serologic

A. Reactive STS (VDRL or FTA-ABS)
B. Reactive FTA-ABS (IgM) test
C. Nonreactive STS (VDRL or FTA-ABS))
D. Reactive STS (VDRL or FTA-ABS that does not revert to nonreactive within 4 mo)
E. Rising VDRL titer over 3 mo

Maternal history

AA-documented history of adequately treated syphilis during pregnancy

Certainty or Diagnosis

Definite: Absolute clinical criterion

Probable: Any of the following: (1) serologic criterion E; (2) serologic criterion D; (3) one major clinical criterion and serologic criterion A or B; (4) two or more minor clinical criteria and serologic criterion A or B; (5) one major and one minor clinica criterion

Possible: Serologic criterion A or B with only one minor criterion, or no criterion+

Unlikely: (1) serologic criterion C with any other criterion; (2) serologic criterion A or B with maternal history AA

* Table is adapted from Kaufman et al.1 STS indicates serologic test for syphilis

+ Modified schema

(Reproduced by permission of the Am J Dis Child.)

APPENDIX B

Lymphocyte transformation values (dpm) in congenital syphilis on day 2 and day 10, and in the healthy control population

No	Congen Syph		Controls		Treated Syph	
	Autologous Serum	AB Serum	Autologous Serum	AB Serum	Autologous Serum	AB Serum
1	29372	43070	40493	40815	35038	34887
2	43308	47504	57954	57464	34181	47897
3	20257	23554	50711	52766	47927	51392
4	40380	49238	56521	42553	44190	40830
5	60374	63377	59482	60056	61279	62767
6	31725	36113	52397	49697	47166	53390
7	49666	45304	34063	44344	52203	49952
8	54056	51774	59134	55618	56465	50789
9	47582	33407	69494	68568	36849	54282
10	45461	51083	35389	52060	35005	46621
11	14904	33424	-	-	44071	35604
12	42127	62127	35578	38537	46399	47664
13	32927	58697	46387	62582	57022	59290
14	27230	29587	63062	66695	-	-
15	16128	32213	48145	62788	29315	34732
16	49003	31307	51583	51810	-	-
17	8331	14114	36872	34856	-	-
18	43113	48711	52090	55264	-	-
19	19967	45461	45975	54432	-	-
Median	40380	45304	51583	52766	45295	48925
IQ range	20257 - 47582	32213 - 51083	38683 - 58544	43449 - 58761	35030 - 53269	39524 - 53613
Mean	35574	42109	49938	52254	44794	47864
SD	14750	13196	10609	9631	9757	8735

APPENDIX C

Data set of total haemolytic complement (CH50) in congenital syphilis and controls

No.	Patients (units/ml)	Controls (units/ml)
1	10	-
2	17.5	17.2
3	31	29
4	16.4	20
5	29.7	28
6	-	17.2
7	-	18.0
8	10	23.5
9	21.9	38.7
10	31	-
11	10	-
12	25.2	-
13	10	-
14	11.3	26.5
15	10	21.4
16	10	15.6
17	12.5	19.2
18	-	26.7
19	8.7	18

APPENDIX D

Data set of C3 levels (i.u./ml) in congenital syphilis and controls

Patient No.	Congenital syphilis	Controls
1	105	-
2	-	-
3	-	-
4	63-	-
5	76.2	76.2
6	-	-
7	-	80.8
8	-	95
9	-	132
10	-	-
11	137	-
12	67.3	-
13	76.2	-
14	-	-
15	-	-
16	-	-
17	-	-
18	-	90
19	76.2	59

APPENDIX E

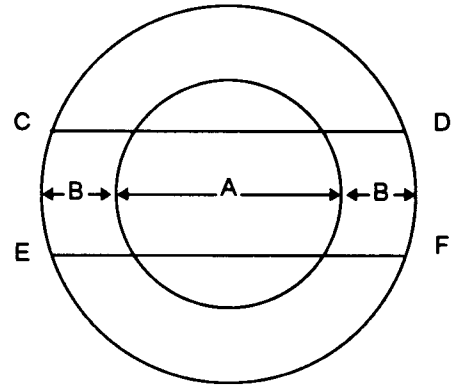


Figure E-1 **Diagrammatic representation of the surface of the placenta; this is divided into central (A) and peripheral (B) zones. (H, Fox, 1978c). Reproduced by permission of WB Saunders: London**

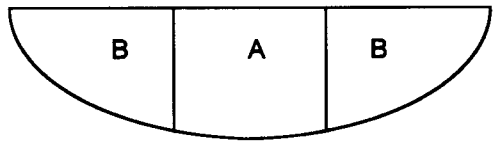


Figure E-2 **Diagrammatic representation of vertical strip of placenta taken from the area lying between the lines CD and EF in Figure E-1. The strip is divided into central (A) and peripheral (B) zones which correspond to those shown in Figure E-1. (H, Fox, 1978,c). Reproduced by permission of WB Saunders: London.**

Figure E-3 **Diagrammatic representation of vertical strip of placenta taken from the area lying between the lines CD and EF in Figure E-1. The strip is divided in the vertical plane into subchorionic, intermediate and maternal (or basal) zones. (H, Fox, 1978,c). Reproduced by permission of WB Saunders: London.**

INTERPRETATION OF RESULTS

The following table interprets results which might be achieved using various staining controls.

Case No.	Pos. Ctrl	Neg. Ctrl	Reag't Ctrl	Unknown	Analysis
1	-	-	-	-	Procedure incorrectly performed.
2	+	+	+	+	Non-specific staining due to protein binding or endogenous enzyme activity.
3	+	+	-	+/-	Negative control contains antigen.
4	-	-	-	+	Positive control does not contain antigen.
5	+	-	-	-	Unknown does not contain antigen.
6	+	-	-	+	Unknown contains antigen.

TROUBLESHOOTING

- Possible causes for negative staining on positive slides:
  1. Steps for the staining procedure were not performed in correct sequence.
  2. Either primary or secondary antibody incubation steps were skipped.
  3. Destruction of labile antigens.
  4. Improper fixation and/or processing of specimens.

- Possible causes for weak staining on all slides:
  1. Specimen retained too much liquid after rinsing steps.
  2. Incubation times are too short or the antibody solution is too dilute.
  3. Poor titer of primary antibody.
  4. Improper substrate preparation or use of old substrate solution.

- Possible causes for high background staining:
  1. Endogenous enzyme activity was not completely blocked.
  2. Non-specific binding of protein to the specimen. Use CAS-Block™ (Zymed Cat. No. 00-8020 or 00-8120) in place of normal blocking reagent or as a direct diluent for the primary antibody solution.
  3. Deparaffinization was not complete.
  4. Excessive application of tissue adhesive.
  5. Drying-out of specimens during staining.
  6. Inadequate rinsing of slides.
  7. Improper antibody dilution.
  8. Over development of substrate.

CONDITIONS FOR USE

Zymed HISTOSTAIN-BULK kits are designed for research use only and are not intended for therapeutic or diagnostic purposes. Zymed Laboratories, Inc., Zymed sales agents and distributors will take no responsibility for HISTOSTAIN-BULK kits used in a way which directly or indirectly violates local regulations or patents. Neither Zymed nor its sales agents can be held responsible for any patent infringement which may occur as the result of improper use of this product.

HANDLING STORAGE AND SHELF LIFE

Store kit at 2-8°C. All performance claims are void after kit expiration date. Observe necessary health and safety precautions when using the kit. Avoid contact with skin and clothes. Since there is a potential hazard of explosion due to the reaction of sodium azide with copper and metal in the plumbing system, flush the drain thoroughly with water after disposal of reagents.

