Qualitative and quantitative aspects of immune responses to polysaccharide vaccines in children with HIV infection

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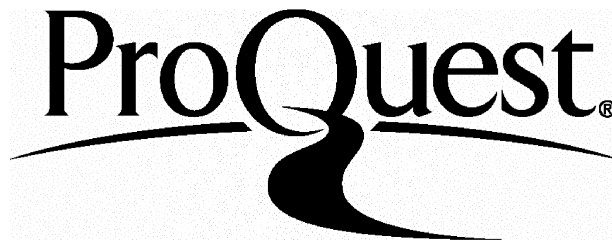

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Abstract

The susceptibility of HIV infected children to bacterial infections, mainly from bacteria with polysaccharide capsule, remains poorly understood. Impaired antibody responses as well as factors related to appropriate isotype production and antibody avidity may be responsible for their vulnerability to infection. Although vaccination with the currently available polysaccharide vaccines is recommended, there is a paucity of data for its real benefits in HIV infected children.

The studies presented in this thesis, investigate the quantitative and qualitative aspects of immune responses to polysaccharide vaccines in HIV infected children by measuring levels, isotypes and avidity of antibodies produced after the administration of 23-valent pneumococcal vaccine and Hib-tetanus conjugated vaccine. Since there is a paucity of data to determine long term immunogenicity of the above vaccines, the study population was followed up for a year and persistence of antibodies was monitored in sequential samples taken at 6 and 12 months post vaccination. For that purpose, solid phase assays were developed for the measurement of pneumococcal antibodies to pneumococcal serotypes (PS) 3, 6B, 19F and 23F and cell wall polysaccharide (cps).

Immunizations against *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* had similar adverse reaction rates in HIV infected children and controls. Immunogenicity of the 23-valent pneumococcal vaccine and the pattern of IgG2 subclass immune responses, were found similar in seropositive children and controls. Furthermore anti pneumococcal antibody levels were similar at 12 months post vaccination in Human Immunodeficiency Virus (HIV) infected and controls. 84% of HIV infected children achieved protective antibody levels against Hib post immunization, however the mean response was 7.6 fold lower than in controls. In

addition, 1 year later only 57% of the initial seropositve responders had persisting titers above the level associated with long term protection (>1μg/ml). For both vaccines there was a significant trend toward poorer responses in children with advanced disease but no correlation with age adjusted CD4 counts.

A relation between avidity for PS19F and 23F, and age and stage of the disease could not be established. However, there was some increase in avidity with time, indicated by the higher avidity indices in the 12 months post vaccination samples. This difference was more prominent, when avidity was measured in serum where antibodies against cell wall polysaccharide antibodies (cps) had not been removed. Removal of cps antibodies, resulted in a reduction of avidity indices in most of the samples tested, indicating that cps are high avidity antibodies.

The data presented in this thesis, support the policy of vaccinating HIV infected children with polysaccharide vaccines soon after the establishment of diagnosis.

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This work would not have been completed without the financial help of the Greek Goverment and the continous support and understanding of my family.

Declaration

The work presented in this thesis is that of the candidate with the following exceptions:

(a) Anti -PRP antibody levels measured at 1 and 12 months post immunization with ActHib vaccine.

(b) Total IgG pneumococcal antibodies measured by whole vaccine ELISA before and after adsorption with cell wall polysaccharide.

The above assays were performed by Dr Helen Griffiths, in the Immunology Department at John Radcliffe Hospital, Oxford.

Statistical analyses in Chapters 3 and 4 were performed by Dr Di Gibb, Senior Lecturer in Epidemiology, at the Institute of Child Health, London.

Abbreviations

ABN	antibody nitrogen
ADCC	antibody-dependent cell-mediated cytotoxicity
AI	avidity index
AIDS	aquired immune deficiency syndrome
APC	antigen presenting cells
AZT	azidothymidine (zidovudine)
BSA	bovine serum albumin
CDC	Center for Disease Control
Con A	staphylococcal Cowan A
cps	cell wall polysaccharide
CR	complement receptor
CTL	cytotoxic immune responses
DEA	diethylamine
ELISA	enzyme linked immunosorbent assay
Fc	fragment crystalline
FcγR	Fc gamma receptor
FDC	folicular dendritic cells
GMC	geometric mean concentration
H. influenzae	*Haemophilus influenzae*
HbOC	capsular oligosaccharide-mutant diptheria toxin, CRM
Hib	*H influenzae* type b
HIV	human immunodeficiency virus
Ig-V	immunoglobulin-variable region
IL	interleucin
INF-γ	interferon gamma
IU	international units
IVIg	intravenous immunoglobulin
LIP	lymphocytic interstitial pneumonitis
MHC	major histocompatibility complex
NH_4SCN	ammonium thiocyanate
OD	optical density
OMP	Neisseria meningitidis outer membrane protein
ON	over night
OPD	o-phenylene diamine
PBS	phosphate buffered saline
PGL	persistent generalized lymphadenopathy
PHA	phytohaemagglutinin
PRP	polyribosylribitol phosphate
PS	pneumococcal serotypes
PWM	pokeweed mitogen
RIA	radioimmunoassay
RNA	ribose nucleic acid
RT	room temperature
S. pneumoniae	*Streptococcus pneumoniae*
STD	standard
TcR	T cell receptor
TD	thymus-dependent
Th	T helper cells
Ts	T suppressor cells
TI	thymus-independent
Tw	Tween 20
URT	upper respiratory tract
U	uninfected
VI	vertically infected

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1

Introduction

The immune system, engaged in a continuous war to defend humans from a hostile environment, is able to recognise foreign antigens, recruit appropriate cells and produce an effective immune response to combat invading pathogens. An immune response to an antigen, involves a complex set of interactions between multiple cell types which allow the immune system to identify the nature of antigen and mount a rapid specific response, utilising the most efficient effector mechanisms as well as retaining specific memory. Cellular and humoral immunity work together during the response to an antigen. While cellular immunity, expressed mainly by thymus derived T cells, involves cell mediated killing, humoral immunity, is associated with the production of specific antibodies expressed by bone marrow derived B cells. Most pathogens are short lived in normal individuals due to the effective way the immune system eliminates them; however, in a few cases the ineffective agent manages to

escape the immune system causing latent, recurrent, or lifelong subclinical infection to the host. At present, we have little understanding on the underlying immunological mechanisms of these infections.

Human Immunodeficiency Virus (HIV), the cause of Aquired Immune Deficiency Syndrome (AIDS) is one of the pathogens that cannot be eliminated effectively by the immune system. Since 1981, when the first AIDS cases were described, HIV infection has become the epidemic of our generation with a dramatic global impact. By the end of 1992 about 2.5 million AIDS cases in men, women and children had occured and 13 million persons had been infected with the Human Immunodeficiency virus (HIV). Estimates of the number of cases of HIV infection likely to have occured by the year 2000, range from 40 milion to more than 110 million which is approximately the 2% of the world's present population. Since 1983 when the first case of paediatric AIDS was described, 1 million children have already been infected and HIV infection has become one of the leading causes of death in paediatric populations.

1.1. HIV INFECTION

1.1.1. The HIV virus

Since the HIV virus (Figure 1.1), an RNA retrovirus, was established as the cause of HIV disease, intensive research has tried to elucidate not only it's complex structure, but also it's primary role in the pathogenic mechanisms associated with the clinical appearance of the new syndrome.

HIV, like all other retroviruses, has 3 main structure genes, the *gag* which encodes for the p17 and p24 core proteins and is highly preserved between HIV different isolates, the *pol* encoding for its transcription enzyme called reverse transcriptase, and the *env* for the envelop proteins gp120 and gp41 which differ between HIV different isolates. The remarkable ability of HIV to become rapidly resistant to various drugs is related to the inaccuracy of its reverse transcriptase, which continiously generates new variants by introducing mutations to the incorporated nucleotides.

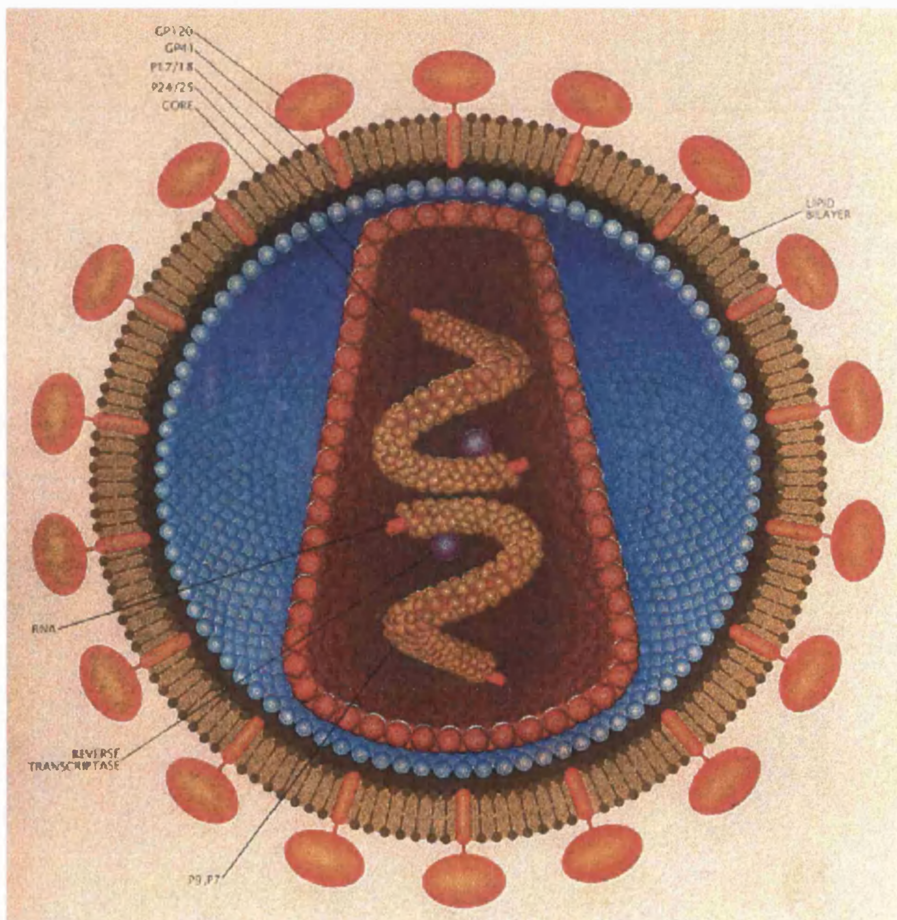

Figure 1.1 The HIV virus

1.1.2. Immunopathogenesis of HIV infection

While *in vitro*, HIV shows tropism for many types of cells including antigen presenting cells (APC), macrophages, monocytes, epithelial and endothelial cells, the main cells infected and depleted *in vivo* are the CD4+ T lymphocytes. The high affinity gp120-CD4+ binding, facilitates the entrance of the virus into the CD4+ T cells and the subsequent intracellular viral proliferation results in cell killing by osmotic lysis. However while only a small percentage of CD4+ T cells are infected by the virus, other cytotoxic mechanisms have also been associated with the massive T cell depletion observed in HIV infection, such as the formation of syncytia and ADCC killing of uninfected cells coated with gp120 protein (Pantaleo et al. 1993).

HIV is a highly antigenic virus inducing not only cytotoxic (CTL) but also humoral immune responses to its many surface and core proteins. While there is evidence that protective immunity to HIV does exist (Newell et al. 1996), much research is going on, to identify immune responses associated to the maintenance of the asymptomatic stage of the disease or even the clearance of the virus. CTL immune responses which precede the appearance of neutralising antibodies, have been associated with the suppression of the initial HIV viremic phase. Although it is unlikely that the highly divergent, between different isolates, gp120 molecule can elicit consistently protective immune responses, neutralizing antibodies to the highly conserved p24 molecule have been associated with the reduction of the viral load *in vivo.* However it is still unclear if anti-p24 antibodies are important in maintaining the asymptomatic stage of the disease. Currently it is believed that increased amounts of Th-1 cytokines (IL-2, IL-4, INF-a) which preserve CTL responses are associated with the maintenance of the asymptomatic period, whereas a shift to Th-2 cytokines (IL-4,

IL-6, IL-10) with the predominance of humoral specific immune responses is associated with progression of the disease to the symptomatic stage (Wasik et al. 1997).

1.1.3. HIV Infection in children

1.1.3.1. Clinical manifestations

Differences in the immune system between adults and children are probably responsible for the specific clinical manifestations and immunological characteristics of paediatric HIV infection. While in children, the first clinical appearence of the disease is usually dramatic with severe opportunistic and bacterial infections in adults, these symptoms appear after a prolonged asymptomatic period and only when the virus has severely damaged their immune system. In paediatric HIV infection, the mean period for progression from the asymptomatic stage of the disease to AIDS, is difficult to estimate. While some children (fast progressors) have a rapid decline in CD4+ counts resulting in progression to full blown AIDS by a mean age of 4.1 months and death by the age of 1 year, others (slow progressors) remain healthy and symptom-free for more than 5 years [(Auger et al. 1988), (Scott et al. 1989)]. The route of transmission, the type of HIV strain causing infection and the viral burden of the host have been associated with the different clinical presentation of the disease in children (Balotta et al. 1997).

The classification of paediatric HIV infection in children, according to the Center for Disease Control (CDC) criteria, appears in table 1.1 (see overleaf).

Table 1.1: CDC classification of HIV infection in children

CLASS P-0. INDETERMINATE INFECTION
Infants younger than 15 months, born to infected mothers, but without evidence of HIV infection

CLASS P-1. ASYMPTOMATIC INFECTION
Subclass A.. Normal immune function
Subclass B. Hypergammaglobulinemia, CD4+ lymphopenia, decreased CD4+/CD8+ ratio, absolute lymphopenia
Subclass C. Immune function not tested

CLASS P-2. SYMPTOMATIC INFECTION
Subclass A. Non specific findings (fever, failure to thrive, generalized lymphadenopathy, hepatomegaly, splenomegaly, enlarged parotids, persistent or recurrent diarrhoea)
Subclass B. Progressive neurologic disease
Subclass C. Lymphoid interstitial pneumonitis
Subclass D. Secondary infectious diseases
Category D-1 Opportunistic infections (bacterial, fungal, parasitic, viral)
Category D-2 Recurrent serious bacterial infections (more than 2 in 2 years)
Category D-3 Other infectious diseases (persistent oral candidiasis, herpes etc)
Subclass E. Secondary cancers associated /possibly associated with HIV
Subclass F. Other conditions possibly associated with HIV (hepatitis, cardiopathy, nephropathy, hematologic disorders, dermatologic diseases)

1.1.3.2. Immunological abnormalities

Paediatric HIV infection acquired peri or postnatally, has distinct immunologic characteristics as compared to those observed in adults and these are probably related to phenotypic and functional differences of the neonatal lymphocyte lineages. Although the viral load in the peripheral blood at different stages of the infection has been reported to be similar to that found in adults, the progression of the disease is usually more rapid. This is probably related to the inability of the immature immune system to

restrict viral replication or to the more profound impact that HIV-induced thymic injury has on the T cell population of the developing host.

Most of the perinatally or postnatally infected slow progressors, develop both cellular and humoral immune responses during the first 2 years of life while they remain clinically and immunologically well. However, in the next 2 years, an attrition of these responses usually occurs associated with the development of several immunological abnormalities (table 1.2) which sometimes precede the onset of clinical symptoms, and their early detection could be of predictive and therapeutic importance (Borkowsky et al. 1992a).

Table 1.2: Immunological abnormalities in Paediatric HIV infection

T cells	Decreased CD4+, Increased CD8+ cells Increased HLA-DR expression Decreased CTL responses
Monocytes/ Macrophages	Decreased HLA -DR expression Impaired antigen presentation
Natural Killer	Increased CD57 + cell subsets
Granulocytes	Increased/decreased chemotaxis, decreased phagocytosis
B cells	Impaired responses to thymus-dependent and thymus-independent antigens
IgG	Increased (at all stages)
IgM	Increased (at early stages)
IgA	Increased (at late stages)
IgD/IgE	Increased
Cytokines	Increased IL-6 production, decreased IL-2, IL-4, IFN-γ (vary at different stages of the disease)

(after P. Pizzo: Pediatric AIDS 1994)

(a) T cell abnormalities

The most pivotal consequence of HIV infection at all ages, is the qualitative and quantitative abnormalities in T lymphocytes. In paediatric, as compared to adult HIV

infection, the T cell related immune abnormalities have different characteristics, probably attributed to the quantitative and qualitative differences of neonatal T cell lineages. Although only 5% of the fetal thymic T cells mature and enter the peripheral circulation expressing T cell receptor (TcR), their numbers in normal children are much higher than in adults (Comans-Bitter et al. 1997). HIV infected neonates also have high CD4 T cell numbers and severe opportunistic infections do frequently occur at much higher counts than in seropositive adults. Furthermore, qualitative differences of neonatal T cells expressed as restricted TcR diversity and low numbers of CD45RA memory T cells, are also responsible for the inability of HIV infected children to deal with tumors and infections that rely on cellular immunity, for control.

While there is a continuous decline in CD4+ T lymphocytes in paediatric HIV infection, there is a lack of data monitoring the rate of T cell attrition. Since direct comparisons from studies in adults cannot be done, the quantitative abnormalities of CD4+ cells are not a reliable marker for the progression of HIV infection in children.

The presence of functional T cell abnormalities expressed as cutaneous anergy or *in vitro* impaired mitogenic responses, which often precede their quantitative defects and occur irrespective of the route or timing of HIV infection have also been described in neonatal HIV infection (Roilides et al., 1991a).

CD8+ T cells increase with progression of the disease and elevated counts early in the course of paediatric HIV infection have been associated with poor prognosis (de Martino et al. 1991). The reduced CTL activity of neonatal CD8+ T cells, deteriorates further in HIV infection, either by the direct effect of the virus on the cells or as a consequence of impaired CD4-induced activation. Studies in HIV infected homosexuals demonstrated a direct correlation between reduced CD4+ numbers and

impaired CTL activity probably mediated by impaired cytokine production. This finding is in accordance with studies showing that replacement of CD4-derived IL-2 could restore CTL responses (Rook et al. 1983).

(b) B cell abnormalities

B cell abnormalities associated with severe and recurrent bacterial infections mainly from encapsulated bacteria, have a major impact in paediatric HIV infection. This is reflected by the inclusion of recurrent episodes of bacterial sepsis (defined as 2 episodes within a 2 year period) in the 1987 CDC surveillance definition of paediatric AIDS (MMWR, 1987).

Quantitative B cell abnormalities have been described in many studies performed in paediatric populations. Hypergammaglobulinemia, is one of the commonest findings amongst symptomatic HIV infected children. It is usually polyclonal, related to the general state of immune activation induced by the virus (Roilides et al. 1991b) and has been associated with poor prognosis.

Abnormalities in all Ig classes have been reported in HIV infection (see table 1.2). In addition, IgG1 and IgG3 subclasses appear to be elevated whereas IgG2 and IgG4 are usually normal or low. However, while it has been suggested that low IgG subclass levels occurring in HIV infected children could be associated with an increased risk of bacterial infections, relevant studies in seropositive paediatric populations, have failed to establish such a relationship (Roilides et al. 1991b).

The qualitative B cell defects observed in HIV infection are expressed as unresponsiveness to new antigenic stimulations (anergy), impaired differentiation to plasma cells and activation of inappropriate signaling leading to programmed cell death

(apoptosis). Several studies provide evidence that the observed B cell functional abnormalities are related either to a direct effect of the virus on B cells or are the consequence of impaired T cell help. Impaired antibody responses to the T-dependent (TD) antigens tetanus toxoid and bacteriophage ϕX 174 (Bernstein et al. 1985a) in association with the finding that B cell unresponsiveness in HIV infection can be overcome by artificial T cell help or exogenous administration of T cell derived cytokines (Borkowsky et al. 1987), provide evidence for T cell involvement to the B cell functional defects. However immune responses to staphylococcal Cowan A, a T-independent (TI) antigen and polysaccharide vaccines (see in detail later in this chapter) have also been found to be impaired, indicating that a direct effect of the HIV virus on B cells, may also be present.

Impaired secondary immune responses have also been reported in paediatric HIV infection with absence of IgM to IgG switch and lack of generation of memory B cell clones. While seropositive adults retain humoral immunity to recall antigens and protective memory even in the later stages of the disease (Janoff et al. 1991), HIV infected infants have deficient antibody responses to recall antigens (Bernstein et al. 1985b). The lack of immunologic memory, is probably one of the multifactorial causes of repeated bacterial infections observed in seropositive children and makes the consequences of B cell abnormalities more prominent in paediatric HIV infection.

1.2 POLYSACCHARIDE ANTIGENS

1.2.1. Introduction

Polysaccharide antigens are high molecular weight polymers which have been characterized as TI antigens because they can activate B cells directly, by cross linking

of their surface antigen receptors without a major histocompatibilty complex (MHC) class II restricted T cell help. Today pneumococcal polysaccharides, are characterized as TI-2 antigens which in contrast to TI-1 antigens, represent a heterogenous group which does not stimulate antibody production by the classical Major Histocompatibility Complex (MHC)/T cell receptor (TcR) interaction, but still requires some T cell involvement (Table 1.3).

Table 1.3: Characteristics of T dependent and T independent antigens

	TI Antigens		TD Antigens
	TI 1	TI 2	
Molecular weight	high		low
Degradation	slow		fast
Ontogeny	early	late	early
T cells required	no		yes
T cell regulation	no	yes	yes
Activation of complement	classical or alternative		classical
Memory	no		yes
Isotype/idiotype restriction	yes		no

1.2.2. Immune responses to polysaccharides

Studies on protection from *Streptococcus pneumoniae (S pneumoniae)*, the most representative pathogenic microorganism with polysaccharide capsule, revealed that non-immunological as well as immunological mechanisms are responsible for protection from invasive disease.

Non immunological factors include the integrity of the epithelial surface of the upper and lower respiratory tract while the immunological defense against

pneumococcal infection depends on type-specific anti-capsular polysaccharide antibody interacting with complement to opsonise pneumococci and prepare them for phagocytosis and clearance by neutrophils and tissue fixed macrophages (Bruyn et al. 1991). Defects on any of these components of host defense are usually associated with an increased risk for invasive disease. However type specific antibody deficiency is the most essential predisposing factor to pneumococcal disease.

While *S pneumoniae* is a normal inhabitant of the URT, mucosal immunity mediated by secretory IgA1 and IgA2 subclasses has also an important role in host protection against invasive disease.

Following colonisation or infection by *S pneumoniae*, an immunocompetent individual mounts an immune response to several components of the pneumococcus, including the capsular polysaccharide, the cell wall polysaccharide (cps), mainly its phosphorylcholine residues, pneumolysin and pneumococcal surface protein A [(Gray et al. 1983), (Musher et al. 1986)]. Of these, antibody to the capsular polysaccharide is believed to be the most important for protection. Antibodies to cell wall polysaccharide although provide some protection in mice, are thought not to be protective in humans (Musher et al. 1990a) (see also in chapter 5).

Experiments by Kehrl and Fauci (1983) in mice have shown that 8-10 days after the antigenic stimulation, 5-12% of the B cells of the host, were antigen binding cells expressing preactivation markers whereas pre-existing anti-capsular antibody levels were inversely correlated with the number of antibody secreting cells. In adults and children older than 4 years of age, the antibodies produced are of IgM, IgG and IgA classes and reach their maximum levels 8 weeks after the antigenic stimulation. The antibody-mediated opsonization of polysaccharide antigen, is necessary to allow

contact between the hydrophilic polysaccharide capsule and phagocytes. Although certain capsular types like pneumococcal serotype 3 (PS3) seem to be able to activate complement by the alternative pathway (Winkelstein and Tomasz, 1978), in general, phagocytosis requires activation of the classical pathway.

There is evidence that polysaccharides stimulate immunoregulatory events that lead to the production of antibody responses of a particular isotype, IgG3 in mice and IgG1 and IgG2 in humans. In children, antibody responses, are of IgM and IgG1 subclasses and shift towards IgG2 as the immune system matures, probably indicating the delayed ontogeny of this subclass (Morell et al. 1990). In adults, immune responses are induced by activation of a restricted pattern of IgG1 and IgG2 precommitted anti-PS, B cell clones (Shackelford and Granoff, 1988). Although individuals with congenital IgG2 subclass deficiency, usually have an impaired immunity to polysaccharides expressed as an increased susceptibility to encapsulated bacteria [(Umetsu et al. 1985), (Plebani et al. 1985)] the association of IgG2 subclass immune responses with protective immunity remains unclear. While Hämmärstrom et al. (1986) showed that protective immune responses in adults were usually restricted to the IgG2 subclass, Mäkelä et al. (1987) found that IgG1 subclass could be equally protective. Lortan et al. (1993) reported that IgG1 and IgG2 anti-pneumococcal antibodies in post immunization sera correlated significantly with phagocytosis in the presence of complement. However after the inactivation of complement, the remaining opsonic activity of sera was correlated only with specific IgG2 levels. This finding indicates that IgG2 subclass may induce phagocytosis by direct binding to the fragment crystalline (Fc) receptors on polymorphs and is in accordance with studies showing that it is the

best activator of the alternative complement pathway when found at high epitope densities (Burton and Ayoub, 1992).

Since anticapsular antibodies are protective against invasive disease, patients with decreased antibody levels, (agammaglobulinaemic, nephritic), defects in the complement system (mainly C3 deficiencies) and impaired phagocytosis (splenectomised, leukaemics) are at increased risk for pneumococcal disease.

1.2.2.1. The role of spleen

The observation that splenectomised patients are at particular risk for developing sepsis mainly from encapsulated bacteria, indicates the important role of spleen in protective immunity to polysaccharides (Holdsworth et al. 1990).

Both compartments of the human spleen the red and the white pulp, are involved in the immune responses to polysaccharides, the former is the area where phagocytosis takes place while the latter is related mainly to the production of anticapsular antibodies. Macrophages located in the marginal zone of the white pulp present polysaccharide antigens to the non circulating preactivated B cells which are responsible for the formation of specific anticapsular antibodies (Kraal et al. 1989). The ligation of CR2 on the surface of mature B cells with the polysaccharide antigen, is necessary for the B cell differentiation to antigen-secreting plasma cells (Timens et al. 1989). It has been suggested that the inability of children younger than 12-18 months of age to mount adequate antibody responses to polysaccharides has been associated with impaired expression of complement receptor (CR) 2. Selective antipolysaccharide antibody deficiency in patients who fail to respond to

polysaccharide antigens has also been associated with defects in maturation of the marginal zone B cell (Geha, 1988).

Studies in splenectomized patients have shown that immune responses to different serotypes differ in their dependency on the spleen. Antibody responses to PS14 localised in the marginal zone, are totally abolished after splenectomy, whereas responses to PS3 usually found in the red pulp, were slightly affected.

1.2.2.2. The role of T cells

Experiments in mice have shown that although antibody response to capsular polysaccharide is largely independent of thymic control, T cells can induce the immunoglobulin class switching and the magnitude of the antibody response (Barker, 1990). Two different T cell subsets have been shown to be involved in the immune responses to polysaccharides, helper (Th) and suppressor (Ts) T cell subsets. $CD4+CD8^-$ Th cells, found in the spleen during the first 24 hours after the antigenic stimulation, amplify B cell activation while $CD8+ CD4^-$ Ts cells found 5-24 hours after the immunisation act competitively to Th cells, on B cell activation (Braley-Mullen, 1982). Several studies have shown that polysaccharide antigens do not activate directly T cells. Instead, idiotypic determinants of type specific antibodies, cytokines and antigen-antibody complexes, all have a role in immunoregulation (Reviewed by Mond, 1995).

In humans, the exact mechanism of T cell activation by polysaccharides and the regulatory role of T cell subsets on the PS antibody production remains unclear. The non-MHC nature of T cell activation has been shown by experiments in mice where both monoclonal anti-MHC class antibodies and interruption of the classical

CD40/CD40 ligand interaction, did not block immune responses to polysaccharides. However there is evidence that T cells may enhance the production of IgG2 antibodies either having a direct effect on the immunoglobulin class switching or by expanding B cell clones that have already been switched (Griffioen et al. 1991).

1.2.2.3. The role of genetic factors

The magnitude of immune responses to polysaccharides has been reported to be influenced by specific Gm allotypes which are genetic variations in the amino acid sequence of the immunoglobulin molecule. In humans, 25 allotypes have been described on the γ heavy chain (Gm) and 3 on the constant region of the κ light chain (Km). Individuals with G2m(n) allotype seem to respond better to the Hib vaccine (Ambrosino et al. 1985) while other studies have shown that G2m(n) homozygotes adults produced higher levels of antipneumococcal antibodies of IgG2 subclass than the controls (Sarvas et al. 1989). The mechanism by which Gm allotypes influence immune responses, is unclear. It has been suggested that they could influence the antigen presentation, which subsequently affects the antibody production and induce the immunoglobulin class switching by increasing the B cell responsiveness to cytokines (Vitteta et al. 1985). However there is evidence from similar studies in paediatric populations that the role of Gm(n) may not be equally important in the immature immune system (Granoff et al. 1986). Furthermore the clinical relevance of allotype association with regard to risk of invasive disease in children, remains unclear.

Allotypic forms of the Fcγ receptor (FcγR) found in phagocytic cells have also been associated with differences in the immune responses to polysaccharides with Fcγ RIIa H131 allotype, carrying Histidine at position 131, considered as the more

advantageous for handling IgG2-opsonised bacteria with polysaccharide capsule *in vivo* (Rijkers et al. 1993).

1.2.3. Epidemiology of bacterial infections caused by encapsulated organisms

S pneumoniae and *Haemophilus influenzae* type b (Hib) both contain polysaccharide capsule, and are important causes of bacterial infections in childhood. Pneumococcus is the major cause of bacterial pneumonia and an important etiologic agent of otitis media, bacteremia and meningitis whereas Hib is a major cause of bacterial meningitis and epiglottis in children. In addition, Hib can cause pneumonia, septic arthritis, cellulitis and bacteremia.

S pneumoniae is a component of the normal nasopharyngeal microflora in humans with an estimated carrier prevalence of 80-100% in the first 2 years of life, 20-35% in preschool and school-aged children, 9% in adolescent and 2-18% in adults (Reviewed by Requejo, 1993). The association between colonization and invasive disease has been related to the infectivity of the type of pneumococcus being carried and the type-specific immunity of the individuals in the population. Longitudinal prospective studies by Gray et al. (1980) have shown that the winter peaks of increased exposure, acquisition and carriage of PS were associated with high incidence of pneumococcal infections.

The attack rates of pneumococcal invasive disease appear to vary widely in different geographic and socioeconomic settings and different age groups. Worldwide pneumococcal infections have been estimated to cause 1.2 million pneumonia deaths

per year and are the causative agent for nearly 40% of all pneumonia deaths in children less than 5 years of age (WHO 1993).

Morbidity associated with pneumococcal disease is also significant. While the attack rates of pneumococcal bacteremia in the entire life span has been estimated between 12-50 per 10^5 persons per year, it has been reported to be four times higher in the first 2 years of life. The incidence of otitis media in US is estimated to be 1.2 x 10^6 cases each year with 61% of the cases to occur in the first year of life, 18% in the second and 7% in the third (Austrian, 1989). Data on the incidence of pneumococcal meningitis are limited. It has been estimated to be about 1.5 to 2.5 cases per 10^5 persons per year with one-half of the cases occurring in children between 1 month and 4 years of age. The annual attack rate of pneumococcal pneumonia in Finland among children up to 4 years of age was 24.3/100000 (Eskola et al,1992). However Davidson et al. (1993) found that the annual incidence in paediatric populations in Alaska was 8-10 times higher than in other US groups. In the elderly, pneumococcus is the predominant cause of community acquired pneumonia accounting for more than 70% of cases of pneumonia (Hedlund, 1995).

Although more than 90 different PS have been identified, only 23 of these, regularly cause invasive disease. The pattern of the most common serotypes causing infection, varies with area, time of the year and the age-related maturation of the immune responses. In Western European countries and US, serotypes 3, 6A, 6B,14, 19A, 19F, and 23F are responsible for approximately 50% -70% of pneumococcal infections [(Klein, 1981), (Butler et al. 1995)] whereas studies in Alaska by Parkinson et al. (1994) showed an additional increased prevalence of types 4, 9V and 18C. Furthermore, the epidemiology of pneumococcal disease changes with age with PS3

causing infections at early ages whereas types 6B, 14, 19F and 23F found mainly in older children.

The attack rates of all types of Hib infections are inversely related to age. While the overall incidence of Hib meningitis in the first 5 years of age has been reported to be between 1.5-3.6 per 10^5 children, epidemiologic data from US have shown a peak incidence of 275 per 10^5 amongst infants 6 to 11 months old. The incidence of Hib meningitis in Alaskan Eskimos, Navajo Indians and Gambians has been reported to be 3-4 times higher that in US and ten times higher than in Europe (Bijlmer, 1991). However in the last five years, in countries where Hib conjugate vaccine has been included in the routine infant immunization schedule, the incidence of invasive Hib disease appears to be dramatically reduced [(Peltola et al. 1992), (Shinefield and Black, 1995), (Herceg, 1997)].

Relevant epidemiologic studies in HIV infected populations have shown an increased incidence of invasive disease caused by encapsulated bacteria. *S pneumoniae* is the leading cause of pneumonia and otitis media in HIV infected children while the incidence of pneumococcal bacteremia has been reported to be 100 times higher than in the general population (Redd et al. 1990). Furthermore, Frankel et al. (1996) reported that HIV infected patients with a history of pneumococcal pneumonia had increased reccurence rates of the disease in comparison with the general population (15% vs 1%). The incidence of invasive *H. influenzae* type b disease in HIV infected populations has been suggested to be 20-40 times higher than in the general population with half of the isolates being type b (Steinhart et al. 1992). In HIV infected children, it is estimated that Hib accounts for nearly 5% of the reported bacterial infections (Pizzo: Pediatric AIDS, 1994).

1.3. POLYSACCHARIDE VACCINES

1.3.1. Unconjugated polysaccharide vaccines

1.3.1.1. Pneumococcal vaccine

Since pneumococcal infections are the major cause of morbidity and mortality in children throughout the world, the development of polyvalent pneumococcal vaccines offers the prospect of reducing the incidence of such infections in normal and high risk paediatric populations. Different pneumococcal formulations have been developed in the past. The first pneumococcal vaccine was 6-valent, followed by an 8-valent and a 12-valent preparation. A 14-valent vaccine, containing serotypes 1, 2, 3, 4, 6A, 7F, 8, 9, 12F, 14, 18C, 19F, 23F and 25, was licensed in 1977. The current formulation of the polysaccharide vaccine was licensed in 1983 and consists of 25μg of purified capsular polysaccharide derived from each of the following 23 serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. These serotypes have been shown to be the most commonly isolated causative agents of pneumococcal pneumonia.

Pneumococcal polysaccharide vaccine has been shown to be immunogenic in healthy young adults. In children however, its immunogenicity is age and serotype-related with good antibody responses to a number of serotypes not being attained until the age of 18 to 24 months (Leinonen et al. 1986). Studies performed in Gambian children by Temple et al. (1991) have shown poor immunogenicity of the vaccine in children aged 2-9 months which according to the authors, would be unlikely to provide substantial protection against invasive pneumococcal disease. In contrast, studies in paediatric populations in Papua New Guinea, have shown that the 23-valent

pneumococcal vaccine elicited significant responses in children older than 6 months of age (Pomat et al. 1994).

Reports on the protective efficacy of the vaccine are contradictory and are influenced by host-related, socioeconomic and environmental factors. Although its licence was based on a the results of a randomized clinical trial on healthy African gold miners acutely exposed to *S pneumoniae* (Smit et al. 1977), subsequent efficacy studies performed in high risk populations have shown reduced protection in high risk populations [(Ahonkhai et al. 1979), (Sims et al. 1987)]. Shapiro et al. (1991) by means of a hospital based case-control study, demonstrated 61% efficacy of the vaccine in immunocompetent hosts but only 21% in the immunocompromised group and concluded that the vaccine was not protective for high risk populations. Butler et al. (1993) reported an overall efficacy of 57% for serotypes included in the vaccine. The efficacy for immunocompetent persons older than 65 years was 75% whereas efficacy for patients with sickle cell disease has not been documented. The authors also demonstrated a declining protection with time. Recently Koivula et al. (1997), reported that pneumococcal vaccination significantly reduced the incidence of pneumococcal pneumonia in elderly persons at increased risk for contracting pneumonia.

Efficacy studies of the pneumococcal polysaccharide vaccine in children have shown that it is age-dependent. Studies from Finland showed reduced protective efficacy of the vaccine for otitis media in children older than two years of age (Makela et al. 1983). However studies in paediatric populations in Papua New Guinea by Riley et al. (1991) showed a 37% reduction in pneumonic illness, and a 87% reduction in mortality after pneumococcal vaccination.

The epidemiological characteristics of pneumococcal disease in association with socioeconomic and immunologic differences of the vaccinated population, may be responsible for the observed discrepancies of the protective efficacy of pneumococcal vaccine in different geographic areas. Parkinson et al. (1994) in a prospective study carried out in Alaska between 1986-1990 found that 92% of the serotypes recovered were represented in the 23-valent pneumococcal vaccine whereas Jorgensen et al. (1991) with similar studies in US had shown that only 74.9% of the isolates were included in the current vaccine formulation.