REFERENCES

1. Elias, J.M. et al. Sensitivity and Detection Efficiency of the Peroxidase Antiperoxidase (PAP) Avidin-Biotin Peroxidase Complex (ABC), and Peroxidase-Labeled Avidin-Biotin (LAB) Methods. AM J Clin Pathol 92:62-67, 1989.
2. Chaiet, L. and Wolf, F.S. The properties of streptavidin, a biotin-binding protein produced by Streptomyces. Arch Biochem Biophys 106: 1-5, 1964.
3. Woods, G.S. and Warnke, R. Suppression of endogenous avidin-binding activity in tissue and its relevance to biotin-avidin detection system. J Histochem Cytochem 29(10): 1196-1204, 1981.
4. Hsu, S.M. and Raine, L. The use of avidin-biotin-peroxidase complex (ABC) in diagnostic and research pathology. In: Advances in Immunochemistry. R.A. DeLellis, ed., Mason Publishing USA, Inc., New York, 1984, pp. 31-42.
5. Warnke, R. and Levy, R. Detection of T and B cell antigens with hybridoma monoclonal antibodies: A biotin-avidin-horseradish peroxidase method. J. Histochem Cytochem 28: 771-776, 1980.
6. Zuo-Rong Shi, Steven H. Itzkowitz, and Young S. Kim. A comparison of three immunoperoxidase techniques for antigen detection in colorectal carcinoma tissues. J. Histochem Cytochem 36(3): 317-322, 1988.
7. Steinberger, L.A. The unlabelled antibody peroxidase-anti-peroxidase (PAP) method. In: Immunocytochemistry, 2nd. ed., John Wiley & Sons, Inc., NY, 1979, pp. 109-169.

GENERAL REFERENCES

Banks, P.M. Diagnostic applications of an immunoperoxidase method in hematopathology. J. Histochem Cytochem 27(8): 1192-1194, 1979.

DeLellis, R.A. Basic techniques of immunochemistry. In: Diagnostic Immunohistochemistry, R.A. DeLellis, ed., Masson Publishing USA, New York, 1981, pp. 7-16.

Elias, J. Immunohistopathology: A Practical Approach to Diagnosis, ASCP Press, Chicago, USA, 1990.

Taylor, C.R. Immunoperoxidase techniques. Practical and theoretical aspects. Arch Pathol Lab Med 102:113-121, 1978.

Taylor C.R. and Kledzik, G. Immunohistologic techniques in surgical pathology-A spectrum of "new" special stains. Hum Pathol 12(7): 590-596, 1981.

HISTOSTAIN KIT REFERENCES

Elias, J.M. et al; J Histotechnology 15:315-320 (1992).
Weaver, D.L.; J Histotechnology 15:27-30 (1992).
McQuaid and Allan; J Histochem Cytochem 40:569-574 (1992).
McMaster, M.T.; J Immunology 148:1699-1705 (1992).
D'Sousa, R.N. et al; J Histochem Cytochem 40(3):359-366 (1992).

WARRANTY

Zymed will replace any product that does not perform as described when received by the investigator. However, the products are sold without warranty if treated or used in a manner not recommended by Zymed.

ZYMED LABORATORIES INC.

458 CARLTON COURT
SOUTH SAN FRANCISCO, CA 94080
TELEPHONE (415) 871-4494
OR (800) 874-4494
FAX (415) 871-4499

MAS941108

HISTOSTAIN-BULK™ KITS

ZYMED LAB-SA SYSTEM For Immuno-Histological Staining

PRIMARY ANTIBODY:	CAT NO.
Histostain-SP™ (Peroxidase) Bulk Kits	
Mouse	95-6543-B
Rabbit	95-6143-B
Broad Spectrum*	95-9943-B
Histostain-SAP™ (Alkaline Phosphatase) Bulk Kits	
Mouse	95-6542-B
Rabbit	95-6142-B
Broad Spectrum*	95-9942-B

Good for 1000 slides

* Will react with Mouse, Rabbit, Guinea Pig and Rat Primary Antibodies

INTRODUCTION

Zymed's HISTOSTAIN-BULK kits stain tissues using the Labeled-[strept]Avidin-Biotin (LAB-SA) method,[1] also known as Streptavidin-Biotin Amplification. Their design employs either HorseRadish Peroxidase (HRP) or Alkaline Phosphatase (AP), streptavidin, and a two-tiered antibody technique to reveal the presence of antigens in a variety of human tissues and cell preparations. After a user-supplied primary antibody has been bound to a target antigen, the LAB-SA method uses an affinity-purified and biotinylated secondary antibody which binds specifically to that primary antibody. HRP or AP labeled streptavidin is then bound to this biotinylated secondary antibody, and the entire complex is revealed by adding a substrate/chromogen mixture which creates an intense color deposit through the activity of the bound enzyme. LAB-SA methodology is now widely used in diagnostic medicine and basic research.

Streptavidin, the protein used in this technique, is isolated from *Streptomyces avidinii*,[2] and is similar to egg white avidin in its binding properties. A molecule of streptavidin (60 kD) has 4 subunits, each of which can bind 1 biotin molecule. Biotin is a water-soluble vitamin (244 D) which can be coupled to a variety of proteins and nucleic acids (e.g., immunoglobulins, hormone receptors, cDNA and DNA probes, etc.). This system is based on the unusually strong binding ($K_d = 10^{-15}$ M) between streptavidin and biotin. The advantage to using streptavidin instead of egg white avidin is the elimination of avidin's inherent non-specific binding properties in certain tissue samples.[3] Streptavidin has an isoelectric point of approximately 6.5 while avidin's is nearly 10. Therefore, streptavidin will have a neutral charge under physiological conditions, and exhibit lower non-specific binding than avidin. In avidin, non-specific binding is also contributed by the interaction of carbohydrate side-chains. The lack of carbohydrate in streptavidin results in lower background and a higher signal-to-noise ratio.

In addition to lower background, LAB-SA provides superior sensitivity to that of the Avidin-Biotin Complex (ABC).[1,4,5,6] The LAB-SA method is also more sensitive than the peroxidase (PAP) method,[1,6] and has the advantage of a single universal labeling reagent (enzyme conjugated streptavidin), instead of specific PAP[7] complexes for each animal system.

INTENDED USE

HISTOSTAIN-BULK kits are designed to reveal antigens complexed with a user-supplied primary antibody on human tissue and cell samples (Zymed has over 170 prediluted and 50x-concentrated primary antibodies titrated for use with Histostain kits). Depending on the reactivity of the primary antibody, antigens such as cell surface markers, hormones, cytoskeletal proteins and tumor markers can be detected on properly prepared samples. Frozen or paraffin-embedded tissues, freshly prepared lymphocytes, and fixed cultured cells are the most common tissue preparations. For research use only.

DESCRIPTION

HISTOSTAIN-BULK kits use a biotinylated second antibody, an enzyme conjugate, and substrate-chromogen mixture to demonstrate the presence of antigen. (Substrate chromogen mixture not provided.)

General protocol for the system:

1) Paraffin-embedded tissue should be deparaffinized and rehydrated.
2) Endogenous enzyme activity may be quenched if necessary.
3) Non-specific background staining is eliminated by incubating the tissue section with non-immune serum.
4) The primary antibody for a specific antigen is applied to the tissue and incubated.
5) Incubation is followed by the addition of a biotinylated second antibody. This serves as a linker between the primary antibody and the streptavidin-enzyme conjugate.
6) Streptavidin, bound to peroxidase or alkaline phosphatase is then added. This binds to the biotin residues on the linking antibody.
7) The presence of the enzyme and the entire complex is now revealed by adding a mixture of substrate-chromogen solution (not provided). Zymed offers a number of common substrate chromogen systems which are ideal for this step in the procedure.

REAGENTS SUPPLIED*

Reagent 1A. One dropper bottle (105ml) of ready-to-use serum blocking solution, 10% non-immune serum (rabbit or goat).

Reagent 1B. One dropper bottle (105 ml) of ready-to-use biotinylated second antibody.

Reagent 2. One dropper bottle (105 ml) of ready-to-use streptavidin-peroxidase (HRP) or Alkaline Phosphatase (AP) conjugate.

REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED

Xylene, ethanol, and absolute methanol
Distilled or deionized water
30% hydrogen peroxide or Levamisole solution
10 mM phosphate-buffered saline, pH 7.5 (PBS)
Primary antibody
Substrate buffer
HRP Substrate/Chromogen solutions:
  AEC (Zymed Cat. No. 00-2007)
  DAB (Zymed Cat. No. 00-2014)
AP Substrate/Chromogen solutions:
  BCIP/NBT (Zymed Cat. No. 00-2211)
  AP-Red (Zymed Cat. No. 00-2203)
Counterstain solutions (Zymed Cat. No. 00-8099)
Mounting Media (Zymed Cat. No. 00-8030)

SUGGESTED STAINING PROTOCOL

A. PRELIMINARY PREPARATION OF SLIDES.

1. SPECIMEN PREPARATION: Appropriate tissue and antigen fixation is required to obtain reproducible performances and reliable interpretations. Fixation methods for the antigen of interest may be available from current literature. The following are commonly used fixatives:
   — 10% Neutral buffered formalin, B5, Boulns, Zinc formalin or alcohol-base fixatives are regarded as excellent fixatives for most antigens of clinical significance.
   — Formalin-fixed tissues, post-fixed in B5 before paraffin embedding, may show improved staining. Cell smears prepared from body fluids should be made to assure a monolayer of cells. Multilayers of cells can trap staining reagents and interfere with the interpretation of the results. Smears should be fixed immediately after preparation. Depending on the properties of the antigen, cell smears are usually stable for one to two weeks when stored at 4°C.
   — Fixation of cytospin or frozen sections can be done with acetone (100%) at 4°C for a period of 10 minutes.
2. SLIDE PREPARATION: Precoat slides with HistoGrip™ (Zymed Cat. # 00-8050). As an alternative, use 0.1% poly-L-lysine in water, then air dry.
3. DEPARAFFINIZATION AND REHYDRATION: Paraffin sections are deparaffinized with xylene and rehydrated in a graded series of ethanol. Wash cell smears or tissue in a PBS bath for 10 minutes before starting the staining procedure. **Note: Do not allow specimens or tissue sections to dry from this point on.**
4. CONTROL SLIDES: Three control slides are necessary for the interpretation of results.
   - **Positive Tissue Control:** A specimen, processed in the same way as the unknown, contains the antigen to be stained.
   - **Negative Tissue Control:** A specimen, processed in the same way as the unknown, does not contain the antigen to be stained [optional].
   - **Reagent Control:** An additional slide that will be treated with a non-immune serum instead of same concentration of primary antibody. Any staining observed on the specimen is probably due to non-specific protein binding or non-specific binding of other reagents.

ENDOGENOUS ENZYME QUENCHING STEPS	PROCEDURES (Do all steps at room temperature)	INCUBATION TIME (Min.)
PEROXIDASE QUENCHING (For Bulk-SP Kits): For paraffin-embedded tissues, add 1 part 30% hydrogen peroxide to 9 parts absolute methanol. Mix well. For frozen tissues:	Submerge slides in PEROXIDASE QUENCHING SOLUTION (not provided). Wash with PBS (2 min., 3 times) Treat with Peroxo-Block™ (Zymed Catalog # 00-2015) for 45 sec. Wash immediately.	10
ALKALINE PHOSPHATASE QUENCHING (For bulk, SAP Kits): Optional. Perform this step if the elimination of endogenous alkaline phosphatase activity is necessary.	Zymed offers pre-prepared Levamisole solution (Zymed Cat. No. 00-2205) for use in the inhibition of endogenous AP activity. Add Levamisole solution to substrate buffer.	

REAGENT PREPARATION	STAINING PROCEDURES (Do all steps at room temperature)	INCUBATION TIME (Min.)
SERUM BLOCKING SOLUTION: Ready-to-use, Reagent 1A.	2. Add 2 drops or 100 µl of SERUM BLOCKING SOLUTION to each section. Incubate. Drain or blot off the solution. DO NOT RINSE. This step can be omitted if the primary antibody is diluted in solution containing 10% non-immune serum.	10
PRIMARY ANTIBODY (Not provided): Optimal dilution and incubation time should be determined by the investigator. (Dilution and incubation time are dependent upon sample preparation, antibody affinity, amount of antigen present, and antigen accessibility).	3. Apply 2 drops or 100 µl of PRIMARY ANTIBODY to each section. Incubate in moist chamber. Rinse well with PBS. (2 min., 3 times)	30-60
BIOTINYLATED SECOND ANTIBODY: Ready-to-use, Reagent 1B.	4. Apply 2 drops or 100 µl of BIOTINYLATED SECOND ANTIBODY to each section. Incubate. Rinse well with PBS. (2 min., 3 times)	10
ENZYME CONJUGATE: Ready-to-use, Reagent 2.	5. Apply 2 drops or 100 µl of ENZYME CONJUGATE to each section. Incubate. Rinse well with PBS (2 min., 3 times)	10

The final steps of this procedure, including addition of enzyme substrate, counterstaining and mounting techniques, have been omitted. From this point on, the investigator should proceed with his or her own established laboratory protocol. In purchasing a bulk kit, Zymed assumes the investigator is experienced with established techniques, and possesses the various reagents necessary to complete the task. If other reagents are required, Zymed carries chromogens and substrates for peroxidase and alkaline phosphatase, counterstaining media, mounting solution, and many other ancillary reagents. Consult your Zymed Immunohistochemical catalog, or call 1-(800) 874-4494.

REFERENCES

Ackerman BD. Congenital syphilis: observations on laboratory diagnosis of intrauterine infection. J Pediatr 1969; 74:459-62.

Aiuti F, Ungari S, Turbessi G, Serra GB. Immunologic aspects of congenital syphilis. Helv Pediatr Acta 1966; 1:66-71.

Alford CA, Jr. A correlative immunologic, microbiologic and clinical approach to the diagnosis of acute and chronic infections in the newborn. N Engl J Med 1967; 277:437-49.

Alford CA, Jr. Immunoglobulin determinations in the diagnosis of fetal infection. Pediatr Clin North Am 1971; 18:99-113.

Altshuler G. Placental villitis of unknown etiology: Harbinger of serious disease. J Reprod Med. 1973; 11:215-222.

Anderson DC, Pickering LK, Feigen RD. Leucocyte function in normal and infected infants. J Pediatr 1974; 85:420-25.

Baker-Zander SA, Sell S, Lukehart SA. Serum regulation of in vitro lymphocyte responses in early syphilis. Infect Immun 1982; 37:568-78.

Baker-Zander SA, Hook III EW, Bonin P, Handsfield HH, Lukehart SA. Antigens of *Treponema pallidum* recognized by IgG and IgM antibodies during syphilis in humans. J Infect Dis 1985; 151:264-72.

Baker-Zander S, Lukehart S. Macrophage-mediated killing of opsonized *T.pallidum*. J Infect Dis 1992; 165:69-74.

Ballard JL, Novak KK, Driver M. A simplified score of assessment of fetal maturation of newly born infants. J Pediatr 1979; 95:769-74.

Baughn RE, Tung K, Musher DM. Detection of circulating immune complexes in the sera of rabbits with experimental syphilis: possible role in immunoregulation Infect Immun 1980; 29:575-82.

Baughn RE, Adams CB, Musher DM. Immune complexes in experimental syphilis: a methodologic evaluation. Sex Transm Dis 1982; 9:170-7.

Baughn RE, Musher DM. Isolation and preliminary characterization of circulating immune complexes from rabbits with experimental syphilis. Infect Immun 1983; 42:579-84.

Baughn RE, Adams CB, Musher DM. Circulating immune complexes in experimental syphilis: identification of treponemal antigens and specific antibodies to treponemal antigens in isolated complexes. Infect Immun 1983; 42:585-93.

Baughn RE, McNeely MC, Jorizzo JL, Musher DM. Characterization of the antigenic determinants and host components in immune complexes from patients with secondary syphilis. J Immunol 1986; 136:1406-14.