1.3.1.2. *H Influenzae type b* vaccine

The immunogenicity and protective efficacy of the unconjugated polyribosylribitol phosphate (PRP) vaccine against *H Influenzae* type b, was studied in a large double-blind field study of 100.000 vaccinees aged 3 months to 5 years old, in Finland (Peltola et al. 1984). This study revealed that the immunogenicity and protective efficacy of the vaccine were strongly age-dependent and children 18 months of age or older were protected from invasive Hib infection following vaccination, whereas no protection was seen amongst children younger than 18 months. The efficacy of PRP vaccine in the Finish field trial for children 24 to 35 months of age was 80%. However in US the true vaccine efficacy was estimated to be only 44% (Shapiro et al. 1990).

1.3.2. Conjugated polysaccharide vaccines

The relatively poor immunogenicity of polysaccharide vaccines in young ages, has been overcome by the development of conjugate vaccine formulations, where the

covalent coupling of the TI polysaccharide to a protein carrier confers TD characteristics to the polysaccharide. It is known that immune responses to TI antigens are of restricted idiotypic diversity and are not associated with the formation of memory B cell clones whereas TD responses can elicit secondary immune responses (Table 1.4). The first report describing conjugation of carbohydrates appeared in the 1930's by Avery and Goebel. They observed that if PS3 was coupled chemically to a protein, it was able to stimulate the production of anti-pneumococcal antibodies in rabbits previously unresponsive to vaccination with purified polysaccharide antigens.

The first attempt to produce a polysaccharide conjugate vaccine was repeated in 1980 where PRP was conjugated to several proteins. Early clinical studies in humans, confirmed the increased immunogenicity of conjugated vaccines in infants, the induction of memory and carrier priming to subsequent administration of the pure polysaccharide alone. The proteins usually used in constructing conjugate vaccines, were diphtheria and tetanus toxoid because of their known immunological properties. More recently, conjugation with macromolecular polysaccharides or oligosaccharides has also been used in vaccine formulations targeting in the quantitative improvement of immune responses (Table 1.4).

Immunogenicity studies using different conjugate vaccines revealed that their structural differences confer markedly different immunologic properties to the conjugates. While a single injection of the PRP-OMP elicits serum anti-PRP antibodies, in infants at 2 months of age, HbOC requires two or three injections before eliciting significant increases in serum Ab concentrations (Lucas and Granoff, 1995). However despite these reported differences in the immunogenicity of different formulations in general, all licensed Hib conjugate vaccines appear adequetly immunogenic in

populations previously showing impaired responses to PRP vaccines [(Granoff et al. 1989), (Gigliotti et al. 1989)].

Table 1.4: *H Influenzae* type b conjugate vaccines

PRP-T	PRP coupled to tetanus toxoid
PRP-D	PRP coupled to diphtheria toxoid
PRP-OMP	PRP coupled to an outer membrane protein complex of Neisseria meningitidis group B.
HbOC	Oligosaccharide derivative of type b capsular polysaccahride coupled to the non toxic mutant diphtheria toxin, CRM $_{197}$.

Efficacy trials have also demonstrated that conjugate Hib vaccines are effective for preventing Hib meningitis. A big trial performed in Finland had shown that PRP-D vaccine administered to infants beginning at the age of 3 months was highly effective (Eskola et al. 1990). Similar results were reported recently by Booy et al.(1997) who estimated the overall efficacy of Hib conjugate vaccine in UK to be 98.1%.

Similar encouraging results for the improved immunogenicity of conjugated pneumococcal vaccines have been reported recently. Lagergard et al. (1990) had shown that PS3 conjugated with tetanus toxoid (PS3-T) when injected in laboratory mice elicited significantly higher type specific antibodies than the PS3 alone and exhibited booster responses; Fattom et al.(1990) showed that PS12F conjugated with diphtheria toxoid (PS12F-D) in healthy adults elicited significantly higher antibody levels than the unconjugated PS12F and Sarnaik et al. (1990) showed that PS6B-T vaccine was immunogenic in 2-5 years old children with sickle cell anaemia who had shown previously impaired responses to pneumococcal polysaccharide vaccine. Following successful trials with monovalent pneumococcal vaccine formulations

manufacturers produced polyvalent formulations in an attempt to provide wider coverage of pneumococcal disease. Pneumococcal conjugate vaccine preparations that have been tested in clinical studies are: 4-valent (6B, 14, 19F and 23F) tetanus and diphtheria conjugate (Dagan et al. 1997), 5-valent (6B, 14, 18C,19 F,23F)- CRM_{197} conjugate (Leach et al. 1996), 7-valent (4, 6B, 9V, 14, 18C, 19F, 23F)-CRM_{197} (Daum et al. 1997).

In general, all tested formulations show improved immunogenicity over the 23-valent polysaccharide vaccine in young children and high risk populations. Furthermore several investigators have shown the priming effect induced by conjugate pneumococcal vaccines in healthy adults and children. O'Brien et al. (1995) and Dagan et al. (1997) reported a significant increase of post vaccination antibody levels after booster doses with pneumococcal polysaccharide vaccine while Obaro et al. (1997) have shown that healthy Gambian children had an increase in avidity and opsonic activity of PS14 antibodies after revaccination with the 23-valent polysaccharide vaccine. Furthermore Dagan et al. (1997) reported a significant reduction in the carriage of vaccine-related serotypes after administration of conjugate pneumococcal vaccines.

1.3.3. Polysaccharide vaccines in HIV infected populations

The increased susceptibility of HIV infected adults and children to infections from encapsulated bacteria with polysaccharide capsules makes the prevention of invasive disease by immunization an important aspect of the care of HIV infected populations. Several studies on the immunogenicity of polysaccharide vaccines in HIV infected individuals have been published and they are summarised in table 1.5. For the

23-valent pneumococcal vaccine the overall impression is that despite differences in the study population, the methodologies employed for measurement of antibodies and the criteria used to assess immune responses, the attrition of the immune system observed with the clinical progression of HIV disease, reduces the vaccine immunogenicity in seropositive populations. However most of the studies are not of sufficient size to be conclusive. The few published studies on the Hib conjugate vaccine in seropositive populations have shown it to be more immunogenic in seropositive adults and children than the pneumococcal vaccine. In general, most of the studies demonstrate that the immunogenicity of Hib conjugate vaccine is between 80-100% in HIV infected adults while for pneumococcal vaccine it has been been reported to be between 63% and 88%. However randomised placebo-controlled efficacy studies of both vaccines in HIV infected populations have not been published.

Immunogenicity of the pneumococcal vaccine is associated with the time of vaccination in relation to the stage of the disease. Most of the studies have shown that recent seroconverters and asymptomatic HIV infected patients, mount similar responses as compared to healthy controls [(Rodriguez-Barradas et al. 1992), (Weiss et al. 1995)]. HIV infected individuals with ARC or AIDS, generally give moderately or severely impaired immune responses expressed either as lower post vaccination antibody levels or a response to a reduced number of serotypes [(Ammann et al. 1984), (Ballet et al. 1987), (Ochs et al. 1988), (Opravil et al. 1991)]. Reported discrepancies where some studies failed to show differences in the immune responses to the vaccine at different stages of the disease, may be attributed to the inappropriate methodology (Vandenbruaene M. et al. 1995) (see also in chapter 5) or the criteria for the assessment of immune responses which were not always indicative of the ability of

the patient to respond to the vaccine. This is apparent in a study by Janoff et al. (1988) who found similar fold increases between HIV infected patients and healthy controls but interpreted the responses in the infected patients as impaired, because seropositive individuals, obtained post vaccination levels lower than the controls.

Furthermore, the interpretation of the results from immunogenicity studies in relation to the potential protective efficacy of pneumococcal vaccination is difficult because of the lack of knowledge of the minimum protective antibody levels from invasive disease. In a study by Klein et al. (1989) seropositive adults had impaired fold increase in antibody responses in comparison with controls, although 88% of them developed type-specific antibody titers >400 ngAbN/ml considered as high, for most serotypes in healthy individuals (Landesman and Schiffman 1981). It is possible that the vaccine is more protective when given early after seroconvertion. However vaccine failures have been reported even in the asymptomatic stage of the disease (Willocks et al. 1995).

Although inadequate methodology related to the measurement of pneumococcal antibody levels without previous adsorption of cps antibodies, hampers the interpretation of the results from some published studies (Ballet et al. 1987), pre-vaccination antibody levels are usually similar in HIV infected asymptomatic patients and controls and lower [(Simberkoff et al. 1984), (Janoff et al. 1988), (Klein et al. 1989)] or higher [(Opravil et al. 1991) (Loeliger et al. 1995)] in adults with symptomatic disease, probably influenced by the history of previous infections. Furthermore, post vaccination levels seem to be comparable in asymptomatics and controls whereas they tend to be significantly lower in vaccinees with advanced disease (Weiss et al. 1995). However Rodrigez-Barradas et al. (1992) reported that patients

with AIDS who retain their ability to respond to the vaccine, express immune responses of the same magnitude, as healthy subjects. With regards to the persistence of antibody responses, conflicting data have been published. Rodriguez-Barradas et al. (1996), reported that HIV infected adults who gave an adequate response to the pneumococcal vaccine, retained high post vaccination titers 1 and 2 years after vaccination. However other investigators reported that most of the seropositive individuals who have been vaccinated with the 23-valent pneumococcal vaccine dropped their titers to pre vaccination levels between 3 and 12 months post vaccination [(Loeliger et al. 1995), (Mascart-Lemone et al. 1995)]. However while in some of these studies immune responses to the vaccine have not been measured immediately after vaccination, it is not known if this finding indicates unresponsiveness to the vaccine or attrition of immune responses caused by the HIV virus.

The few studies on the class and subclass profile of immune responses to polysaccharides have shown that HIV-infected patients had no specific defect in class and IgG2 subclass production after immunization and there was no compensation for impaired IgG responses by other isotypes or Ig classes [(Ballet et al. 1987), (Carson et al. 1995)]. Mascart-Lemone et al. (1995) reported that asymptomatic or mildly symptomatic seropositive individuals mounted nearly normal IgG and IgA responses while most of the IgG antibodies belonged to the IgG2 subclass. Only one study by Unsworth et al. (1993) has shown lower post vaccination IgG2 subclass levels in HIV infected individuals in comparison with the controls; however this could be attributed to the lower pre vaccination IgG2 levels found in the infected population.

Correlation between impairment of antibody responses and laboratory markers of the progression of the disease are conflicting. This is probably related to the fact that

polysaccharides are considered mainly as TI antigens, and since there are no other reliable markers for the progression of the disease than CD4 counts, the interpretation of such correlations is questionable. This is demonstrated clearly in a study by Steinhoff et al (1991), who compared antibody responses to the unconjugated TI and to the conjugated TD Hib vaccine and found that only the responses to the TD conjugate vaccine were associated with CD4+ counts. Most of the studies could not establish correlation between immune responses to pneumococcal polysaccharide vaccine and CD4 counts [(Kroon et al. 1994), (Vandenbruaene et al. 1995)]. However it is possible that patients with CD4 counts less that 150-200/mm^3 respond to less serotypes as had been demonstrated in a study by Rodrigez-Barradas et al. (1992).

Association between impaired responses to the vaccine and *in vitro* T cell function tests are also contradictory. Ballet et al. (1987) showed that patients with persistent generalized lymphadenopathy (PGL), had normal *in vitro* lymphocyte proliferative responses to phytohaemagglutinin (PHA), pokeweed mitigen (PWM) and staphylococcal Cowan A (Con A) whereas Opravil et al. (1991) found a trend towards correlation between reduced pneumococcal responses and impaired PHA proliferation in ARC and AIDS patients.

In HIV infected children, although the few published studies on the immunogenicity of polysaccharide vaccines are too small to give statistically significant results, the overall impression is that pneumococcal vaccine shows low immunogenicity especially in children with advanced disease.

In contrast, conjugate polysaccharide vaccines appear to be more immunogenic especially when they are given soon after birth. Indacochea et al. (1992) reported similar immunogenicity of the Hib conjugate vaccine in uninfected (U) and vertically

infected (VI) children born to HIV positive mothers when the later were vaccinated immediately after birth. However in children with advanced HIV disease immmunogenicity of the vaccine seems to be impaired (Kale et al. 1995).

Most of immunogenicity studies of polysaccharide conjugate vaccines in paediatric as well as in adult HIV infected populations establish a relationship between CD4 counts and the magnitude of the immune response [(Steinhoff et al.1991), (Kroon et al. 1997)] indicating the TD nature of immune responses to conjugate polysaccharide vaccines. Furthermore the priming effect of Hib conjugate vaccine was demonstrated in a study by Rutstein et al. (1996) who reported significantly increased antibody concentrations after booster responses in HIV infected individuals.

Recently two pilot studies on the immunogenicity of a 5-valent CRM conjugate pneumococcal vaccine preparation in HIV infected populations have shown conflicting results. While Ahmet et al. (1996) reported similar immune responses of the 23-valent pneumococcal polysaccharide vaccine and PS-CRM vaccine in seropositive adults, King et al. (1997) found improved immunogenicity of this vaccine, after 3 doses in HIV infected children and controls (see also in discussion).

Table 1.5: Studies on immunogenicity of polysaccharide vaccines in HIV infected populations

PNEUMOCOCCAL VACCINE

(a) Adults

STUDY	STAGE	RESPONSE	COMMENTS
Masur (1981)	AIDS	Normal	No control group
Ammann (1984)	AIDS	Impaired	
Simberkoff (1984)	AIDS	Impaired	Later pneumococcal pneumonia
Ballet (1987)	Asympt/ PGL	Impaired	No correlation with CD4 counts
Huang (1987)	Asympt PGL	Normal Impaired	
Ragni (1987)	Asympt ARC	Impaired	Correlation with impaired PKW response
Janoff (1988)	Asympt PGL/ AIDS	Normal Impaired	All Ig classes lower in symptomatic vaccinees
Ochs (1988)	Asympt ARC	Normal Impaired	
Klein (1989)	Asympt PGL	Impaired Impaired	
Rodrigez-Barradas (1992)	Asympt AIDS	Normal Impaired	
Unsworth (1993)	NS	Impaired IgG2	No correlation with CD4 counts
Kroon (1994)	NS	Normal	No correlation with CD4 counts
Weiss (1995)	Asympt	Normal	
Vandenbruaene (1995)	Asymp ARC/AIDS	Normal	No correlation with CD4 counts
Carson (1995)	NS	Normal	Similar IgG class and subclass responses
Loeliger (1995)	CD>300 CD<300	Normal Impaired	Higher pre vaccination levels
Mascart (1995)	Asympt	Normal	Normal IgG reduced IgM responses
Ahmed (1996)*	CD>500 CD<500	Normal Impaired	One dose of a 5valent CRM conjugate vaccine
Janoff (1997)*			Decreased levels of naturally occuring antibody

NS =not stated * pneumococcal conjugate vaccine preparation

Table 1.5: Studies on immunogenicity of polysaccharide vaccines in HIV infected populations (*continued*)

PNEUMOCOCCAL VACCINE
(b) Children

STUDY	STAGE	RESPONSE	COMMENTS
Bernstein (1985)	AIDS	Impaired	Correlation with impaired responses to ϕx174
Apradi (1994)	P-1* P-2*	NS	12 months post vaccination levels, similar to the unvaccinated group
Peters (1994)	P-1 P-2	Impaired	Response was defined as 4 fold increase in post vaccination titers. No control group
King (1997*)		Normal	Three doses of 5valent conjugate vaccine

NS =not stated * pneumococcal conjugate vaccine preparation

Hib CONJUGATE VACCINE

(a) Adults

STUDY	STAGE	RESPONSE	COMMENTS
Steinhoff (1991)	Asympt ARC AIDS	Impaired Impaired Impaired	Correlation with CD4 counts
Kroon (1997)	CD4 >300 CD4 100-300 CD4 <100	Normal Normal Impaired	Correlation with CD4 counts

(b) Children

Indacochea (1992)	P0	Normal	When vaccine given after birth
Walter (1992)		Impaired	
Peters (1994)	P1/P2A*	NS	4-85 months post vaccine only 37% had levels> 1mg/ml
Kale (1995)	PO/P1/P2A*	Normal after 3 doses	Correlation with CD4 counts
Rutstein (1996)		Impaired	No correlation with CD4 counts

NS =not stated

1.4. QUALITATIVE ASPECTS OF THE IMMUNE RESPONSE

1.4.1. Antibody avidity

While most of the studies on immunity to different pathogens focus on quantitative aspects of the immune response, there is increasing evidence that the investigation of qualitative aspects related to the affinity of antibody to antigen is biologically significant. Antibody affinity is the strength of the interaction between an antibody combining site (paratope) and a specific antigenic determinant (epitope). Functional affinity or antibody avidity is a term used to characterize multivalent interactions between antibody and the complete antigen molecule and it is probably more relevant to the biological activities of antibody. Several studies have shown that high avidity antibodies are more effective in many biological processes *in vitro*, including toxin neutralization, complement fixation and virus neutralization (Steward M. 1981). Furthermore, *in vivo*, high avidity antibodies have been associated with better protection from invasive disease. Ahlstedt et al. (1974) have shown that mice producing high avidity antibodies, could clear more effectively intraperitoneal infection from *E coli*, whereas Pincus et al. (1988) noted that the amount of high avidity antibodies bound to group B streptococci was 10-15 times greater than that of the low avidity antibodies. In contrast, the production of low avidity antibodies has been associated with the failure of the immune system to eliminate the ineffective agent. Bruderer et al. (1992) reported that in patients with cystic fibrosis, naturally acquired antibodies against *Pseudomonas aeruginosa* which do not protect against bronchopulmonary exacerbation are of much lower avidity compared with the protective vaccine-induced antibodies. Similar findings have been reported in animal

studies where excessive production of low avidity antibodies has been associated with the development of immune complexes and chronic disease (Steward M, 1981).

A range of different factors are known to influence the avidity of immune responses. These are related to both the nature of the immunogenic stimulus and the antigen processing and presentation by local APC. Experiments in mice have shown that injection via the subcutaneous route resulted in the production of significantly higher avidity antibodies than intraperitoneal administration of the same antigen. Furthermore the coupling of the antigen to different carriers results in variability in the avidity of the produced antibodies. This is apparent in a study by Schlesinger and Granoff (1992) who compared three different Hib conjugate vaccines and found that although they had similar immunogenicity in young children, the anti- PRP antibodies were of different avidity depending on the carrier protein contained in the conjugate formulation. These data have been confirmed recently by Lucas and Granoff (1995), who found that the mean anti-PRP antibody avidity of the serum pool from infants vaccinated with HbOC vaccine, was three-fold higher than that of the pool from infants vaccinated with PRP-OMP.

Host related factors may also influence the immune responses, as has been demonstrated in studies by Hetherington and Rutkowski (1990) who showed that healthy infants produced antibodies of variable avidity in response to vaccination with PRP-OMP vaccine. It has been suggested that age related factors may influence antibody avidity and adult type of immune responses may be associated with the production of higher avidity of immune responses. Studies on the relative avidity of antibodies to polysaccharides have shown that the adult type IgG2 immune responses were of higher avidity than the IgG1 responses, mainly given by young children

(Persson et al. 1988). Furthermore it has been suggested that the absence of the high avidity IgG3 antibodies specific for *Moraxella catarrhalis* in children under the age of four, could be a predisposing factor for their increased susceptibility to infection with this organism (Goldblatt et al. 1990).

1.4.2. Maturation of immune responses and immunologic memory

1.4.2.1. T dependent antigens

The increase of antibody avidity in response to an antigen, described mainly for TD responses, is known as avidity maturation. Although the precise mechanism for this process has not been fully elucidated, experiments in the murine model have shown that this is related to the emergence of B cells clones which have undergone somatic mutation in their rearranged Immunoglobulin-variable (Ig-V) region genes (MacLennan et al. 1992). This process has been shown to take place in germinal centers developed in the B cell follicles of secondary lymphoid tissues following an antigenic stimulation. Resting follicles consist of a number of re-circulating B cells and a rich network of follicular dendritic cells (FDC) which are APCs of haemopoietic origin.

In primary immune responses, B cell blasts activated by the antigen outside follicles, migrate and colonize follicles where they undergo massive clonal expansion resulting in the formation of germinal centers. Mature germinal centers with the characteristic dark and light zones emerge at the end of the exponential growth of B blasts. They contain two populations of B cells expressing different surface markers, proliferating blasts called *centroblasts* which occupy the dark zone and continue to be

in rapid cell cycle although they do not increase their overall numbers and *centrocytes* which move to the heart of the FDC network found in the light zone (Berek, 1992).

Centroblasts, triggered by the antigenic stimulation, activate a hypermutation mechanism which introduces individual point mutations into their Ig-V-region genes and results in the diversification of the B cell antibody repertoire. Studies on hybridoma cell lines have shown that somatic mutation starts in lymph nodes 4 days after the antigenic stimulation introducing successive single nucleotide exchanges at a high rate, mainly in the CDR regions. In the next 14 days, B cells expressing VDJ rearrangements characteristic of the high affinity "key mutations" dominate the response (Berek, 1993). Avidity measurements at that point, have shown an 8-10 fold increase in the pool of antigen-activated B cells indicating that only those B cells with high affinity receptors are clonally expanded whereas those that have picked up mutations leading to a loss of specificity for the antigen, seem to be effectively eliminated.

Centrocytes are high avidity B blasts which are probably selected by their ability to interact with the antigen held as immune complex on the surface of FDC. It has been suggested that FDCs play a crucial role in the process of affinity maturation, although it is not clear if they actively participate in the initial proliferative phase of B cells, where high affinity B cell clones are preferentially expanded. They bind circulating antigen through their complement and Fc receptors expressed on their surface which they subsequently present in an unprocessed form to the B cell receptors. This intimate contact is believed to be necessary for the selection of centrocytes with high affinity receptors to become plasma or memory B cells. Centrocytes which have not positively been selected die quickly through apoptosis.

Germinal centers decline in size progressively and they are no longer visible, about 3 weeks following immunization. At this stage however, small numbers of centroblasts continue to proliferate and are probably the source of plasma cells and memory cells which maintain the antibody production for long periods of time. Although the requirements for the maintenance of memory B cell clones are still unclear there is evidence that this is related to the presence of antigen on the surface of FDC which is retained within their elongated extensions in an unprocessed form for a long time (MacLennan M, 1994). The interaction of centroblasts with the retained antigen provides the necessary stimulation required for their continuous proliferation which is probably necessary to maintain the output of plasma and memory cells during the months or years of established TD antibody responses.

1.4.2.2. T independent antigens

It has been suggested that while TD immune responses generate new antibody specificity by promoting somatic mutation, TI responses have been evolved to deal with antigens that have long been present in the environment for which germline genes exist and they therefore induce minimal or no germinal center formation and no memory response after booster immunization [(Weissman et al. 1976), (Lane et al. 1986)]. However later experiments by Gearhart et al. (1981) showed some degree of idiotypic diversification in secondary responses to certain TI-2 antigens. Later, TI-2 memory responses studied in a number of different systems, were found not only to be greater in magnitude than the primary responses, but also to display a different pattern of immunoglobulin isotypes (class switch from IgM to IgG or IgA) (Zhang et al. 1988).

1.5. THE AIMS OF THE STUDIES DESCRIBED

While much progress has been made in understanding the pathophysiology of HIV infection and the mechanisms associated with the immunity to polysaccharides, the susceptibility of HIV infected children to encapsulated bacteria with polysaccharide capsules remains poorly understood. Polysaccharide vaccines against *S pneumoniae* and *H influenzae*, if immunogenic, could reduce the morbidity and mortality from bacterial infections in paediatric HIV infected populations. Furthermore the study of immune responses to the TD and TI form of polysaccharide antigens could provide a further insight into the effects of the derangement of the B and T cell immunity observed in HIV infection.

In the studies described in this thesis, by investigating the immunogenicity of pneumococcal polysaccharide and Hib conjugate vaccines in a group of HIV infected children and uninfected controls, and monitoring persistence of immune responses over time, it was hoped that essential information for the establishment of realistic vaccination strategies in HIV infected paediatric populations could be obtained and an insight into the mechanisms responsible for the increased susceptibility of seropositive children, to bacteria with polysaccharide capsules may become apparent.

During the immunogenicity study of the 23-valent pneumococcal vaccine, by comparing immune responses to the whole vaccine (before and after adsorption with cps) and to specific serotypes, we had the opportunity to evaluate the effect that different methodological approaches had in the assessment of pneumococcal vaccine.

The assessment of vaccines has traditionally relied on the measurement of total serum antibody concentrations and to some extent on the assessment of isotype distribution of the antibody response. While recent evidence suggests that qualitative

aspects of the immune response may be responsible for inadequate protection of the vaccinated individuals from invasive disease, we further evaluated the immune responses to pneumococcal vaccine, by comparing the avidity of the produced antibodies in HIV infected children and controls.

Aspects of the project presented in the following chapters include:

1. Immunogenicity and IgG and IgG2 subclass responses to the 23-valent pneumococcal vaccine and Hib-T conjugate vaccine, in HIV infected children and uninfected born to HIV positive mothers and were used as controls.
2. Persistence of the immune responses to polysaccharide vaccines in the above populations.
3. A comparison of two different solid phase assays measuring pneumococcal antibodies for the assessment of immune responses to pneumococcal vaccine.
4. Evaluation of the pneumococcal antibody avidity in response to vaccination in HIV infected children and controls.

CHAPTER 2

Materials and General methods

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2

2.1 MATERIALS

2.1.1. General Reagents

Reagent		Supplier	Code
Ammonium thiocyanate		Sigma	A0302
Bovine serum albumin (BSA)		Sigma	A4503
Citric acid		BDH	10081
di-Sodium hydrogen orthophosphate		BDH	102494
Hydrogen peroxide		Sigma	H1009
o-Phenylenediamine		Sigma	P8287
Phosphate buffered saline (PBS) tablets		Unipath	BR14a
Sodium carbonate		BDH	10240
Sodium hydrogen carbonate		BDH	10247
Streptavidin peroxidase conjugate		Zymed	434323
Tween-20		BDH	66368
Sodium azide		BDH	1687
Cell wall polysaccharide		Statens Seruminstitut	
Pneumococcal polysaccharide	serotype 3	ATCC	169-x
	serotype 6B		181-x
	serotype 19F		205-x
	serotype 23F		217-x

2.1.2. Buffers

Coating Buffer (pH 9.6)	Sodium carbonate Sodium hydrogen carbonate Sodium azide	15mM (1.59g /litter) 35Mm (2.93g /litter) (0.2g/litre)
Citrate buffer (pH 5.2)	Citric acid	0.1M (21g /litter)
Phosphate buffered saline (pH 7.3)	Sodium chloride Potassium chloride di-Sodium hydrogen phosphate Potassium di-hydrogen phosphate	140mM (8g/litre) 2.7 mM (0.2g/litre) 8mM (1.15g/litre) 1.5 mM (0.2g/litre)
Wash buffer (PBS-Tw) (pH 7.3)	PBS /Tween 20	0.05%
OPD substrate (pH 5.2)	o-Phenylenediamine Citrate buffer Phosphate buffer 30% Hydrogen peroxide	10mg 10ml 10ml 10μl

2.1.3. Antibodies

Biotinylated monoclonal mouse anti-human IgG2 subclass and anti-total IgG antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA) and were of the following clones: IgG2: clone HP6002, total IgG: clone HP6045. The same set of antibodies were used in the development of the enzyme linked immunosorbent assay (ELISA) for measurement of antibody avidity.

2.1.4. Sources of Sera

Standard serum: Clotted blood from sixty apparently healthy adults was collected, centrifuged and the serum aliquoted and stored at -70° C. This pool was included in all assays and referred to as standard (std) serum. Antibody levels in an unknown serum, were extrapolated from a std curve derived from this std. Antibody concentration for total IgG (IgGT) was expressed in μg/ml, and for IgG2 subclass, as a percentage of the std.

Sources of clinical sera used in the individual studies are described in detail in the relevant chapters. The handling of sera from HIV infected children was done in appropriately designed hoods without previous pretreatment for removal of viral particles in order to avoid possible interference of the neutralization procedure with the accurate measurement of antibody levels.

2.2 METHODS

2.2.1. General ELISA technique for detection of antigen specific antibodies

Flat bottomed ELISA plates (Immulon 2, Dynatech,USA) were coated with 100μ l/well of the antigen of choice diluted in carbonate buffer and incubated for 72hrs at +4°C. Coating buffer was discarded, the plates were washed with wash buffer (PBS-Tw) once and used immediately. Aliquots of the test sera previously adsorbed for removal of cps antibodies (detailed description of the adsorption procedure in chapter 3) and serial dilutions of std diluted in 1% BSA in PBS-Tw were added to the wells (80μl/well) in duplicates; two wells were left empty of serum and used as blank. After 2 hours incubation at room temperature (RT), in humidified atmosphere, the sera were

discarded and the plates were washed four times in PBS-Tw. Biotinylated mouse anti-human IgG2 and IgGT monoclonal antibodies were diluted in 1% BSA with PBS-Tw, as follows: IgG2 at 1:4000 for all assayed serotypes and at 1:3000 for cps and IgGT at 1:3000 for serotypes 3 and 6B and 1:1000 for 19F, 23F and cps and added to the plates (80µl/well). After 2 hours incubation at RT, plates were washed three times in wash buffer and a 1:4000 dilution of peroxidase conjugated streptavidin (Zymed) was added (80µl/well). The plates were left for another hour at RT and washed three times. Substrate solution containing 0.5mg/ml o-phenylenediamine dihydrochloride (Sigma Chemical, UK) in a solution of 0.05% H_2O_2/0.1M citric acid/0.2M Na_2HPO_4 added on the plate (80µl/well) to develop the color of the assay. The reaction was stopped with 4M H_2SO_4 (40µl/well). Optical densities (OD) were measured at 492nm using an ELISA reader (Titretek Multiskan, Flow ICN, UK). Results were expressed as the average of the duplicates after subtraction of the background.

A range of dilutions of the std (1/10-1/2450 depending on the assay) was included in each plate and the results of the unknown sera were expressed as a percentage of this internal standard, for IgG2 subclass antibody levels whereas total IgG antibody concentrations were analyzed by a computer program (Titretek Multiscan) which derived the levels by extrapolating them from the log transformed std curve with known antibody concentrations.

2.2.2. Development of type-specific ELISAs for the measurement of antibodies against pneumococcal serotypes 3, 6B, 19F and 23F.

Type-specific ELISAs with specificity for PS3, 6B, 19F and 23F were developed for the measurement of IgGT and IgG2 subclass pneumococcal antibodies. Capsular

polysaccharides from PS3, 6B, 19F and 23F used as solid phase capture antigens in the assay, were obtained from the American Type Culture Collection (ATCC Rockville, Maryland, USA). Each ELISA plate included a std curve derived from std containing known concentrations of IgGT antibodies for all 4 assayed serotypes. Calibration of the std was done in collaboration with Dr D. Musher in Houston, and was found to contain the following amounts of IgGT antibodies:

PS3	33.67 μg/ml
PS6B	21.97 μg/ml
PS 19F	7.13 μg/ml
PS23F	7.34 μg/ml

Std curves were constructed by a series of five fourfold dilutions of the std (range 1:10- 1:2560), added in duplicates in each plate. Pairs of pre and post vaccination sera from each subject were applied in duplicates and analyzed on the same plate. All sera were tested after adsorption with 100μg/ml free cps antigen. The ELISA was performed as described in detail above. Duplicate results within a 10% variation were averaged. The lower detectable concentration for each tested serotype was taken as twice the background of the assay.

The levels of IgGT concentrations contained in the tested sera were extrapolated from the std curve and expressed in μg/ml. For IgG2 antibodies results were calculated by a computer program which generated a value for the tested samples on the basis of the std curve with an arbitrary value of 100 international units (IU).

2.2.2.1. Antigen binding to the solid phase

We compared different incubation times to achieve the optimal binding for all tested serotypes applied at coating concentration of 12.5μg/ml in the solid phase. As

shown in figure 2.1, incubation for 72 hours at +4°C, was found to give the best binding curves for IgGT antibodies to PS3. Similar incubation times were found to give the best binding for the other serotypes used as capture antigens (data not shown).

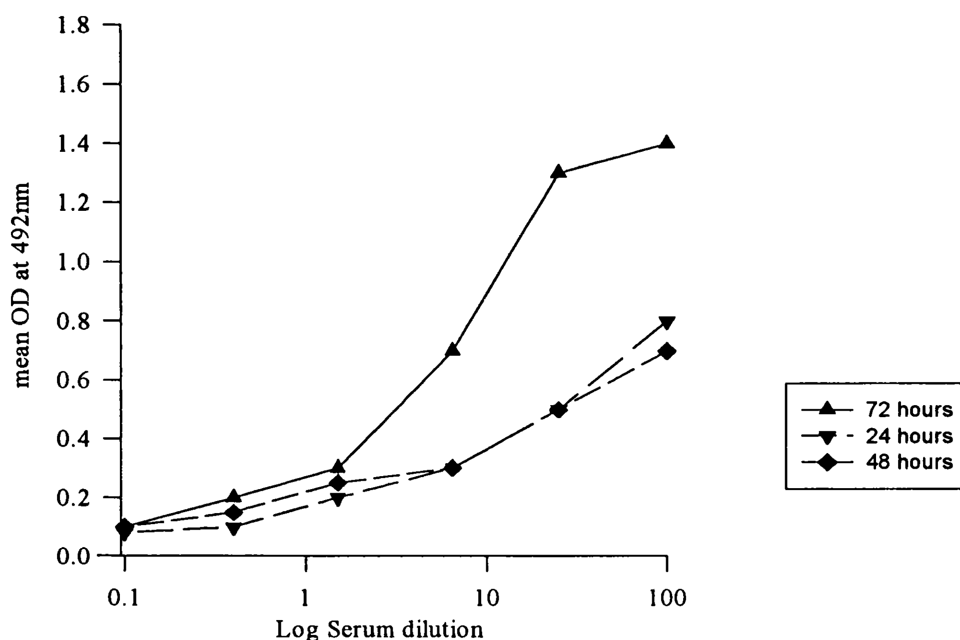

Figure 2.1: Antigen binding to the solid phase in type-specific ELISA.
Standard curves (dilution range from 1:10-1:2560) obtained from the binding of PS3, coating at 12.5 mg/ml and incubated for different times at +4°C.

2.2.2.2. Detector antibody

Different monoclonal antibodies were tested to achieve optical densities (OD) within a similar range (1.5-0.25) for all 4 serotypes and cps (data not shown). Monoclonal antibodies IgG2 HP6002 (Zymed) and IgGT HP6045 (Zymed), were found to give the best std curves at the following dilutions (see overleaf):

PS	IgG2	IgGT
3	1:4000	1:3000
6B	1:4000	1:3000
19F	1:4000	1:1000
23F	1:4000	1:1000

The IgG2 and IgGT std curves for all four serotypes are shown in Figure 2.2:

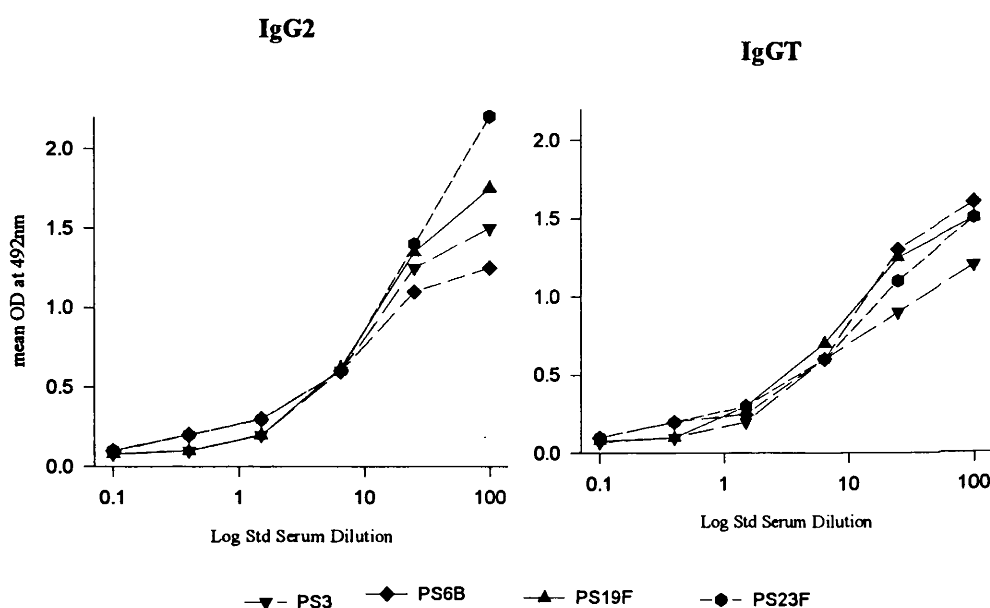

Figure 2.2: Standard curves for IgG2 and IgGT antibodies in serotype specific ELISAs.
Detector IgG2 and IgGT monoclonal antibodies were used in appropriate dilutions to give OD values within a similar range for PS3, 6B, 19F and 23F std curves.