Baum J, Stasney P, Ziff M. Effects of rheumatoid factor and antigen-antibody complexes on the vessels of the rat mesentery. J Immunol. 1964; 93:985-92.

Beatty DW, Dowdle EB. The effects of kwashiorkor serum on lymphocyte transformation in vitro. Clin Exp Immunol 1978; 32:134-43.

Beatty DW, Dowdle EB. Deficiency in kwashiorkor serum factors required for optimal lymphocyte transformation in vitro. Clin Exp Immunol 1979; 35:433-42.

Beatty DW, Handzel ZT, Pecht M, Ryder CR, Hugh J, McCabe K,et al. A controlled trial of treatment of acquired immunodeficiency in severe measles with thymic humoral factor. Clin Exp Immunol 1984; 56:479-85.

Beck-Sague C, Alexander ER. Failure of benzathine penicillin G therapy in early congenital syphilis. Pediatr Infect Dis J 1987; 6:1061-4.

Benirschke K. Syphilis-the placenta and the fetus. Am J Dis Child 1974; 128:142-143.

Benirschke K, Driscoll SG. Infections. In: Benirschke K, Driscoll SG, editors. The Pathology of the Human Placenta.Springer-Verlag.Berlin-Heidelberg-New York. 1967:269-270.

Benirschke K, Kaufmann P. Infectious Diseases. In: Pathology of the Human Placenta. 2nd ed. New York:Springer-Verlag, 1990:542-635.

Benirschke K, Mendoza GR, Bazely PL. Placental and fetal manifestations of cytomegalovirus infection. Virchows Arch B Cell Pathol 1974; 16:121-34.

Bey RF, Johnson RC, Fitzgerald TJ. Suppression of lymphocyte response to concanavalin A by mucopolysaccharide material from *Treponema pallidum* infected rabbits. Infect Immun 1979; 26:64-9.

Bhorade MS, Carag HB, Lee J, Potter EV, Dunea G. Nephropathy of secondary syphilis: a clinical and pathological spectrum. JAMA 1971; 216:1159-66.

Blanc WA. Pathology of the placenta and cord in some viral infections. In: Hanshaw JB, Dudgeon JH, editors. Viral disease of the fetus and newborn. Philadelphia: W.B.Saunders,1978:237-58.

Blandford G. Immune complex diseases. In: Waldman RH, editor. Clinical concepts of immunology. Baltimore: Williams and Wilkins, Co. 1979:113-33

Blumberg N, Heal JM. Effects of transfusion on immune function. Arch Pathol Lab Med 1994; 118:371-9.

Boackle R. The Complement system. In: Virella G, editor.Introduction to Medical Immunology.3rd ed. New York: Marcell Dekker Inc. 1993:135-59.

Bonforte RJ, Topilsky M, Siltzbach LE. Phytohaemagglutinin (PHA) skin test: a possible in vivo measure of cell mediated immunity. J Pediatr 1972; 81:775-80.

Borobio MV, Negales MC, Palomares JC. Value of serological diagnosis in congenital syphilis. Br J Vener Dis 1980; 56:377-80.

Bos JD, Hamerlink F, Cormane RH. Antitreponemal IgE in early syphilis. Br J Vener Dis 1980a; 56:20-5.

Bos JD, Hamerlink F, Cormane RH. Immunoglobulin-bearing lymphoid cells in primary syphilis. Br J Vener Dis 1980b; 56:69-73.

Bos JD, Hamerlink F, Cormane RH. T lymphoid cells in primary syphilis. Br J Vener Dis 1980c; 56:74-6.

Bourne GL. Human amnion and chorion. Chicago:Year Book Med Publishers, 1962:94-112.

Boyd JD, Hamilton WJ. The Human Placenta.Cambridge: Heffer,1970.

Braunstein GD, Lewis EJ, Galvaneck EG, Hamilton A, Bell WR. The nephrotic syndrome associated with secondary syphilis: an immune deposit disease. Am J Med 1970; 48:643-48.

Bromberg K, Rawstron S, Tannis G. Diagnosis of congenital syphilis by combining *Treponema pallidum*-specific IgM detection with immunofluorescent antigen detection for *T.pallidum*. J Infect Dis 1993; 168:238-42.

Bulmer JN, Johnson PM. Macrophage population in the human placenta and amniochorion. Clin Exp Immunol 1984; 57:393-403.

Bulmer JN, Morrison L, Smith JC. Expression of class 2 MHC gene products by macrophages in human uteroplacental tissue. Immunology 1988; 63:707-14.

Campbell AC, Waller C, Wood J, Aynsley-Green A, Yu V. Lymphocyte subpopulations in the blood of newborn infants. Clin Exp Immunol 1974; 18:469-82.

Cannefax GR. Immunity in syphilis. Br J Vener Dis 1965; 4:260-74

Cannefax GR, Norins LC, Gillespie EJ. Immunology of syphilis. Ann Rev Med 1967; 18:471-82

Castellucci M, Zaccheo D, Pescetto G. A three-dimensional study of the normal human placental villous core. I. The Hofbauer cells. Cell Tiss Res. 1980; 210:235-247.

Castellucci M, Kaufmann P. A three-dimensional study of the normal human placental villous core. II. Stromal architecture. Placenta 1982; 3:260-286.

Castellucci M, Richter A, Steininger B, Celona A, Schneider J. Light and electron microscopy identification of mitotic Hofbauer cells in the human placenta. Arch Gynecol Obstet 1985; 237(Suppl):235-

Castellucci M, Celona A, Bartels H, Steininger B, Benedetto V, Kaufmann P. Mitosis of the Hofbauer cell: possible implications for a fetal macrophage. Placenta 1987; 8:65-76.

Centers for Disease Control. STD treatment guidelines. MMWR 1985; 34 (4S):965-95.

Centers for Disease Control. Guidelines for the prevention and control of congenital syphilis. MMWR 1988; 37 (suppl 1):15-135.

Centers for Disease Control. Congenital syphilis - New York City, 1986 - 1988. MMWR 1989a; 38:825-9.

Centers for Disease Control. STD treatment guidelines. MMWR 1989b; 38 (58):1-43.

Chandler BD, Kapoor H, Barker BE, Boyle RJ, Oh W. Nitroblue tetrazolium test in neonates. J Pediatr 1978; 92:638-40.

Chandra RK. T lymphocytes and cell mediated immunity (CMI) in low birth weight (LBW) infant (abstract). Pediatr Res 1975; 9:328.

Chandwani S, Greco A, Mital K, Antoine C, Krasinski K, Borskowsky W. Pathology and HIV expression in placentas of seropositive women. J Infect Dis.1991; 163:1134-8.

Chawla V, Gupta K, Raghu MP. Congenital syphilis: a clinical profile. J Trop Pediatr 1985; 31:204-8.

Cocchi P, Mori S, Becattini A. Nitroblue tetrazolium reduction by neutrophils of newborn infants in in vitro phagocytosis test. Acta Pediatr Scand 1971; 60:475-8.

De Horatius RJ, Williams RC.Jr. Rheumatoid factor accentuation of pulmonary lesions associated with experimental diffuse proliferative lung disease. Arthritis Rheum 1972; 15:293-301.

De Sousa M. Blood transfusions and allograft survival: iron-related immunosuppression. Lancet 1983; 2:681-2.

Dietrich M, Hoosen AA, Moodley J, Moodley S. Urogenital tract infections in pregnancy at King Edward VIII Hospital, Durban South Africa. Genitourin Med 1992; 68:39-41.

Dobson SRM, Taber LH, Baughn RE. Characterization of the components in circulating immune complexes from infants with congenital syphilis.J Infect Dis 1988a; 158:940-47.

Dobson SRM, Taber LH, Baughn RE. Recognition of *Treponema pallidum* antigens by IgM and IgG antibodies in congenitally infected newborns and their mothers. J Infect Dis 1988b; 157:903-10.

Dorman HG, Sahyun PF. Identification and significance of spirochaetes in the placenta. Am J Obstet Gynecol 1937; 33:954-967.