2.2.2.3. Assay variation

Inter-assay and intra-assay variation were determined by testing a serum with known antibody concentration in 18 replicates in addition to the std and on the same batch of antigen coated plates, for three consecutive days. Results were expressed as the mean of coefficient of variation which was calculated according to the formula:

Coefficient of variation=*standard deviation/mean OD value x 100*

As shown below while the inter-assay variation was different for the five assays, it was below 20% for all assays.

PS	IgG2	IgGT
3	15.2	15.3
6B	5.3	14.3
19F	10.1	11.1
23F	14.4	3.6

Intra-assay variation was measured by testing a serum in 12 replicates on three different plates, together with a std curve. Coefficient of variation has been calculated as <9% for PS3 and 19F, < 6% for PS 6B and 23F.

2.2.3. Solid phase assays for measurement of avidity

2.2.3.1. Ammonium Thiocyanate elution ELISA

Antigen diluted in carbonate coating buffer (100μl/well) was adsorbed onto plates Immulon 2 (Dynatech, USA), for 72 hours at +4°C. The plates were then washed once with PBS-Tw. Test sera diluted in 1% BSA in PBS-Tw were added (80μ l/well) at a dilution predetermined to give an OD of approximately 1.0 and incubated for 2 hours at RT and in humidified atmosphere, to prevent evaporation. The plates were then washed four times with PBS-Tw. Ammonium Thiocynate (NH_4SCN) diluted in PBS-Tw-BSA at different molarities (0.1M to 5M) was added to appropriate wells (80 μl/well) leaving one set of wells with PBS-Tw, and left for 15 min at RT. The plates were washed four times with PBS-Tw and biotinylated murine anti-human antibodies (Zymed) were then added (80μl/well) and left for 2 hours at RT. After three

washings with PBS-Tw, peroxidase conjugated streptavidin (Zymed) in 1/4000 dilution, was added and left for 1 hour at RT. The plates then were washed three times with PBS-Tw before addition of substrate solution consisted of 0.5mg/ml o-phenylene diamine (OPD) in solution of 0.05% H_2O_2/0.1M citric acid/0.2M Na_2HPO_4. Color reaction was stopped with 4M H_2SO_4 and ODs of the wells read at 492nm using an ELISA reader (Titretek Multiskan, Flow).

Results were extrapolated by plotting the log % inhibition as derived from the change in OD at 492nm at the different molarities of NH_4SCN and the antibody avidity was expressed as the molarity required to cause 50% inhibition of binding to the solid phage called avidity index (AI).

2.2.3.2. Competition-inhibition ELISA

ELISA plates were coated with the antigen of choice in carbonate buffer (100μ l/well) and left for 72 hours at +4°C before the coating mixture was discarded and the plates washed once with PBS -Tw. Serial dilutions of free antigen in 1% BSA in PBS-Tw were added (40μl/well), leaving one set of wells empty. Same volume of the clinical sera (40μl/well), diluted to give a predetermined OD of 1.0, was added to each dilution of the free antigen in duplicates and the plates were incubated for 2 hours at 37°C. After washing, monoclonal antibodies were added and the ELISA processed as already described.

The log % inhibition in OD values was plotted against the free antigen concentration and the AI was expressed as the concentration required to cause 50% inhibition.

2.2.4. Development of an elution ELISA for measuring avidity of antibodies against pneumococcal serotypes 19F and 23F.

A modification of the NH_4SCN elution ELISA described above was employed for the measurement of avidity of anticapsular antibodies against PS19F and 23F. NH_4SCN in a concentration range from 0.5M to 4 M was added to a constant dilution of the test serum chosen to give an absorbance of approximately 1.0 and an ELISA was performed as described in detail above. The effect of NH_4SCN was demonstrated as a shift in the binding curve which was inversely correlated to the strength of the antigen-antibody binding.

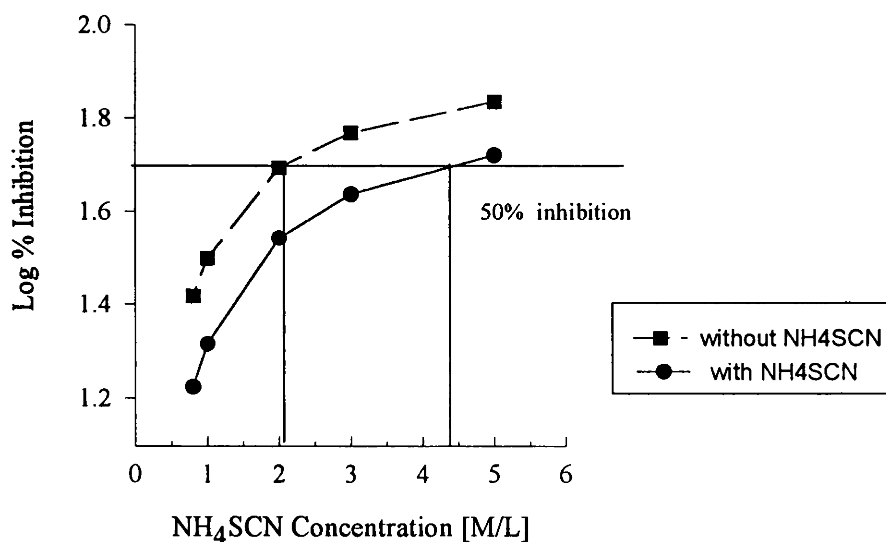

Figure 2.3: Ammonium thiocyanate elution ELISA for measurement of pneumococcal antibody avidity
The method has been applied for the estimation of the average PS19F antibody avidity in a clinical serum. The shift in the curve produced by the chaotropic agent is proportionally correlated to the avidity of PS19F antibodies in the serum tested. AI has been calculated as the 50% inhibition point after curve fitting

AI for the ranking of antibody avidity, was calculated as the log_{10} concentration of NH_4SCN which gave a 50% reduction in the adsorbance of the tested sera at 492nm (Figure 2.3). The 50% reduction point was estimated after curve fitting generated by a computer program (Sigma plot, Jandel Scientific). The interassay coefficient of variation was found to be 5.5% and 12.7% for PS19F and 23F assays respectively.

CHAPTER 3

Immunogenicity of the 23-valent pneumococcal vaccine and *H influenzae* type b conjugate vaccine in children with HIV infection

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3

3.1. Introduction

Severe or recurrent bacterial infections with bacteria containing polysaccharide capsules are common among HIV infected paediatric populations and have shown to be the first indicator of the disease in 16% to 23% of annual paediatric AIDS reports in US between 1989 and 1992 (CDC,1994). Data from prospective trials of placebo-controlled intravenous immunoglobulin therapy (IVIg), suggest that approximately 20% of HIV infected children with low CD4 counts have a serious bacterial infection during a 2 year period (Spector et al. 1995) while otitis media has been reported to be 3 times more frequent in HIV infected than in uninfected (U) children (Barnett et al. 1992).

Epidemiological data from relevant studies have shown that *S pneumoniae* and Hib are the main causative agents of bacterial infections, and prophylactic antibiotics, IVIg infusions in association with the universal administration of polysaccharide vaccines in all HIV infected individuals, has been recommended for their prophylaxis (Mofensen and Spector 1994). However, as already discussed in detail (see chapter 1) there is a disagreement regarding the immunogenicity of pneumococcal vaccine in symptomatic seropositive adults while no immunogenicity studies of sufficient size in HIV infected paediatric populations, have been published. Furthermore, although the

protein-conjugated Hib vaccines have shown improved immunogenicity in children with conditions associated with impaired responses to polysaccharides, it is possible that the functional impairment and gradual depletion of CD4+ lymphocytes observed in HIV infection could minimize the T cell-related enhanced immunogenicity in seropositive populations.

To assess the immunogenicity of conjugated and unconjugated polysaccharide vaccines in HIV infected children, we compared antibody responses to the 23-valent pneumococcal vaccine and the Hib tetanus conjugate (ActHib) vaccine in vertically infected (VI) children at different stages of the disease and U children born to HIV positive women. In order to have a detailed assessment of the immunogenicity of the 23-valent pneumococcal vaccine in our study population, we measured IgGT and IgG2 subclass immune responses against PS3, 6B, 19F and 23F, all included in the vaccine and reported as commonly causing pneumococcal disease. In addition we monitored adverse reactions associated with the administration of the vaccines.

3.2. Study Population

HIV infected children and controls were recruited from 2 centers: The Hospital for Sick Children, Great Ormond Street, UK and the Instituto di Clinica Paediatrica, Italy. The Hospital for Sick Children, is a tertiary paediatric referral center, and has established an HIV family outpatient clinic following up HIV infected and U children born to HIV infected mothers and referred from all parts of UK. Half of the children for the study were recruited here and the remaining from those attending the Instituto di Clinica Paediatrica in Padua, Italy. Both centers participate in the European Collaborative Study, an ongoing European prospective study on the natural history of

paediatric HIV disease (Ades et al. 1991). The vaccine study protocol received ethical approval from the ethical committes of both hospitals. Written informed consent was obtained from all parents and children, where appropriate, prior to participation.

Children VI and U born to HIV infected mothers were eligible for the study. VI children were classified at enrollment into 3 groups: AIDS, symptomatic and asymptomatic. Children with mild lymphocytic intestitial pneumonitis (LIP) were excluded from the AIDS group and put into the category of symptomatic HIV infection. Children with lymphadenopathy, hepatomegaly and/or HIV related thrombocytopenia only, were assigned to the asymptomatic group.

3.2.1. Schedule of Immunization

All children more than 6 months of age were offered 0.5 ml of the 23-valent pneumococcal vaccine (Pneumovax II, Morson) subcutaneously, those 6-18 months of age at recruitment received 2 doses, 6 months apart and those older than 18 months a single dose. When the study started, Hib vaccine was not routinely given to infants or children in UK or Italy. Children older than 18 months of age were immunized with a single dose of ActHIB vaccine (Pasteur-Merieux), whereas infants less than 6 months of age received 3 doses of ActHIB at monthly intervals coinciding when possible with other routine immunizations; children aged 6-18 months at the time of first dose received 2 doses one month apart and a third dose at 18 months. Adverse reactions associated with the administration of the vaccine were monitored by the parents.

Information on HIV infection status, clinical disease status and history of previous bacterial infections were recorded at recruitment. Parents were asked to record local or systemic adverse reactions in the 72 hours following immunization.

Blood was taken for measurement of T lymphocyte cell subsets and antibody was taken pre vaccination and 4 weeks after completing the immunization schedule in both VI and U children. In VI children receiving monthly IVIg as prophylaxis against bacterial infections, immunizations were given 4-5 weeks after the last IVIg and a dose of IVIg was omitted. Post-immunization bloods were taken before the next infusion of IVIg.

3.3. Methods

3.3.1. Measurement of type specific anti-pneumococcal antibodies

Anti-pneumococcal IgG2 and IgGT capsular antibodies against PS3, 6B 19F and 23F were measured by the ELISA described in detail in chapter 2, after serum adsorption for removal of cps antibodies. The adsorption of serum with free cps antigen can eliminate the interference of cps antibodies in the measurement of type specific antibodies (see also in chapter 5). We tested different concentrations of free cps antigen derived from an unencapsulated mutant of *S pneumoniae* (Statenserum Institut, Denmark) in our std pool (previously shown to contain anti cps antibodies) and we found that by adding 100μg/ml free cps antigen and incubating ON at +4°C, we could elute completely the cps binding on the solid phase (Figure 3.1).

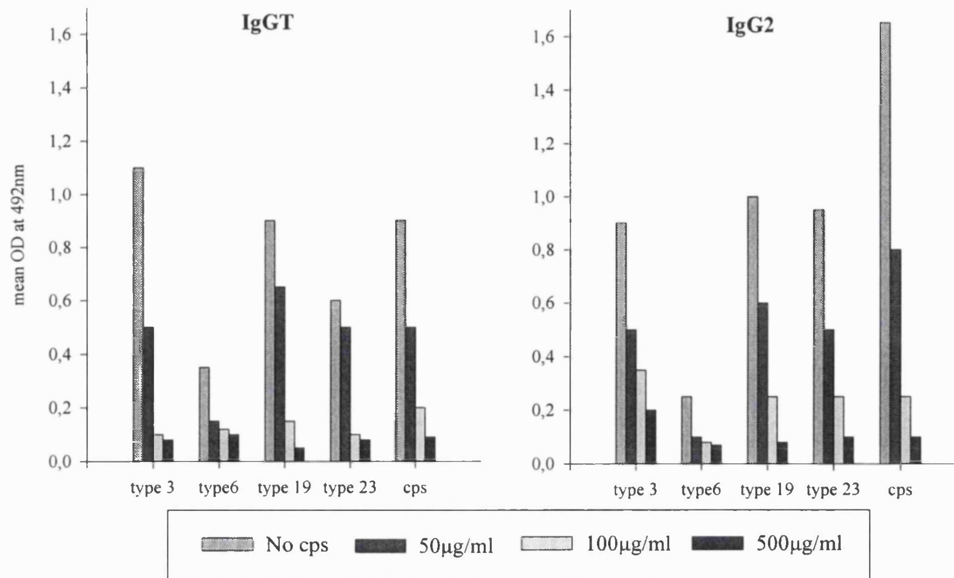

Figure3.1: Serum adsorption of IgGT and IgG2 cell wall polysaccharide antibodies

Different amounts of free cps antigen were added to the std serum (diluted at 1:40) Addition of 100μg/ml free cps antigen was sufficient to abolish completely the binding of IgGT (a) and IgG2 (b) cps antibodies to the solid phase.

While the dilution curves from the test sera were approximately parallel both to each other and to the std curve, in all serotype specific ELISAs, some sera taken from children younger than 2.5 years, showed lack of parallelism with the std curve (Figure 3.2). However since 95 % of children included in our study were however older than 2.5 years, the lack of parallelism was not a significant problem.

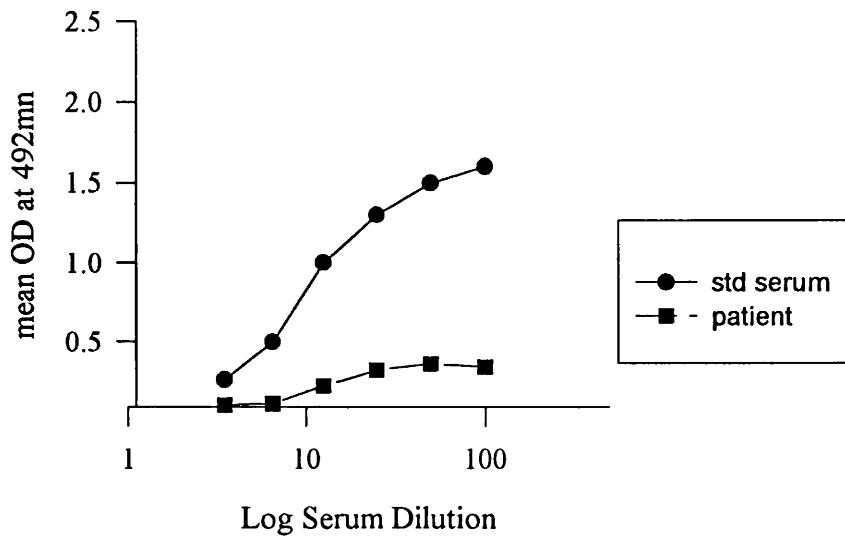

Figure 3.2: Comparison of dilution curve from a young patient's serum with standard curve
The curve obtained from serial dilutions of serum taken from a child aged 19 months shows lack of parallelism with the std curve.

Many of the seropositive children were on prophylactic IVIg therapy for bacterial infections. To investigate if IVIg preparations contain pneumococcal antibodies that could interfere with the immune responses to vaccination and the accurate measurement of antibody levels we tested three different IVIg samples (diluted at 5g/l), before and after adsorption with cps. As shown in Figure 3.3, the test sample contained considerable amounts of IgG2 and IgGT antibodies for some serotypes which were not completely eliminated after cps adsorption. Similar results were obtained from the other two samples (results not shown).

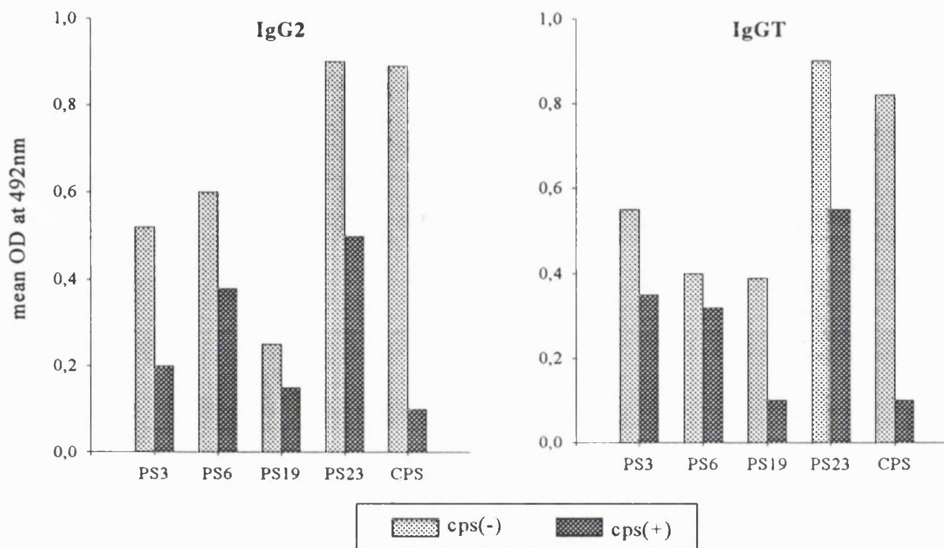

Figure 3.3: IgG2 and IgGT pneumococcal antibodies in intravenous immunoglobuln preparations before and after adsorption with cell wall polysaccharide antigen

Detectable amounts of IgG2 and IgGT pneumococcal antibodies against serotypes 3, 6B, 19F and 23F were found in the IVIg sample (diluted at 1:40) before and after adsorption with 100μg/ml free cps antigen.

3.3.2. Measurement of anti-PRP antibodies and T cell subsets

Antibodies against PRP were measured in Oxford, at the Immunology laboratory of John Radcliff Hospital which participates in the European quality control scheme for the standardization of measurement of PRP antibodies. The ELISA employed used PRP (Pasteur-Merieux Serum et Vaccins, France) as capture antigen, coupled to poly-L-lysine, according to the method described by Gray et al. (1979). The assay was performed according to the general ELISA technique described in chapter 2. A log linear std curve was plotted with antibody concentration against OD, from which the antibody concentration of the test samples were calculated. The lower limit of sensitivity of the assay was 0.16 μg/ml.

Measurement of T cell subsets was done by flow cytometry.

3.4. Statistical Power and Analysis

Assuming that approximately 70% of immunocompetent children respond to Pneumovax, a reduction to 40% in the VI children would be detected with 48 children in each group with an alpha error of 0.05 and a beta error of 0.20. Estimation of sample size with the use of the variance in antibody response in normal children (Booy et al, 1992), suggested that a total of 50 VI and 50 U children would allow the detection of at least a 2.5-fold difference in mean anti-PRP antibody titer with the same alpha and beta error.

Statistical analyses were performed using the statistical packages Statistical Analysis System (SAS). Egret was used for logistic regression. As T cell subsets in children vary with age, CD4 counts and percentages were expressed as standard deviation scores (Z score), based on age related values from U children born to HIV positive mothers (European Collaborative Study, 1992).

The number of children responding to one or more of the serotypes was determined in VI and U children. Overall response to Pneumovax was defined as a 2 fold or greater increase in IgGT or IgG2 subclass to at least one of the four (3, 6B, 19F, 23F) assayed serotypes 4 weeks post completion of the vaccination schedule. A concentration of anti-PRP antibody of 1μg/ml was taken as indicating long term protection.

Overall response defined as the ratio of post/pre immunization titer, was compared using a chi square test and a logistic regression model, allowing for age, ethnicity, IVIg and AZT (azidothymidine) therapy.

Absolute anti-PRP antibody titers and post vaccination fold increase of antibody levels (as defined above) were positively skewed and therefore log-transformed for the analysis. The proportions of children with post-immunization antibody levels >1μg/ml in the groups of VI and U, were compared using the chi-square test. The increase in antibody titers in these two groups was also compared using a regression model allowing for age and ethnicity and in the VI group, for IVIg and AZT treatment.

3.5. Results

A total of 67 VI and 57 U children born to HIV positive mothers were immunized, of whom all but 8 VI and 4 U children were more than 18 months of age. Adequate pre and post-immunization serum samples were obtained from all but 24 children (11 VI, 13 U). This group included infants who had interruptions of their schedule either because of illness (in 5 of the 8 VI infants) or missed appointments (4 U infants). The immunogenicity analysis was therefore restricted to VI and U children more than 18 months of age from whom there were adequate serum samples (56 VI, 44 U children).

3.5.1. Baseline Characteristics

The age distribution in VI and U children was similar. This together with numbers of VI children in each disease stage is shown in Table 3.1. More U (51/53) children were Caucasian. In the 18 VI children receiving regular IVIg therapy, the median time between their last IVIg infusion and immunization was 32 days (range 23-68 days). The median interval between immunization and measurement of post-immunization antibodies was 35 days (range 23-98) in VI and 37 days (range 21-91) in

U children. Local adverse reactions to Pneumovax II were reported in 4 (6%) of VI and 3 (5%) of U children. A further 5 (7%) VI and 2 (4%) U children had a mild fever in the 24 hours following the administration of the vaccine.

Table 3.1: Characteristics of HIV infected and uninfected children

Clinical status	No of children	Age	CD4 Z score Median Range	Ethnicity* Non Caucasian	IVIg	AZT
Total HIV +ve	56	4.4 (1.7-13.6)		23 (41) †	18 (32)	23 (41)
AIDS	13	4.2 (2.5-13.6)	-5.8 (<-7 to -1.7)	7 (54)	8 (62)	12 (92)
Symptomatic	24	4.1 (1.7-8.1)	-3.0 (< -7 to + 0.7)	12 (50)	6 (25)	7(29)
Asymptomatic	19	5.9 (1.8-10.1)	-2.4 (<-7 to +0.3)	4 (21)	4 (21)	4(21)
Uninfected	44	4.9 (1.7-9.5)	+0.24 (-3.0 to +1.8)	2 (5)		

*all but one of the non-Caucasian children were Black African

† Numbers in parentheses are percent

3.5.2. Response to pneumococcal vaccine

The immunogenicity of all assayed serotypes was similar amongst U and VI children with PS19F and 23F being the most and PS6 the least immunogenic.

41% of U and 59% of VI children had no antibodies to 1 or more serotypes (p=0.07). Post vaccination, 61% of U and 54% of VI children responded to 1 or more of the 4 assayed serotypes (p=0.4). However, 45% of the U compared with only 20% of VI responded to 2 or more serotypes. IgG2 subclass responses in relation to the number of responded serotypes, were similar to total IgG responses in the two groups of children where 26/56 (27%) of the VI and 42/44 (48%) of U children responded to 1 or more of the assayed serotypes, with IgG2 subclass serotype specific

pneumococcal antibodies. However 20.5% of the U compared with only 7% of VI, responded to 2 or more serotypes (Table 3.2).

Table 3.2: IgGT and IgG2 responses* to pneumococcal vaccine according to the number of serotypes responded.

Serotypes	IgGT		IgG2	
	VI(%)	U(%)	VI (%)	U(%)
None	26 (46)	17 (39)	41 (73)	23 (52)
1 or more	29 (54)	27 (61)	15 (27)	21 (48)
1	19 (34)	7 (16)	11 (20)	12 (27)
2	4 (7)	14 (32)	4 (7)	7 (16)
3	3 (7)	5 (11)	0 (0)	2 (4.5)
4	3 (6)	1 (2)	0 (0)	0 (0)
Total	**56 (100)**	**44 (100)**	**56 (100)**	**44 (100)**

* Defined as a 2 or more fold increase in post vaccination titer

A comparison between IgGT and IgG2 immune responses to Pneumovax, revealed that most of the U children (39%) responded with both IgG2 and IgGT subclass immune responses to the vaccine while the majority of the VI children (32%) responded with IgGT type specific pneumococcal antibodies only (Table 3.3).

Table 3.3: IgGT and IgG2 subclass immune responses to pneumococcal vaccine

IgG Response	VI Children (%)	U Children (%)
None	23 (41)	13 (29)
IgGT only	17 (32)	10 (23)
IgG2 only*	3 (5)	4 (9)
IgG2 and IgGT	12 (22)	17 (39)
Total	**56 (100)**	**44 (100)**

* the 7 children who gave IgG2 subclass response only, had a nearly two fold increase in the post vaccination IgGT levels to at least one of the 4 assayed serotypes.

In a logistic regression model allowing for age and ethnicity, the U children had a 1.5-fold (95% CI 0.6-3.8) adjusted relative response compared with VI children. Relative response allowing for age, ethnicity and IVIg therapy in children with AIDS compared with those who were asymptomatic was lower but the trend was not significant ($p>0.05$). There was no association between immune response and AZT therapy and between IgG2 subclass responses and age of the vaccinated children.

3.5.3. Response to ActHib vaccine

The vaccine was well tolerated and the only adverse reaction reported was a mild local reaction in the site of immunization in a VI child.

Prior to immunization, all but one VI and 5 U children had anti-PRP antibody titers above 0.15µg/ml which has been associated with short-term protection (Booy et al. 1992) whereas 21% U and 20% VI children, had anti-PRP antibody levels >1µg/ml, indicating long term protection. Amongst the VI children, those with long term protective titers were evenly distributed between children with AIDS, symptomatic and asymptomatic disease and were not significantly different in infected children who had received IVIg a month or more previously (7/38, 18%) compared with those who had not (5/18, 27%) ($p=0.5$).

Caucasian children responded in a similar way to ActHib, although VI children had a reduced response to the vaccine. While 100% of U children acquired anti-PRP levels >1µg/ml post immunization, this was achieved in only 86% of VI children ($p=0.008$). The geometric mean increase in anti-PRP antibody was 7.6 (95% CI: 3.5-16.3) times higher in U than in VI children ($p<0.001$) after adjusting for age and ethnicity (Table 3.4).

Table 3.4: Anti-PRP antibody titers*1 month after ActHIB immunization.

	Mean Fold increase	Relative Crude	Response Adjusted**	*p*
Infection status				
HIV infected	7.6	1.0	1.0	
Uninfected	73.3	9.6	7.6 (3.5-16.3)†	**0.0001**
Age		0.99	1.03(0.9-1.2)	0.7
Ethnicity				
Black	5.9	1.0	1.0	
White	31.2	5.6	2.0 (0.8-4.9)	0.11

* Geometric mean (post/pre immunization levels)
** Responses were adjusted for age and ethnicity
†Numbers in parentheses, range.

There was a significant trend towards decreased responses among patients with progressed disease compared with those observed in asymptomatic children which was not significantly affected after controlling for age, ethnicity and IVIg therapy (Table 3.5).

Table 3.5: Immune responses to ActHIB* in HIV infected children by clinical disease status.

	Mean Fold increase	**Relative** Crude	**Response** Adjusted**	**p**
Disease status				
AIDS	3.0	1.0	1.0	
Symptomatic	5.6	1.9	3.0 (1.2-8.1)†	
Asymptomatic	20.4	6.8	3.8 (1.1-13.4)	0.05
Age (per year)		0.99	1.03 (0.88-1.2)	0.7
Ethnicity				
Black	9.3	1.0	1.0	
White	5.7	0.6	0.7 (0.27-1.7)	0.4
IVIg				
Yes	3.6	1.0	1.0	
No	11.1	3.1	2.7 (0.98-7.4)	0.06

* Geometric mean (post/pre immunisation anti PRP levels)
** Responses were adjusted for age, ethnicity and IVIg therapy
† Numbers in parentheses, range.

No relationship was observed between age-adjusted CD4 counts and response to ActHib when either all children (p=0.9) or only VI children (p=0.5) were included in the regression model (Figure 3.4). In addition, no relationship was observed between response and AZT therapy after adjusting for disease stage (p=0.9).

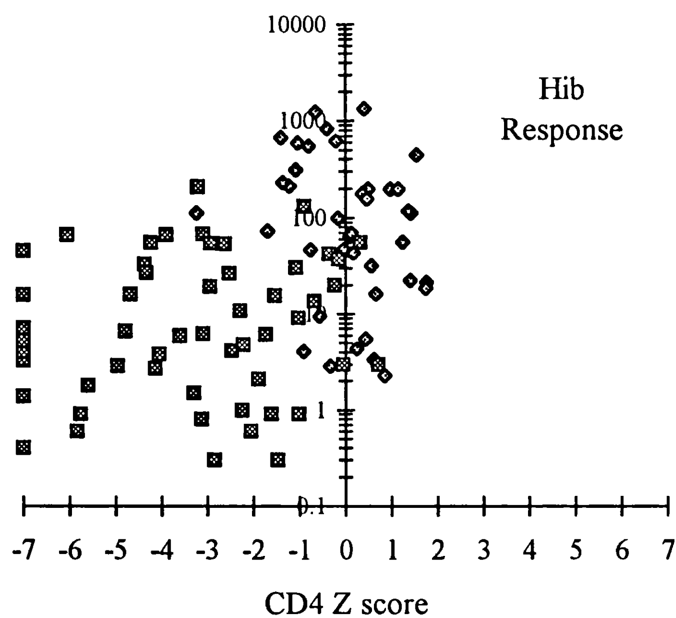

Figure 3.4: Correlation between age-adjusted CD4 counts and response to ActHib
Hib response (post/pre immunization levels) on a log scale in HIV infected (■) and uninfected (◆) children, plotted against CD4 counts expressed as an age adjusted standard deviation score (Z score) based on CD4 counts in uninfected children born to HIV positive women.

3.6. Discussion

We evaluated the immunogenicity of polysaccharide vaccines in VI and U children born to HIV positive mothers by measuring type specific antibodies to 4 clinically relevant serotypes after removal of the non-specific cps antibodies and anti PRP antibodies.

VI children were found to have naturally occurring pneumococcal antibodies against fewer serotypes, compared to the controls, a finding that could be attributed either to the effect of the virus on the development of natural immunity to pneumococcus, or to the attrition of the immune system with progression of the disease. The immunogenicity of pneumococcal vaccine, was relatively low in all vaccinated children, as expected for a polysaccharide vaccine. We could not establish statistically significant differences in antibody responses between VI children and

controls. However, more detailed analysis showed that the group of U responded to more serotypes than the VI children.

The pattern of immunogenicity of the 4 assayed serotypes was similar in the VI and U children. In addition we could not establish statistically significant differences in the IgG2 responses to the four serotypes between infected and U children although overall IgG2 responses were higher in the U children, providing evidence that the underlying mechanism for the increased susceptibility of HIV infected children to encapsulated organisms may not lie solely in their inability to produce specific IgG2 antibodies as has been suggested by Parkin et al. (1989) who found that low total IgG2 levels in HIV infected individuals. In our study we did not measure total serum IgG2 values however we consider it as a poor marker of the overall ability of the immune system to produce antigen-specific IgG subclass responses, a more sensitive marker being the production of appropriate antibody subclass responses to immunization or infection (Shackelford et al. 1990).

All U children responded to the ActHib vaccine, which is in accordance with previous reports for the improved immunogenicity of conjugate Hib vaccine in young children. However the HIV infected children gave significantly impaired responses in comparison with the controls, and only 86% of them obtained long term protective (>1μg/ml) post vaccination antibody titers. Similar findings have been reported by Steinhoff et al. (1991) in seropositive adults vaccinated with conjugate or unconjugated Hib vaccine. Although the percentage of men achieving titers >1μg/ml following immunization is not stated in the paper, the fold (post/pre) responses were significantly lower in HIV infected than in U individuals and those with AIDS had significantly lower responses than those with asymptomatic disease. Similar studies in

HIV infected paediatric populations are small and show contradictory results. Indacochea et al. (1992), reported that infected and U children born to HIV positive mothers gave similar antibody responses to HbOC. A further study by Walter et al. (1992) reported significantly lower anti-PRP antibody concentrations following 2 doses of PRP-OMP vaccine in 9 HIV infected infants (GMC 0.46 μg/ml) compared with 12 U infants (GMC 4.06 μg/ml) whereas only 2/9 HIV-infected and 7/12 U infants achieved titers >1μg/ml. In our study although similar differences in response were observed between HIV infected and U children, the immunogenicity of the vaccine and the post vaccination antibody levels were much higher in both groups. These differences could be attributed to the older age of our study population and the different cojugate vaccine we used, as it is known that antibody responses to the different Hib conjugates vary in immunogenicity and avidity of the produced antibodies (Schlesinger et al. 1992).

The possibility that ActHIB does not behave as a TD antigen in HIV infected children, as has been suggested by Peters and Sood (1997), is not considered possible since the response to the vaccine given by U and VI children, was much higher than what somebody could expect for a polysaccharide vaccine in young children. Furthermore differences in anti-PRP responses between HIV-infected and U children could be explained on the basis of impaired cellular immunity. However although the magnitude of immune responses to vaccination were significantly related to the disease stage, we could not establish a linear correlation between antibody responses to the vaccine with the age adjusted CD4 counts in infected children. Kale et al. (1995) had also shown reduced anti PRP responses only in HIV infected children with CD4 counts <100 x 10^6/L. In contrast, Steinhoff et al. (1991) observed a direct correlation between

CD4 counts and antibody responses to conjugate Hib vaccine in HIV infected adults. These discrepancies could be explained by the relative lack of a clear-cut association between CD4 counts and the progression of paediatric HIV infection (European Collaborative Study, 1994). A recent study by Kroon et al. (1997) who have shown that adults patients with CD4 counts between 100-300x 10^6 cells/L respond to Hib conjugate vaccine equally well as asymptomatics and controls, may indicate that the presently used antiretroviral regimens with protease inhibitors which preserve CD4 cell function, may have favorable effects on the antibody response to conjugate polysaccharide vaccines.

It has been reported that genetic factors may affect the immune responses to Hib vaccine and Km(1) or G2m(n) immunoglobulin allotypes have been associated with increased immunogenicity of the Hib vaccine (Granoff and Holmes, 1992). Rodriguez-Barradas et al. (1996) have shown that responsiveness of HIV infected adults to pneumococcal vaccine was significantly associated with the Km(1) negative allotype. The possibility that the observed differences in immune responses between infected and U children could be attributed to genetic factors because most of our infected children were black Africans while the majority of the controls were Caucasians, was excluded from our regression model allowing for ethnicity. These discrepancies could be a futher evidence that the role of genetic allotypes on immune responses to polysacharides is not so important in childhood (see chapter 1).

Children on IVIg treatment showed lower responses to both vaccines, although differences were not statistically significant in the multivariate model after allowing for disease stage, age and ethnicity. Since we have shown that intravenous immunoglobulin preparations contain pneumococcal antibodies, this could be

attributed to the attenuation of the antigenic stimulation even 5 weeks following the last IVIg infusion. In our study, children were vaccinated on average nearly 5 weeks following their last IVIg infusion and received no further infusion for another 4 weeks. Since the titers of specific antibodies in different IVIg preparations may vary up to four times, these children might benefit from a longer interval between the last IVIg infusion and vaccine administration. Our findings are in accordance with Siber 's suggestions for interruption of IVIg treatment for as long as 3 months before vaccination especially when larger doses of IVIg (40 to 80 mg/kg) are used.

In addition we observed no differences in the immunogenicity of both vaccines in children on AZT, after controlling for disease status. This contrasts with a small study by Glaser et al (1991), suggesting that AZT might increase antibody responses to vaccines. However in our study, immune responses to pneumococcal vaccine were not significantly impaired and anti-PRP prevaccination titers were similar in VI and U children, an indication that in most of the infected children, the disease had not been progressed causing attrition of their immune system. In contrast in Glaser's study, immunogenicity of the pneumococcal vaccine was greatly impaired in the study population, indicating that antiretroviral therapy may improve immune responses in more advanced stages of the disease.

In conclusion, our data on the immunogenicity of polysaccharide vaccines support the policy of vaccinating with both vaccines, all HIV infected children more than 18 months of age. Even children with AIDS may mount adequate antibody responses, hence the stage of disease should not be a contraindication to immunization.

Although we have not reported data on children less than 18 months of age, adequate immunogenicity of all conjugate Hib vaccines has been well documented in

immunocompetent infants of that age and early immunization of HIV infected children with Hib vaccine may well be appropriate. However further research is required to clarify the need for and optimal timing of reimmunisation when the vaccine is given soon after birth.

In contrast, alternative strategies are probably required for the prevention of invasive pneumococcal disease in very young children. Administration of prophylactic penicillin although has the disadvantage of the development of drug-resistant strains will be undoubtedly beneficial for preventing infections in age groups. A study by Peters et al. (1995), confirmed the beneficial prophylactic effects of IVIg in seropositive children. Finally the introduction of the new and more immunogenic conjugate pneumococcal vaccine formulations may provide an optimal opportunity for a better protection of HIV infected children in the future (for more discussion see chapter 7).

CHAPTER 4

Persistence of antibody responses to the *23-valent pneumococcal* and *H. influenzae type b* conjugate vaccine, in children with HIV infection.