Dosset JH, Williams RC, Quie PG. Studies on interaction of bacterial, serum factors, and polymorphonuclear leucocytes in mothers and newborns. Pediatrics 1969; 44:49-57.

Dudley DJ, Weidmeier S. The ontogeny of the immune system: perinatal perspectives. Sem Perinatol 1991; 15:184-95.

Durandy A, Fischer A, Griscelli C. Active suppression of B lymphocyte maturation by two different newborn T lymphocyte subsets. J Immunol 1979; 123:2644-50.

Enders AC, King BF. The cytology of Hofbauer cells. Anat Rec 1970; 167:231-52.

Engel S, Diezel W. Persistent serum immune complexes in syphilis. Br J Vener Dis 1980; 56:221-2.

Farshy CE, Hunter EE, Larsen SA, Cerney EH. Double conjugate enzyme linked immunosorbent assay for immunoglobulins G and M against *Treponema pallidum*. J Clin Microbiol 1984; 20:1109-13.

Faulk WP, Jarret R, Keane M, Johnson PM, Boackle RJ Immunological studies of human placentae:complement components in immature and mature chorionic villi. Clin Exp Immunol 1980; 40:299-305.

Faulk WP, Johnson PM. Immunological studies in human placentae:basic and practical implications. Recent Adv Clin Immunol 1980; 2:1-31.

Fenton LJ, Light IJ. Syphilis after maternal treatment with erythromycin. Obstet Gynecol 1976; 47:492-4.

Festenstein H, Abrahams C, Bokkenheuser B. Runting syndrome in neonatal rats infected with *Treponema pallidum*. Clin Exp Immunol 1967; 7:311-20.

Fleisher TA, Luckhasen JR, Sabad A, Gehrtz RC, Kersey JH. T and B lymphocyte subpopulations in children. Pediatrics 1975; 55:162-5.

Floyd M, Tesar JT. The role of IgM rheumatoid factor in experimental immune vasculitis. Clin Exp Immunol 1979; 36:165-174.

Folds JD, Maret SM, Rauchbach AS. Lymphocyte transformation and the effect of circulating immune complexes in humans with syphilis. Sex Transm Dis 1982; 9:109-14.

Ford PM. Interaction of rheumatoid factor with immune complexes in experimental glomerulonephritis - possible role of antiglobulins in chronicity. J Rheumatol 1983; 10(Suppl 11):81-4.

Forman ML, Stiehm ER. Impaired opsonic activity but normal phagocytosis in low birth weight infants. N Engl J Med 1969; 281:926-31.

Fox H. The significance of villous syncytial knots in the normal placenta. J Obstet Gynecol Brit Cwlth 1965; 72:347-355.

Fox H. The incidence and significance of Hofbauer cells in the mature human placenta. J Path Bact 1967; 93:710-717.

Fox H, Kharkonger NF. Enzyme histochemistry of the Hofbauer cells of the human placenta. J Obstet Gynecol Brit Cwlth 1969; 76:918-921.

Fox H. The development and structure of the placenta. In: Pathology of the Placenta. Major problems in Pathology series. London: WB Saunders 1978a; 7:1-37.

Fox H. Infection of the placenta. In: Pathology of the Placenta. Major problems in Pathology series. London: WB Saunders 1978b; 7:286-325.

Fox H. Pathological examination of the placenta. In: Pathology of the Placenta. Major problems in Pathology series. London: WB Saunders 1978c; 7:473-476.

Frauli M, Ludwig H. Immunocytochemical identification of mitotic Hofbauer cells in cultures of the first trimester human placental villi. Arch Gynecol Obstet. 1987; 241:47-51.

Friedmann PS, Turk JL. A spectrum of lymphocyte responsiveness in human syphilis. Clin Exp Immunol 1975; 21:59-64.

Friedmann PS. Cell-mediated immunological reactivity in neonates and infants with congenital syphilis. Clin Exp Immunol 1977; 30:271-6.

Fulford KWM, Brostoff J. Leucocyte migration and cell mediated immunity in syphilis. Br J Vener Dis 1972; 48:483-8.

Gahr M, Blanke R, Speer CP. Polymorphonuclear leucocyte function in term and preterm newborn infants. Biol Neonate 1985; 48:15-20.

Gajewska E. Role of T and B lymphocytes in the intrauterine infections of newborns. Arch Immunol Ther Exp (Warsz) 1982; 30:71-7

Gamble CN, Reardon JB. Immunopathogenesis of syphilitic glomerulonephritis. Elution of antitreponemal antibody from glomerular immune complex deposits. N Engl J Med. 1975; 292:449-54.

Gamboa D, Miller JN, Lukehart SA, Baker-Zander SA, Sell S. Experimental neonatal syphilis. II. Immunological responses of neonatal rabbits to intradermal inoculation with *Treponema pallidum* (Nichols strain). Pediatr Res 1984; 18:972-9

Garcia AGP, Marques RLS, Lobato YY, Fonseca MEF, Wigg MD. Placental pathology in congenital rubella. Placenta 1985; 6:281-295.

Garcia AGP, Fonseca EF, De Souza Marques RL, Lobato YY. Placental morphology in cytomegalovirus infection. Placenta 1989; 10:1-18.

Gitlin D, Kumate J, Urrusti J, Morales C. The selectivity of the human placenta in the transfer of plasma proteins to the fetus. J Clin Invest 1964; 43:1938-51.

Gjestland T. The Oslo Study of untreated syphilis - an epidemiological investigation of the natural course of untreated syphilis based on a study of the Boeck-Bruusgard material. Acta Dermato Venereologica 1955; 35 (suppl 34):1-365.

Goddard EA, Hughes EJ, Duys PJ, Hoffman EB, Beatty DW. Treatment of chronic granulomatous disease with recombinant gamma interferon. S Afr Med J. 1992:81:81-3.

Goldman AS, Ham Pong AJ, Goldblum RM. Host Defenses: Development and maternal contributions. In: Barness LA, editor. Advances in Pediatrics. Chicago: Year Book Medical, 1985; 32:71-100.

Gonin JM. Syphilis - an appraisal of the Groote Schuur Hospital antenatal screening programme. S Afr Med J. 1985; 67:962-5.

Griffen FM. Opsonizations In: Day NK, Good RA,editors. Biological Amplication Systems in Immunology. New York: Plenum Medical, 1977.

Grimpel E, Sanchez PJ, Wendel GD, Burstain JM, McCracken GH Jr, Radold JD, Norgard MV. Use of polymerase chain reaction and rabbit infectivity testing to detection *Treponema pallidum* in amniotic fluid, fetal and neonatal sera, and cerebrospinal fluid. J Clin Microbiol 1991; 29:1711-8.

Haeney MR. Tests for circulating immune complexes. In: Thompson RA, editor. Techniques in Clinical Immunology (2nd ed.). Blackwell Scientific Publications, Oxford 1981:170-202.

Handsfield HH, Lukehart SA. Prevention of congenital syphilis (editorial). JAMA 1984; 252:1750-1.

Hardy PH, Graham DJ, Nell EE, Dannenberg AM. Macrophages in immunity to syphilis: suppressive effect of concurrent infection with Mycobacterium bovis BCG on the development of syphilitic lesions and growth of T pallidum in tuberculin positive rabbits. Infect Immun 1979; 26:751-3.

Harris MC, Stroobaut J, Cody CS, Douglas SD, Polin RA. Phagocytosis of group B streptococcus by neutrophils from newborn infants. Pediatr Res 1983; 17:358-61.

Harter CA, Benirschke K. Fetal syphilis in the first trimester. Am J Obstet Gynecol 1976; 124:705-11.

Hashiasaki P, Wertzberger GG, Conrad GL et al. Erythromycin failure in the treatment of syphilis in the pregnant woman. Sex Transm Dis 1983; 10:36-8.

Hay FC, Hull MGR, Torrigiani G. The transfer of human IgG subclasses from mother to fetus. Clin Exp Immunol 1971; 9:355-58.