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4

4.1. Introduction

While the results of the immunogenicity study of the 23-valent pneumococcal vaccine and Hib-T conjugate vaccine in HIV infected children and U born to HIV infected mothers, revealed that both vaccines were immunogenic in seropositive children, post vaccination titers obtained by both vaccines were lower in the infected population than in the controls. For Hib vaccine, the magnitude of the immune response was 7.6 fold lower in VI than in U children, raising concerns about the longevity of protection, since as already discussed, anti-PRP antibody > 1μg/ml is thought to be critical for the long-term protection against invasive Hib disease.

Little is known regarding the persistence of immune responses to polysaccharide vaccines in relation to the titers obtained post vaccination. In this chapter, the results of anti-pneumococcal and anti-PRP antibody monitoring, 12 months after the administration of the vaccines in the population described in chapter 3 are reported. It was hoped that the monitoring of antibody levels, could provide essential information for deciding on the need for and timing of booster doses of both vaccines.

4.2. Follow-up of the study population

Of the original study population described in chapter 3 [56 VI infected and 44 U children, born to HIV infected women] who received Hib-tetanus conjugate vaccine (ActHIB, Merieux) and 23-valent pneumococcal vaccine (Pneumovax II, Morson), a total of 48 VI and 36 U children had samples taken approximately 1 year later. Forty one VI and 9 U children also had anti-PRP and antipneumococcal antibodies measured 6 months after immunization.

All children were over 15 months of age and were prospectively classified into 3 groups: AIDS, symptomatic and asymptomatic, according to the CDC criteria. The vaccination schedule was described in chapter 3. Information on HIV clinical disease status in VI children were recorded at 6 and 12 months post-immunization. Blood for measurement of antibody levels and T lymphocyte subsets on all children was collected at the same time. In VI children receiving monthly IVIg therapy as prophylaxis against bacterial infections, blood was drawn 4 weeks after the last infusion.

4.3. Methods

Type-specific pneumococcal antibodies, anti-PRP and T lymphocyte subsets were measured as described in chapter 3.

4.4. Statistical Analysis

Anti pneumococcal antibody titers and changes in anti-PRP antibody levels defined as the 1 month divided by the 12 month post immunization titer, were positively skewed and were therefore log transformed prior to analysis.

CD4 counts were expressed as Z scores (described in chapter 3). Comparisons of pneumococcal GMC between U and VI children were performed using unpaired *t* test. The proportions of children with ≥0.15μg/ml and ≥1μg/ml antibody titers at different time points were compared in VI and U children using the chi square test. The rates of antibody titer decline were compared in VI and U children using a regression model allowing for age, ethnicity and the titer achieved one month post immunization. The rates of antibody decline in both groups, were compared with a regression model allowing for age. ethnicity and the titer achieved 1 month post immunization. In addition, in VI children rates of antibody decline were compared by disease status, age-adjusted baseline CD4 count and CD4 count change, allowing for IVIg or antiretroviral therapy received.

Statistical calculations were performed using SAS.

4.5. Results

Of the original 56 VI children, 48 were evaluated at a median of 419 (range 315-693) days after immunization. Five of the 8 remaining VI children who were immunized had died of AIDS and 3 were lost to follow up. Thirty six U children were evaluated a median of 393 (range 280-658) days (13.1 months) after immunization; eight U children had been lost to follow up or were reluctant to have blood taken. Details of disease status, age distribution and CD4 Z-scores at the time of immunization and changes in CD4 Z-score over the 12 month period of follow up are shown in table 4.1. More VI compared with U children were of black African origin. In the 19 VI children receiving IVIg therapy as prophylaxis against bacterial infections, the median time between their last IVIg infusion and the 12-month post-immunization evaluation was 32 days (range 28-175 days).

Table 4.1: Characteristics of vertically infected and uninfected children

Clinical Status at Immunization	No of children	Age at immunization Median Range	CD4 Z-Score Median Range	Fall in CD4 Z Score* Median Range	Ethnicity**	IVIg†	AZT†
Total HIV-infected	48	5.0 (1.7-13.6)	-6.0 (-80 to 0.38)	0.54 (-13.9 to 66.1)	23	19	17
AIDS	8	5.5 (1.7-13.6)	-20 (-80 to -2.6)	6.8 (-8.5 to 66.1)	3	7	8
Symptomatic	22	4.6 (1.7-8.1)	-3.8 (-12.7 to 0.38)	-0.93 (-13.9 to 5.5)	11	7	6
Asymptomatic	18	5.3 (1.8-10.1)	-3.0 (-15.4 to 0.28)	-0.7 (-2.6 to 1.0)	4	5	3
Uninfected	36	4.7 (1.7-9.5)	-0.16 (-3.6 to 1.7)	0.01 (-2.1 to 2.7)	2	-	-

* A negative sign denotes an *increase* in median CD4 Z-score

** All but one of the non-Caucasian children were Black African

†At time of immunization

4.5.1. Anti pneumococcal antibody levels-Changes with time

The monitoring of GMC for all 4 assayed PS, as measured in 36 U and 48 VI children who were followed up for 12 months post vaccination, are shown in table 4.2. There was a fall in antibody levels in the group of U children at 12 months for all except one serotypes. In contrast, in VI children, there was a continuous increase in antibody levels for all assayed serotypes at 12 months.

Table 4.2: Acquisition and persistence of IgGT antibody levels against pneumococcal serotypes 3, 6B, 19F and 23F

PS	Pre Immunization		1 Month Post		12 Months Post	
	U	VI	U	VI	U	VI
3	0.56	0.36	1.09	0.47	5.25	3.19
6B	0.27	0.13	0.23	0.16	0.25	0.43
19F	1.99	0.14*	14.10	0.28 *	6.16	1.77
23F	1.62	0.23*	8.12	0.64 *	6.30	1.77

*Differences between U and VI children, statistically significant *($p<0.001$)*

Similar patterns were found for IgG2 subclass responses for all 4 assayed serotypes as measured in the 12 months samples (Table 4.3). While one month post vaccination, IgG2 antibody levels were significantly lower in VI than in U children for 2 of the 4 assayed serotypes (19F and 23F) ($p<0.05$), the increase of antibody titers in the group of VI children at 12 months, resulted in a narrowing of the observed differences (table 4.3).

Table 4.3: Acquisition and persistence of IgG2 antibody levels against pneumococcal serotypes 3, 6B, 19F and 23F

PS	Pre Immunization		1Month Post		12 Months Post	
	U	VI	U	VI	U	VI
3	3.27	0.21	2.28	0.28	7.34	1.34
6B	0.14	0.11	0.15	0.08	0.60	0.32
19F	0.43	0.04	6.16	0.05*	4.17	0.27*
23F	0.84	0.06	3.64	0.13*	3.59	0.21

*Differences between U and VI, statistically significant *($p<0.05$)*

4.5.2. Anti PRP levels-Changes with time

All 36U and all but 1 VI children, at 12 months post immunization, had titers over 0.15 μg/ml, associated with short term protection. While however, one month post immunization, 100% of U and 87.5% of VI children had anti-PRP antibody titers >1 μg/ml, 12 months later, only 24 (57%) VI who had responded to the initial vaccination, compared with 32 (89%) U retained levels >1μg/ml associated with long term protection (chi-square 9.7, p=0.002) (Table 4.4). Nearly half (11/24) of VI children with long term protective titers at 12 months, were amongst the asymptomatics. The proportion of children with long term protective titers was not significantly different in infected children who were on IVIg prophylactic treatment from those who were not (p>0.05).

Table 4.4: Acquisition and persistence of protective* anti-PRP titers

Infection Status	1 month Post		1 year Post	
	GMC (μg/ml)	No(%) ≥1μg/ml	GMC (μg/ml)	No (%) ≥1μg/ml*
Total HIV-infected (n=48)	4.9	42 (88%)	1.2	24 (57%)
AIDS (n=8)	3.6	7 (88%)	0.9	4 (57%)
Symptomatic (n=22)	3.1	17 (77%)	0.8	9 (53%)
Asymptomatic (n=18)	10.0	18 (100%)	1.9	11 (61%)
HIV-uninfected (n=36)	38.3	36 (100%)	4.6	32 (89%)

* data refers only to the children who were ≥1μg/ml at 1 month.

As already reported in chapter 3, the geometric mean increase in anti-PRP antibody following immunization was 7.6 (95% CI 3.5-16.3) times higher in U compared with VI children after adjusting for age and ethnicity. The rate of fall from these post-immunization levels during the subsequent year was not related to age, ethnicity or whether a child was receiving IVIg therapy. It was greater by a factor of 1.4 (95% CI, 0.7-3.) in the U compared with the VI children after adjusting for these variables. However, there was a significant correlation between the 1 month and 1 year post immunization titers in both VI and U children (Figure 4.1). If the 1 month post immunization titers were also allowed for in the regression, the rate of fall was greater in VI than in U children, although this did not reach statistical significance (Table 4.5).

Table 4.5: Changes in anti-PRP IgG titers, 12 months post vaccination

	Mean Fold Fall*	Relative Response		p
		Crude	Adjusted	
Infection Status				
HIV -infected	4.3	1.0	1.0	
HIV-uninfected	8.3	1.92	0.6 (0.3-1.1)**	0.07
HIV-infected only				
Asymptomatic	5.3	1.32	0.99(0.23-4.31)	0.99
Symptomatic	3.7	0.7	0.7 (0.29-2.14)	0.63
AIDS	4.0	1.0	1.0	
Age		1.03	0.99 (0.95-1.03)	0.8
Ethnicity				
White	8.4	1.7	1.1 (0.6-2.2)	
Black	3.7	1.0	1.0	0.5
Post immunization titers		3.0	3.4 (2.4-4.9)	**0.0001**
IVIg therapy				
Yes	3.6	1.0	1.0	
No	6.5	1.8	1.2 (0.4 -3.2)	0.6

*Geometric mean (1month/12 months post-immunization levels)

** Numbers in parenthesis, range

Amongst VI children, the rate of fall in antibody levels was not significantly different in children with AIDS or symptomatic disease compared with those with asymptomatic disease at the time of immunization after adjusting for age, ethnicity, 1 month post immunization titers and whether or not a child was receiving IVIg. Similarly, neither the age adjusted CD4 count (CD4 Z-score) at the time of immunization, nor the rate of fall of age adjusted CD4 count (i.e. difference in CD4 Z-score) over the year following immunization were significantly related to the rate of fall of anti-PRP antibody titers in the VI children.

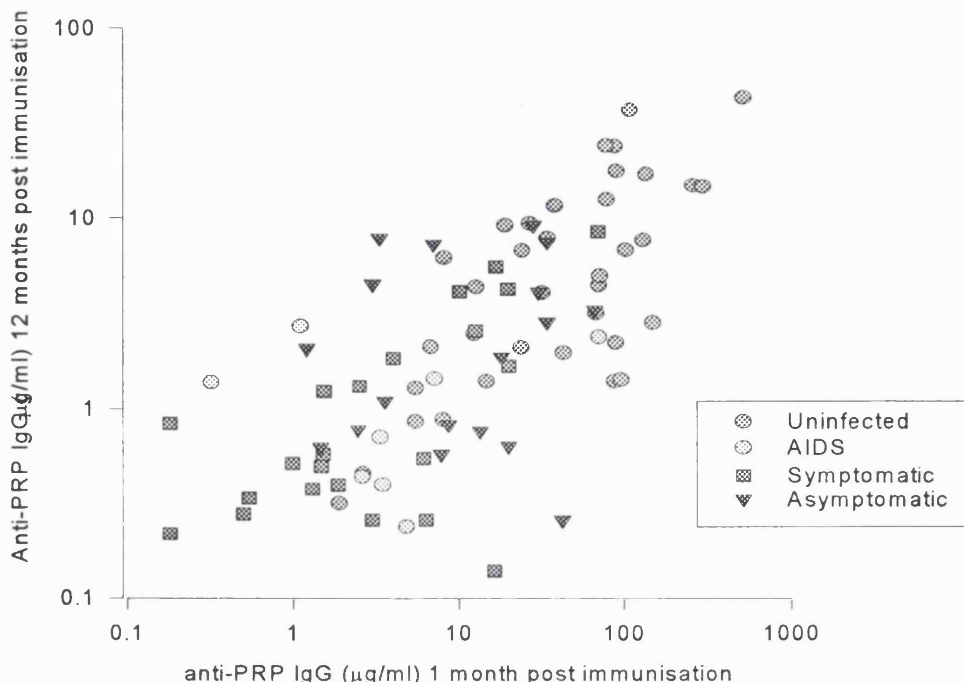

Figure 4.1: Correlation between the 1 month and 1 year post immunization anti-PRP titers in HIV infected children and controls.
Anti-PRP antibody levels measured 1 and 12 months after administration of ActHIB vaccine in 36 U and 48 VI patients at the time of immunization. There was a significant correlation between the 1 month and 1 year post vaccination titers.

4.6. Discussion

There were no differences between HIV infected children and U born to HIV infected mothers, on pneumococcal antibody levels 12 months after vaccination with the 23-valent pneumococcal vaccine. However mean antibody levels at 12 months post vaccination were much lower in HIV infected children for all assayed serotypes, than what has been reported in the elderly (Sankilampi et al. 1997). While it has been estimated that revaccination with the pneumococcal vaccine in the elderly is necessary every 3-4 years, this period is probably shorter in seropositive children.

Interestingly in the group of VI children, in contrast with the controls, there was a continuous increase in antibody levels during the whole follow up period. However while actual post GMC levels were much lower than in controls it is questionable if the observed differences are biologically significant. It is possible that the high sensitivity of our pneumococcal ELISAs (see chapter 5) enabled us to detect even small differences associated either with the hypergammaglobulinaemia observed in HIV infection or the IVIg treatment, some of these children were receiving as prophylaxis. Arpadi et al. (1994), reported no differences in antibody titers to 7 common pneumococcal capsular polysaccharides assayed 1 to 16 months post immunization between symptomatic children and age-matched unvaccinated HIV-infected and controls and concluded that pneumococcal vaccine does not confer long lasting immunity to seropositive children. However in that study, antibody levels had not been measured after the administration of the vaccine; while all vaccinated children except one were symptomatic, it is possible that these children failed to respond to the initial vaccination. In contrast, in our study nearly half of the infected children were asymptomatic and we have shown that the response to polysaccharide vaccines is better at the earlier stages of the disease.

While the minimum protective antipneumococcal antibody level from invasive disease is not known, it is premature to assume that the persistence of pneumococcal antibodies in seropositive children will lead to long lasting protection. Although Ragni et al (1987) have shown that none of the 24 vaccinated seropositive adults developed pneumococcal pneumonia in the year following the vaccination whereas there were 3 cases of pneumococcal disease in 60 HIV infected unvaccinated individuals during the

same period, the study was not of considerable size to establish statistically significant results.

Antibody responses to the Hib conjugate vaccine were preserved in HIV infected children and even the majority of children with AIDS were able to mount an antibody titer above the long term protective level of 1μg/ml immediately post immunization. Although the GMC of anti-PRP antibody one month post immunization was significantly higher in controls (7.6 fold) at 12 months, this difference had fallen to just under 2 fold. This narrowing of the difference in GMC is related to the more rapid decline in antibody titers in those with higher titers immediately post immunization. When allowing for the GMC values 1 month post immunization of the two study groups it can be seen that the rate of decline of specific IgG was similar in both groups and HIV infection did not appear to correlate with an increase in the turnover rate of specific IgG. This is in contrast to the findings and conclusions of Al-Attar et al. (1994) who studied the decline of measles antibody titers after the immunization of HIV infected children. They concluded that a major factor in the previously reported low prevalence of measles antibody in this population was loss of antibody after an initial response. Unfortunately, in their study, no control group was included. They found no significant association between CD4 counts at the time of vaccination and the slope of decline of measles antibody, a finding similar to that reported here.

The persistence of PRP antibody following vaccination has been studied by a number of groups although these studies have predominantly taken place in healthy infants who have received the vaccine in the first year of life [(Claesson et al. 1991), (Berkowitz et al, 1990), (Walter et al, 1994)]. In general, most of the studies demonstrate that one year following immunization with a Hib conjugate vaccine 100%

of healthy infants have maintained an antibody titter above the minimum protective level of 0.15μg/ml. Figures for those maintaining titers over 1μg/ml at one year range from 90-100%. Despite the difference in study designs, antibody persistence in our control group is similar to that in the published literature.

Peters and Sood (1994b) have studied the immunogenicity of Hib vaccine in HIV infected children given a single dose of PRP or PRP-conjugate vaccine. The 19 children described in their study received vaccines 15 to 56 months prior to analysis. No immediate postimmunisation serological data is presented so it is difficult to ascertain whether the low percentage of children with persisting antibody titers ≥1 μg/ml is due to loss of antibody over time or to an initial failure to seroconvert.

The relevance of the circulating anti-PRP antibody concentration as a surrogate marker of protection against Hib when immunity has been induced by a TD vaccine has been questioned (Kurikka et al, 1995). Protective antibody titers against invasive Hib disease were deduced from studies of a pure polysaccharide vaccine which, in contrast to the conjugate vaccines, does not induce memory (Peltola et al, 1977). In theory, even in the absence of circulating antibody, an individual primed with a conjugate vaccine should be protected from infection due to an ability to produce high avidity IgG (anamnestic response) rapidly following subsequent contact with the bacterium or cross-reacting antigen. While this may be true for vaccinees with a normal immune system, circulating specific antibody titers may be particularly important for the HIV infected individual in whom the anamnestic response may be impaired. This impairment may be due to a variety of factors including the reported loss of CD45RO positive memory cells (Borkowsky et al, 1992b).

In conclusion, HIV infection, did not seem to increase the turnover of antibody levels obtained after vaccination with polysaccharide vaccines; however while 12

months later, a smaller proportion of infected children had anti-PRP antibody levels associated with long term protection, it is possible that a booster dose is probably required in a shorter period of time. Further data are required on the longevity of the antibody response beyond this period, in order to allow the rational timing of any booster immunization. Furthermore the comparison of the boostability of antibody responses induced by polysaccharide conjugate vaccines, in VI children and controls, will give us an idea on the effects of HIV infection on the establishment of immunologic memory.

CHAPTER 5

A comparison of whole-vaccine and type-specific solid phase assays for the assessment of immune responses to pneumococcal vaccine

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5

5.1. Introduction

Natural and vaccine induced immunity to *S pneumoniae,* is associated with the production of antibodies specific for its capsular polysaccharide (see chapter 1). The accurate measurement of pneumococcal antibodies is of fundamental importance for the understanding of the nature of immunity to pneumococcal infection and the optimal evaluation of the currently available 23-valent pneumococcal vaccine. While quantitative precipitin analysis and Farr assay have been employed for the determination of pneumococcal antibodies in the past, at present the most commonly used assays are a modified radioimmunoassay (RIA) and ELISA. RIA developed by Schiffman et al. (1980) employs a known amount of radiolabeled antigen which reacts with the unknown antibody and the extent of primary binding of Ag to Ab is determined after separating bound and unbound antigen, typically by antibody precipitation with ammonium sulfate. However, RIA compared to ELISA, is a more labor intensive (since it requires labeling of the antigens) and therefore is more time consuming method, it requires the use of relatively large amounts of sera and most importantly, it does not distinguish between immunoglobulin classes and IgG subclasses.