Hayward AR, Lawton AR. Induction of plasma cell differentiation of human fetal lymphocytes: evidence for functional immaturity of T and B cells. J Immunol 1977; 119:1213-7.

Hill LL, Singer DB, Falleta J, Stasney R. The nephrotic syndrome in congenital syphilis. Pediatrics 1972; 49:260-66.

Humbert JR, Kurtz ML, Hathaway WE. Increased reduction of nitroblue tetrazolium by neutrophils of newborn infants. Pediatrics 1970; 45:125-8.

Ikeda MK, Jenson HB. Evaluation and treatment of congenital syphilis. J Pediatr 1990; 117:843-52.

Ingall D, Musher D. Syphilis. In: Remington JS, Klein JO (eds). Infectious diseases of the fetus. 2nd edn. WB Saunders. 1983:335-74.

Jauniaux E, Nessman C, Imbert MC, Meuris S, Puissant F, Hustin J. Morphological aspects of the placenta in HIV placentas. Placenta 1988; 9:633-42.

Jensen JR, From E. Alterations in T lymphocytes and T lymphocyte subpopulations in patients with syphilis. Br J Vener Dis 1982; 58:18-22.

Johnson PM, Trencher P, Faulk WP. Immunological studies of the human placentae: binding of complexed immunoglobulin by stromal endothelial cells. Clin Exp Immunol 1975; 22:133-8.

Johnson PM, Natvig JB, Ystehede UA, Faulk WP. Immunological studies of human placentae: the distributions and character of immunoglobulins in chorionic villi. Clin Exp Immunol 1977; 30:145-53.

Johnson PM, Matre R. Membrane receptors for IgG in the human placenta. In: Hemmings WA, editor. Protein transmission through living membranes.Amsterdam:Elsieriver 1979:45-54.

Johnson PM, Brown PJ. Fcy receptors in the human placenta. Placenta 1981; 2:355-70

Johnston RB Jr. The complement system in host defense and inflammation: the cutting edges of a double edged sword. Pediatr Infect Dis J 1993; 12:933-41.

Jones CJP, Fox H. Syncytial knots and intervillous bridges in the human placenta:an ultrastructural study. J Anat 1977; 124:275-86.

Jorizzo JL, McNeely MC, Baughn RE, Solomon AR, Cavallo T, Smith EB. Role of circulating immune complexes in human secondary syphilis. J Infect Dis 1986; 153:1014-22

Kabat E, Mayer M. Complement and complement fixation. In: Kabat EA, Mayer MM, editors. Experimental Immunochemistry. 2nd ed. Springfield, IL.: Charles C Thomas, 1971:133.

Kantor FS. Infection, anergy, and cell-mediated immunity. N Engl J Med 1975; 297:629-34.

Kaplan BS, Wiglesworth FW, Marles MI, Drummond KN. The glomerulopathy of congenital syphilis - an immune deposit disease. J Pediatr 1972; 81:1154-56.

Karayalcin G, Khanijou A, Kim KY, Aballi AJ, Lanzkowski P. Monocytosis in congenital syphilis. Am Dis Child 1977; 131:782-3.

Kaufman RE, Olansky DC, Wiesner PJ. The FTA-ABS (IgM) test for neonatal congenital syphilis. A critical review. J Am Vener Dis Assoc 1974; 1:79-84.

Kaufman RE, Jones OG, Blount JH et al. Questionaire survey of reported early congenital syphilis: problems in diagnosis, prevention and treatment. Sex Transm Dis 1977; 4:135-9.

Kaufmann P, Stark J, Stagner HE. The villous stroma of the human placenta 1. The ultrastructure of fixed connective tissue cells.Cell Tiss Res 1977; 177:105-21.

Kaufmann P, Sen DK, Schweikhart G. Classification of human placental villi. I .Histology. Cell Tiss Res. 1979; 200:409-23.

Kaufmann P. Development and differentiation of the human placental villous tree. Bibl Anat 1982; 22:29-39.

Kaufmann P, Luckhardt M, Schweikhart G, Cantle SJ. Cross-sectional features and three - dimensional structure of human placental villi.Placenta 1987; 8:235-47.

Kaufmann P, Scheffen I. Placental Development. In: Polin R, Fox W A., editors. Fetal and Neonatal Physiology. Philadelphia: Saunders W B., 1992:47-55.

Laird SM, Thorburn AL. Assessment of the "Luotest" in late syphilis. Br J Vener Dis 1966; 42:119-21.

Lawton AR. Ontogeny of the immune system. In: Ogra PL, editor. Neonatal infections : nutritional and immunologic interactions. Orlando, Florida: Grune and Stratton,1984:3-20.

Levene GM, Turk JL, Wright DJM, Grimble AGS. Reduced lymphocyte transformation due to a plasma factor in patients with active syphilis. Lancet 1969; 2:246-7.

Levene GM, Wright DJM, Turk JL. Cell-mediated immunity and lymphocyte transformation in syphilis. Proc Roy Soc Med 1971; 64:426-8.

Lewis LL, Taber LH, Baughn RE. Evaluation of Immunoglobulin M Western blot analysis in the diagnosis of congenital syphilis. J Clin Microbiol 1990; 28:296-302.

Loveday C, Bingham JS. Changes in intravascular complement, kininogen, and histamine during Jarisch-Herxheimer reaction in secondary syphilis. Genitourin Med 1985; 61:27-32.

Lukehart SA, Baker-Zander SA, Sell S. Characterization of lymphocyte responsiveness in early experimental syphilis. I. In vitro response to mitogens and Treponema pallidum antigens. J Immunol 1980; 124:454-60.

Malan AF, Woods DL, van der Elst CW, Meyer MP. Relative placental weight in congenital syphilis. Placenta 1990; 11:3-6.

Maret SM, Baseman JB, Folds JD. Cell-mediated immunity in *Treponema pallidum* infected rabbits: in vitro response of splenic and lymph node lymphocytes to mitogens and specific antigens. Clin Exp Immunol 1980; 39:38-43.

Mariani G, Strober W. Immunoglobulin metabolism. In: Metzger H, editor. Fc receptors and the action of antibodies. Washington D.C.: American Society of Microbiology, 1990: 94-177.

Marshack LC, Rothman S. Skin testing with a purified suspension of *Treponema pallidum*. Am J Syph Gonorr Ven Dis 1951; 35:35-41.

Mascola L, Pelosi R, Blount JH, Alexander CE, Cates W. Congenital syphilis revisited. Am J Dis Child 1985; 139:575-80

Maszkiewicz W. Results of studies on circulating immune complexes in newborn infants with infections. Pediatr Pol 1983; 58:221-7.

Matre R. Similarities of Fc receptors on trophoblasts and placental endothelial cells. Scand J Immunol 1977; 6:953-8.

Matre R, Johnson PM. Multiple Fc receptors in the human placenta. Acta Pathol Microbiol Scand 1977; 85:314-6

Matre R, Haugen A. The placental Fc receptors studied using immune complexes of peroxidase. Scand J Immunol 1978; 8:187-93.

Martensson L, Furdenberg HH. Gm genes and yG globulin synthesis in the human fetus. J Immunol 1965; 94:514-20.

McCord JR. Syphilis of the placenta. Am J Obstet Gynecol 1934; 28:743-50.

McCormick JN, Day J, Morris CJ, Hill AGS. The potentiating effect of rheumatoid arthritis serum in the immediate phase of nephrotoxic nephritis. Clin Exp Immunol 1969; 4:17-28.

McCracken GH, Hard JB, Chen TC, Sever JL. Evaluation of a radial diffusion plate method for determining serum immunoglobulin levels in normal and congenital infected infants. J Pediatr 1969; 75:1204-10.

McKay DG, Hertig AT, Adams EC, Richardson MV. Histochemical observations on the human placenta. Obstet Gynecol 1958; 12:1-36.

Metzger M. Role of humoral versus cellular mechanisms of resistance in the pathogenesis of syphilis. Br J Vener Dis 1979; 55:94-8.

Meyer MP, Malan AF. Rheumatoid factor in congenital syphilis. Genitourin Med 1989; 65:304-7.

Meyer MP. Congenital syphilis and rheumatoid factor [MD Thesis]. Cape Town, South Africa: University of Cape Town, 1990.

Meyer MP, Beatty DW. IgM rheumatoid factor in congenital syphilis: associations with clinical and laboratory findings. Clin Exp Immunol 1991; 86:43-8.

Meyer MP, Eddy T, Baughn RE. Analysis of Western blotting (immunoblotting) technique in diagnosis of congenital syphilis. J Clin Microbiol 1994; 32:629-33.

Miller JN. Immunity in experimental syphilis. VI. Successful vaccination of rabbits with *Treponema pallidum*, Nichols strain attenuated by y-radiation. J Immunol 1973; 110:1206-15.

Miyawaki T, Kubagawa H, Butler JL, Cooper MD. Ig isotypes produced by EBV transformed B cells as a function of age and tissue distribution. J Immunol 1988; 140:3887-92.

Mlisana KP, Monokoane S, Hoosen AA, Moodley J, Adhikari M, Taylor T. Syphilis in the "unbooked" pregnant woman. S Afr Med J 1992; 82:18-20.

Moscicki RA, Luttinger AL, Colvin RB. Analysis of human T cells in blood with monoclonal antibodies and flow cytometry. In: Yoshida T, editor. Investigation of cell-mediated immunity. 1st ed. Practical Methods in Immunology Churchill Livingstone,1985:6-36.

Moskalewski S, Ptak W, Czannik Z. Demonstration of cells with IgG receptor in the human placenta. Biol Neon 1975; 26:268-73.

Motala C, Ireland JD, Hil ID, Bowie MD. Cholestatic disorders of infancy - aetiology and outcome. J Trop Pediatr 1990; 36:218-22.

Muller F, Moskophidis M, Borkhardt HL. Detection of immunoglobulin M antibodies to *Treponema pallidum* in a modified enzyme-linked immunosorbent assay. Eur J Clin Microbiol 1987; 6:35-9.

Musher DM, Schell RF, Knox JM. In vitro lymphocyte response to Treponema refringens in human syphilis. Infect Immun 1974; 9:654-7.

Musher DM, Schell RF, Jones RH, Jones AM. Lymphocyte transformation in syphilis: an in vitro correlate of immune suppression in vivo? Infect Immun 1975; 11:1261-5.

Musher DM, Schell RF, Knox JM. Immunologic mechanisms of infectious syphilis. Cutis 1976; 17:739-42.

Musher DM, Hague-Park M, Gyorkey F, Anderson DC, Baughn RE. The interaction between *Treponema pallidum* and human polymorphonuclear leucocytes. J Infect Dis 1983; 147:77-86.

Naicker S, Moodley J. Serological diagnosis of syphilis in pregnancy. S Afr Med J 1983; 63:536-7.

Noguchi N. The Luetin reaction. JAMA 1912; 59:1262-3

Opai-Tetteh ET, Hoosen AA, Moodley J. Re-screening for syphilis at the time of delivery in areas of high prevalence. S Afr Med J 1993; 83:725-6.

Oppenheim JJ. Modulation of in vitro lymphocyte transformation by antibodies: enhancement by antigen-antibody complexes and inhibition by antibody excess. Cell Immunol 1972; 3:341-60.

Oski FA, Naiman JL. Haematologic problems in the newborn. 2nd Edition. WB Saunders. 1972.

Pahwa S, Sia C, Harper R, Pahwa R. Lymphocyte subpopulation in high risk infants; influence of age and blood transfusions. Pediatrics 1985; 76:914-7.

Park BH, Fibrig SM, Smithwick EM. Infection and nitroblue tetrazolium reduction by neutrophils: a diagnostic aid. Lancet 1968; 2:532-4.

Pavia CS, Folds JD, Baseman JB. Depression of lymphocyte response to concanavalin A in rabbits infected with *Treponema pallidum* (Nichols strain). Infect Immun 1976; 14:320-2.

Pavia CS, Baseman JB, Folds JD. Selective response of lymphocytes from *Treponema pallidum* infected rabbits to mitogens and *Treponema reiteri*. Infect Immun 1977; 15:417-22.

Pavia CS, Folds JD, Baseman JB. Cell-mediated immunity during syphilis. A review. Br J Vener Dis 1978; 54:144-50.

Pedersen SA, Petersen J, Andersen V. Suppression of B lymphocytes in mature newborn infants. Acta Pediatr Scand 1983; 72:441-7.

Phimister GM, Whaley K. Measurement of complement. In: Gooi HC, Chapel H,editors. Clinical Immunology - a practical approach. Oxford University Press, 1990:81-109.

Punnonen J. The role of interleukin 2 in the regulation of proliferation and IgM synthesis of human newborn mononuclear cells. Clin Exp Immunol 1989; 75:421-6.

Qureshi F, Suzanne MJ, Milagros PR. Placental histopathology in syphilis. Hum Pathol 1993; 24:779-84.

Radolf JD, Fehniger TE, Silverblatt FJ, Miller JN, Lovett MA. The surface of virulent *Treponema pallidum*: resistance to antibody binding in the absence of complement and surface association of recombinant antigen 4D. Infect Immun 1986; 52:579-85.

Rice M, Fitzgerald TJ. Immune immobilization of *Treponema pallidum*: antibody and complement interactions revisited. Can J Microbiol 1985; 31:1147-51.

Rosen EW, Richardson NJ. A reappraisal of the value of the IgM fluorescent treponemal antibody absorption test in the diagnosis of congenital syphilis. J Pediatr 1975; 87:38-42.

Ross SM. Sexually transmitted disease in pregnancy. Clin Obstet Gynecol 1982; 9:565-91.

Russel P, Altshuler G. Placental abnormalities of congenital syphilis. Am J Dis Child 1974; 128:160-3.

Russel P. Inflammatory lesion of the human placenta III. The histopathology of villitis of unknown etiology. Placenta 1980; 1:227-44.

Ryan JL, Arbeit JD, Dickler HB, Henkart PA. Inhibition of lymphocyte mitogenesis by immobilized antigen-antibody immune complexes. J Exp Med 1975; 142:814-26.

Salvatore CA. The placenta in acute toxaemia. Am J Obstet Gynecol 1968; 102:347-52

Samson GR, Beatty DW, Malan A. Immune studies in infants with congenital syphilis. Clin Exp Immunol 1990; 81:315-8.

Sanchez PJ, Wendel GD, Grimprel E, Goldberg M , Hall M. Arencibia-Mireles O, Radolf JD, Norgard MV. Evaluation of molecular methodologies and rabbit infectivity testing for the diagnosis of congenital syphilis and neonatal central nervous system invasion by *Treponema pallidum*. J Infect Dis 1993; 167:148-57.

Sartin P. The anaemia of congenital syphilis. South Med J 1965; 58:27-31.

Saxoni F, Lapatsanis P, Pantelakis SN. Congenital syphilis: a description of 18 cases and re-examination of an old but ever present disease. Clin Pediatr 1967; 6:687-91.

Schell RF, Musher DM, Jacobson K, Schwethelm P. Induction of acquired cellular resistance following transfer of thymus dependent lymphocytes from syphilitic rabbits. J Immunol 1975; 114:550-3.

Schulz KF, Cates W Jr., O'Mara PR. Pregnancy loss, infant death and suffering; legacy of syphilis and gonorrhoea in Africa. Genitourin Med 1987; 63:320-5.

Schwartz DA, Khan R, Stoll B. Characterization of the fetal inflammatory response to cytomegalovirus placentitis. Arch Pathol Lab Med 1992; 116:21-7.

Scotti AT, Logan L. A specific IgM antibody test in neonatal congenital syphilis. J Pediatr 1968; 73:242-3.

Sen DK, Kaufmann P, Schweikhart G. Classification of human placental villi. II. Morphometry. Cell Tiss Res 1979; 200:425-34

Shahin B, Papadopoulou ZL, Janis EH. Congenital nephrotic syndrome associated with congenital toxoplasmosis. J Pediatr 1974; 85:366-70.

Shigeoka AO, Santos JI, Hill JR. Functional analysis of neutrophil granulocytes from healthy, infected, and stressed neonates. J Pediatr 1979; 95:454-60.

Shigeoka AO, Jensen C, Pincus SH, Hill HR.Absolute requirement for complement in IgM-mediated protection in experimental type III group B streptococcal infection. J Infect Dis 1984; 150:63-70.

Silverstein AM. Congenital syphilis and the timing of immunogenesis in the human fetus. Nature 1962; 194:196

Silverstein AM, Lukes RJ. Fetal response to antigenic stimulus. Lab Invest 1962; 11:918-32.

Solling J, Solling K, Jakobsen K, From E. Circulating immune complexes in syphilis. Acta Dermatol (Stockh.) 1978; 58:263-7.

South MA, Short DH, Knox JM. Failure of erythromycin estolate therapy in in-utero syphilis. JAMA 1964; 190:70-1.

Stachowski J, Michalkiewicz J, Burczynska B, Walczak M, Maciejewski J, Madalinski K. Activity of B cell lineage in the cord blood of newborns. Allerg Immunol (Leipz) 1991; 37:75-82.

Stagno S, Volanakis JE, Reynolds DW,Stroud R, Alford CA. Immune complexes in congenital and natal cytomegalovirus in congenital infected children and their mothers. J Clin Invest 1977; 60:838-45.

Stevens MCG, Derbyshire PJ, Brown SM. Early congenital syphilis and severe hematological disturbance. Arch Dis Child 1987; 62:1073-5.

Stiehm ER. Fetal defense mechanisms. Am J Dis Child 1975; 129:438-43.

Stoll BJ, Lee FK, Larsen S, Hale E, Schwartz D, Rice RJ, Asby R, Holmes R, Nahmias AJ. Clinical and serologic evaluation of neonates for congenital syphilis: a continuing diagnostic dilemma. J Infect Dis 1993; 167:1093-9.

Stray-Pedersen B. Economic evaluation of maternal screening to prevent congenital syphilis. Sex Transm Dis 1983; 10:167-72.

Swingler GH, Van Coeverden de Groot HA. The antenatal prevention of congenital syphilis in a periurban settlement. S Afr Med J 1993; 83:34-5.

Tardien M, Grospierre B, Durandy A, Griscelli C. Circulating immune complexes containing rubella antigens in late onset rubella syndrome. J Pediatr 1980; 97:370-3.

Theofilopolous AN, Dixon FJ. Immune complexes in human diseases. Am J Pathol 1980; 100:530-91.

Thomas RM, Linch DC. Identification of lymphocyte subsets in the newborn using a variety of monoclonal antibodies. Arch Dis Child 1983; 58:34-8.

Thompson JJ, Mangi RJ, Lee R, Dwyer JM. Immunoregulatory properties of serum from patients with different stages of syphilis. Br J Vener 1980; 56:210-7.

Thompson RA, editor. Laboratory investigations in clinical immunology. V. Complement. In: The practice of clinical immunology. 2nd ed. London: Edward Arnold, 1978:119-27.

Tourville DR, Lawrence HB, Kim DU, Zajd D, Lee J, Reichmann LB, Baskin SE. Treponemal antigen in immunopathogenesis of syphilitic glomerulonephritis. Am J Pathol 1976; 82:479-92.

Tramont EC. Persistence of *Treponema pallidum* following penicillin therapy. JAMA 1976; 236:2206-7.

Turner DR, Wright DJM. Lymphadenopathy in early syphilis. J Path 1973; 110:305-8.

Van Coeverden de Groot HA, Davey DA, Smith JA, Vader CG, Van der Merwe FW. The midwife obstetric unit. S Afr Med J 1978; 53:706-8.

Venter A, Pettifor JM, Duursma J, Pudifin DJ, Smyth A, Becker PJ. Liver function in early congenital syphilis: does penicillin cause a deterioration? J Pediatr Gastroenterol Nutrition 1991; 12:310-4.

Virella G, editor. The humoral immune response and its induction by active immunization. In: Introduction to Medical Immunology. 3rd ed. New York: Marcell Dekker Inc.:1993a:213-32.

Virella G. Immune complex diseases. In: Virella G, editor. Introduction to Medical Immunology. 3rd Ed. New York: Marcel Dekker Inc. 1993b:379-96.

Virella G. Diagnostic evaluation of phagocytic function. In: Virella G, editor. Introduction to Medical Immunology. 3rd Ed. Marcel Dekker Inc. 1993c:311-27.

Walter P, Blot P, Ivanoff F. The placental lesions in congenital syphilis - a study of six cases. Virchows Arch A Pathol Anat Histopathol 1982; 397:313-26

Watson W, Oen K, Ramdahin R, Harman C. Immunoglobulin and cytokine production by neonatal lymphocytes. Clin Exp Immunol 1991; 83:169-74.

Webster LA, Rolfs RT. Surveillance for primary and secondary syphilis - United States, 1991. MMWR 1993; 42:13-9.

Weinberg AG, Rosenfeld CR, Manroe BL, Browne R. Neonatal blood cell count in health and disease. II. Values for lymphocytes, monocytes and eosinophils. J Pediatr 1985; 106:462-6.

Whaley K,editor. Measurement of complement. In: Methods in complement for clinical immunologists. Edinburgh: Churchill Livingstone, 1985:77-139.

Whitaker JA, Sartain P, Shaheedy M. Hematological aspects of congenital syphilis. J Pediatr 1965; 66:629-36.

Wicher V, Wicher K. Cell response in rabbits infected with T pallidum as measured by the leucocyte migration inhibition test. Br J Vener Dis 1975; 51:240-5.

Wigfield AS. Immunological phenomenon of syphilis. Br J Vener Dis. 1965; 41:275-84.

Wiggelinkhuizen J, Kaschula ROC, Uys CJ, Kuitjen RH, Dale J. Congenital syphilis and glomerulonephritis with evidence for immune pathogenesis. Arch Dis Child 1973; 48:375-81.

Wilson M. Immunology of the fetus and newborn: lymphocyte phenotype and function. Clin Immunol Allergy. 1985; 5:271-86.

Wood GW. Mononuclear phagocytes in the human placenta. Placenta 1980; 1:113-23.

Wood G, King GR Jr. Trapping antigen - antibody complexes within the human placenta. Cell Immunol 1982; 69:347-62.

Wright D, Berry JM, Colin L. Liver involvement in congenital syphilis (letter). Br J Vener Dis 1974:50:241.

Wright DJM, Grimble AS. Why is the infectious stage of syphilis prolonged? Br J Vener Dis 1974; 50:45-9.

Wright WC Jr., Ank BJ, Herbert J, Stiehm RE. Decreased bactericidal activity of leucocytes of stressed newborns. Pediatrics 1975; 56:579-584.

Wynn RM. Derivation and ultrastructure of the so-called Hofbauer cell. Am J Obstet Gynecol 1967; 97:235-48.

Wynn RM. Development and ultrastructural adaptations of the human placenta. In: Brosens IA, Dixon G, Robertson WB (editors). Human Placentation. Excerpta Medica 1975; 3-21.

Xanthou M, Valassi-Adam E, Kintzonidou E, Matsaniotis N. Phagocytosis and killing ability of *Candida albicans* by blood leucocytes of healthy term and preterm infants. Arch Dis Child 1975; 50:72-5.

Zubler RH, Lange G, Lambert PH, Mieschler PA. Detection of immune complexes in heated sera by a modified ^{125}I-C1q binding test. Effect of heating on the binding of C1q by immune complexes and application of the test to systemic lupus erythematosus. J Immunol 1976; 116:232-5.