Appropriately designed ELISAs, have become increasingly attractive, because of their convenience and their ability to measure specific IgG subclasses (Pedersen and Henrichsen 1982). This methodology is particularly appropriate for studying immune responses in children in whom delayed maturation of immune response to polysaccharides is expressed as an inability to produce an IgG2 subclass response following vaccination.

One of the ongoing complications of the pneumococcal ELISA is the contamination of the capsular polysaccharides used in the assay, with cps, proved by liquid chromatography (Musher et al. 1990a).

Cell wall is one of the three main components of *S pneumoniae,* located between the capsule and the plasma membrane of pneumococcus and is common to all serotypes. It is composed of peptidoglycan and techoid acid which is the most immunogenic component and induces antibodies *in vivo* against the cell wall. While it has been reported that cps antibodies are protective against pneumococcal infection in mice (Aaberge et al. 1992), there is little evidence for an *in vivo* protective activity from invasive pneumococcal disease in humans. Studies in patients with chronic bronchitis have shown that a substantial proportion of anti-pneumococcal antibodies induced by vaccination, were against the cps yet failed to protect patients from exacerbation of chronic bronchitis (Musher et al. 1990b). Furthermore studies in otitis media prone children have shown that they have significantly higher concentrations of anti-cps IgG in their middle ear effusions and the authors suggested that anti-cps antibodies may induce immune complex formation resulting in exacerbation of the local inflammation (Koskela et al. 1987).

Antibodies against the cell wall capsule interfere with the measurement of anti-capsular type-specific antibodies in both adults and children (Musher et al. 1990c) (Goldblatt D. et al. 1992), and probably account for discrepancies, between an apparently good response to the vaccine and lack of clinical efficacy from invasive disease. Therefore it is recommended, that the evaluation of the vaccine should be done by measuring antibody responses in adsorbed for cps antibodies, sera.

The currently available polyvalent pneumococcal vaccine contains 23 different serotypes with distinct immunological properties. Since type-specific antibodies are not cross protective, the immunogenicity of the vaccine should be assessed by measuring type-specific responses. However at present, a methodological protocol has not been standardized, and many laboratories rely on measuring antibodies to the entire vaccine, for the general assessment of vaccine immunogenicity [(Windebank et al. 1987), (Vandenbruaene et al. 1995)]. This is partly due to the time consuming nature of measuring responses to different serotypes. In addition, whole-vaccine ELISAs have been used for the establishment of normal ranges of the naturally acquired pneumococcal antibody levels in children, which could be used for the identification of individuals with impaired immunity to encapsulated bacteria (Hazlewood et al. 1993).

Whilst comparison of pneumococcal antibody levels measured by investigators in different laboratories could facilitate the optimal evaluation of pneumococcal vaccine, little effort has been made to compare type-specific and whole-vaccine ELISA assays. During the immunogenicity study of the 23-valent pneumococcal vaccine described in previous chapters, we had the opportunity to compare the immunogenicity of pneumococcal vaccine and persistence of antibodies obtained post vaccination as measured by both methods.

5.2. Methods

5.2.1. Whole-vaccine ELISA

Whole-vaccine pneumococcal assay was performed in Oxford as follows: Pneumovax II (Morson) diluted in 0.05M carbonate buffer (pH 9.6) to approximately 1μg/ml was added the plates (100 μl per well) and left overnight at 4^0C. Double dilutions of std sera and single dilutions of test sera (1:50 or 1:100) in PBS-Tw added to the wells (100 μl/well) and incubated for 1 h at 37^0C. Further steps were conducted as described for the type-specific ELISAs. Results were extrapolated from a std curve prepared by in-house standards and expressed in UI. Each clinical sample was analyzed before and after cps adsorption (see chapter 3).

Type-specific ELISAs for PS3, 6B, 19F and 23F were performed as described in detail in chapter 3.

5.3. Statistics

The proportion of children who responded to 2 or more serotypes were compared with the responders to one serotype or non responders by the chi square test. Overall response to the vaccine, when assessed by the whole-vaccine ELISA, was defined as a 2 fold increase in IgG levels 4 weeks post vaccination. If no antibody was detectable pre vaccination, response was defined as post vaccination titers above 10 IU. Responses to specific serotypes were defined as described in chapter 3.

Comparison between the two methods were performed by Pearson correlation test.

5.4. Results

5.4.1. The effect of cell wall polysaccharide adsorption on whole-vaccine ELISA

To investigate the interference of cps antibodies in whole-vaccine ELISA, we measured whole-vaccine IgGT pneumococcal antibody levels pre and at 1 and 12 months post vaccination before and after adsorption with 100μg/ml free cps antigen. As shown in figure 5.1, pre vaccination IgG pneumococcal antibodies in all 74 vaccinated children, were mainly against cps.

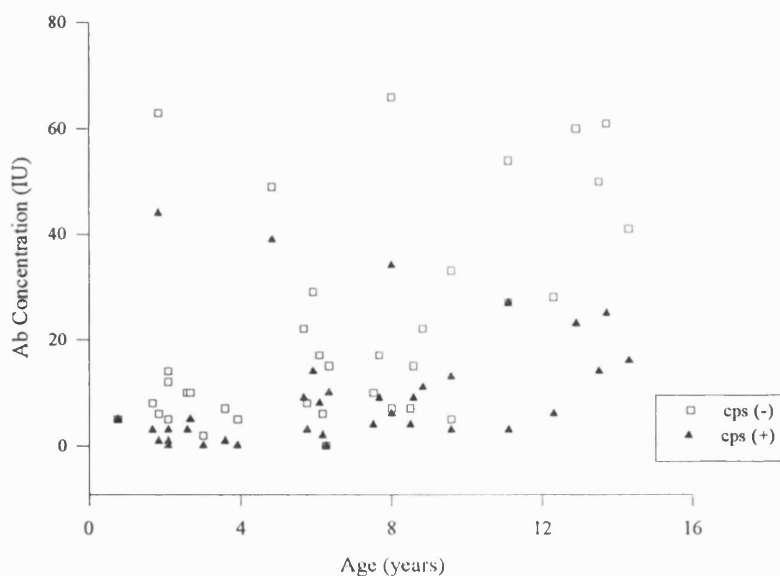

Figure 5.1: The effect of adsorption on the measurement of pre vaccination whole-vaccine pneumococcal antibodies
IgGT antibody levels (IU) before and after removal of cps antibodies as measured by whole-vaccine ELISA in 74 clinical sera taken before vaccination. Pre vaccination IgGT antibodies were mainly against cps.

Furthermore the effect of adsorption in antibody levels induced by vaccination, was investigated in 36 sequential samples obtained at 1, 6 and 12 months post

vaccination. GMC for unadsorbed and adsorbed sera, as measured by whole-vaccine ELISA, are shown in table 5.1. Adsorption reduced significantly *($p<0.001$)* GMCs at all time points.

Table 5.1: Effect of adsorption on IgGT pneumococcal antibody levels

	Pre Vaccine	1 Month	6 Months	12 Months	p
CPS (-)	13.3	24.1	20.64	24.9	
CPS(+)	4.2	13.76	9.74	10.7	<0.001

5.4.2. Comparison of the pneumococcal vaccine immunogenicity assessed by whole-vaccine and type-specific ELISA

The immunogenicity of pneumococcal vaccine assessed by whole-vaccine and type-specific ELISA assays, revealed concordance of the two methods in 38/70 (54.5%) responders and 9/70 (13%) non responders. 17 vaccinees appeared to have responded to the vaccine by means of the whole-vaccine assay only while 6 responders were detected by the serotype-specific ELISA only (Table 5.2).

Table 5.2: Immunogenicity of the 23 valent pneumococcal vaccine assessed by type-specific and whole-vaccine ELISA

Whole-vaccine Responders	Type-specific Responders		Total
	No	Yes	
No	9	6	15
Yes	17	38	55
Total	36	34	70

The magnitude of immune responses to vaccination was defined as fold increase of total IgG levels, post vaccination, as measured by whole-vaccine ELISA, or the number of serotypes responded by each vaccinated individual, assessed by the type-specific assay. Comparison of the two methodologies, in 70 clinical sera, revealed a significant correlation (f=3.8, p=0.008) between the two assays (Figure 5.2). Patients who responded to 2 or more serotypes, had a mean fold increase of total IgG levels detected by the whole-vaccine ELISA of 13.06, one month post vaccination, whereas responders to one serotype or non responders according to the type-specific ELISA, had a mean fold increase of 4.94 (p=0.04).

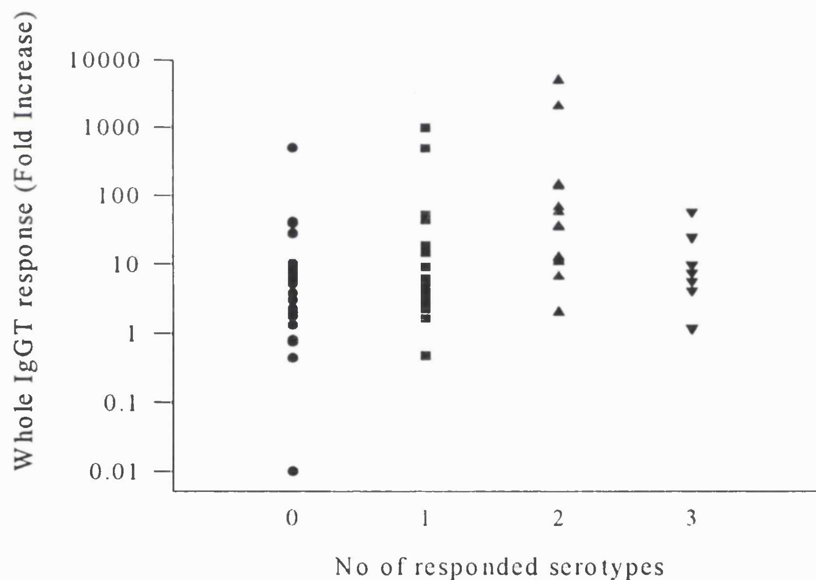

Figure 5.2: Comparison of the magnitude of immune responses to 23-valent pneumococcal vaccine assessed by whole-vaccine and type-specific ELISA
The magnitude of immune responses to vaccination was assessed in 70 clinical sera taken one month post vaccination. Comparison of fold increase of antibody levels as defined by whole-vaccine ELISA with the number of serotypes responded by each vaccinated individual revealed a statistically significant correlation (p=0.008)

5.4.3. Comparison of whole-vaccine and type-specific antipneumococcal antibody levels.

The correlation of IgGT antibody levels (IU) measured by the whole-vaccine ELISA with the sum of serotype-specific concentrations (μg/ml) as measured by the type-specific assay, one month post vaccination, revealed a rather weak correlation between the two assays (r=0.57). (Figure 5.3). Nine children with detectable whole-vaccine pneumococcal antibodies, were excluded from the analysis because they had no detectable type-specific pneumococcal antibodies to any of the 4 assayed serotypes.

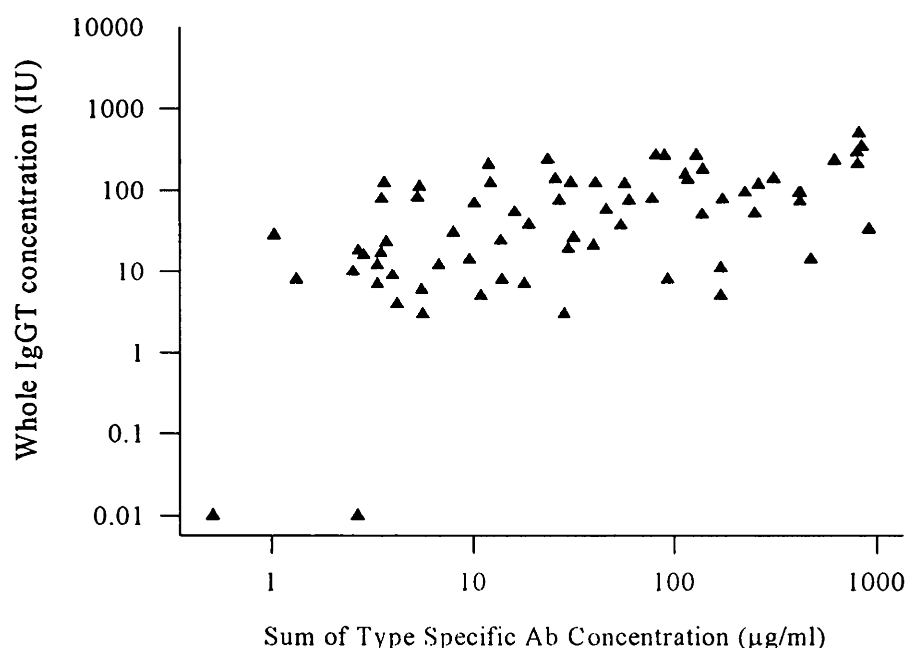

Figure 5.3: Correlation of pneumococcal antibody levels measured by whole-vaccine and type-specific ELISA.
Pneumococcal antibody levels measured in 63 clinical sera taken one month post vaccination. Comparison of log whole-vaccine IgGT (IU) concentration with the sum of type-specific (μg/ml) antibody levels for each vaccinated child, revealed a correlation of r=0.57 between the two assays.

The monitoring of IgGT antibodies measured by the two methods in 29 U and 42 VI children 12 months post vaccination, revealed different patterns of persistence of whole-vaccine and type-specific pneumococcal antibodies in the group of VI children. While whole-vaccine IgGT titers obtained one month post vaccination, declined in the succeeding 12 months in all children, there was a continuous increase throughout the follow up period in VI children for all 4 type-specific IgGT pneumococcal antibodies (table 5.3).

Table 5.3: Concentrations of whole-vaccine and type-specific IgGT pneumococcal antibodies

	Pre Vaccine		1 Month post		12 Months post	
	U	VI	U	VI	U	VI
Whole-vaccine IgG (UI)	7.2	3.77	109.2	11.9	60.4	6.73
PS3 (μg/ml)	0.71	0.31	0.73	0.47	7.88	6.52
PS6B (μg/ml)	0.41	0.19	0.34	0.26	0.38	0.7
PS 19F (μg/ml)	2.45	0.17	13.8	0.34	7.19	3.43
PS23F (μg/ml)	1.49	0.23	6.7	0.62	8.4	4.2

5.5. Discussion

Although there was a good correlation between the two assays in relation to the crude assessment of the magnitude of immune response to pneumococcal vaccine, comparison of the vaccine immunogenicity defined by both assays, revealed significant differences, demonstrating the extent to which the choice of the assay can influence the evaluation of a vaccine. There was agreement of both methods in detecting vaccine

response in 54.5 % of our study population; however type-specific ELISA failed to detect 17 individuals said to have responded when analyzed by means of the whole-vaccine assay. It is possible that these individuals may well have responded to types other than the four mentioned, indicating that the panel of the assayed serotypes should be increased, for a more accurate estimation of the vaccine immunogenicity when this is defined as type-specific responses. Whole-vaccine ELISA on the other hand, was not sensitive enough to detect 8 individuals who were assessed as responders by the type-specific assay. This is probably related to the lower concentration of the capture antigen in the whole-vaccine ELISA (0.05μg/ml versus 12.5 μg/ml in the type-specific assay) which reduces its sensitivity to detect low affinity antibodies (Griswold et al. 1989).

Furthermore, although there was some correlation between whole-vaccine and sum of type-specific pneumococcal antibody levels obtained post vaccination, the detailed monitoring of antibody levels in sequential samples taken from U and VI children at 6 and 12 months post vaccination, revealed differences between the two assays. While pneumococcal antibodies measured by the whole-vaccine ELISA in the group of U children, fell in the12 months following vaccination, antibody titers for all 4 assayed serotypes had a continuous increase in the group of VI children during the follow up period indicating that small changes in type-specific levels can be missed when pneumococcal antibodies are measured by whole-vaccine ELISA.

The measurement of whole-vaccine pneumococcal antibodies has been used to assess immunogenicity of the vaccine in populations with suspected impaired immunity to polysaccharides (Vandenbruaene et al. 1995). Our data indicate that this is probably not a sensitive enough method for a detailed characterization of the immune response.

Furthermore, attempts to establish normal ranges of anti-pneumococcal antibody levels in paediatric populations that could be used for the evaluation of immune function in children with increased susceptibility to infections from encapsulated bacteria were defined by whole-vaccine ELISAs (Hazlewood et al.1993). While these measurements may be of some value for the general assessment of immunity to carbohydrates, they probably have little role for a detailed evaluation of pneumococcal immunity.

In our study, whole-vaccine ELISA was profoundly affected by the presence of antibodies against cps. Pre and post vaccination titers were significantly lower in adsorbed than in unadsorbed sera. Similar findings have been reported previously for type-specific ELISA (Goldblatt et al. 1993) and indicate that adsorption is necessary when evaluating the pneumococcal vaccine in adults and children, in order to avoid overestimation of its immunogenicity. However this procedure may have some limitations because the amount of cps added may not be sufficient for some samples.

Since the emergence of multiple drug-resistant strains of *S pneumoniae* accelerates the need for the imminent use of the new generation of conjugate pneumococcal vaccines in high risk populations, the accurate estimation of their immunogenicity and the establishment of their clinical efficacy with confidence is necessary. Clearly for a detailed analysis of the capacity of the vaccines to induce type-specific immunity in high risk populations, only assays designed to measure type-specific antibodies will suffice. Furthermore, it is clear that type-specific ELISA is the appropriate methodology for the establishment of minimum protective antibody levels for each individual serotype included in the vaccine which may ultimately be a useful tool for prediction of the clinical efficacy of pneumococcal vaccine in a given individual. However it is essential that the procedures for assaying type-specific

antibodies should be standardized, using an international pneumococcal antibody standard and a well defined protocol. Efforts to standardize such a protocol are currently under way.

CHAPTER 6

Qualitative aspects of immune responses to *23-valent pneumococcal vaccine* in children with HIV infection.

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6

6.1. Introduction

The increase of antibody avidity following immunization has been well documented not only in experimental animals but also in humans (Goldblatt et al. 1998). The biological significance of antibody affinity discussed in detail in chapter 1, indicates that the production of high affinity antibodies in association with an increase in post vaccination titers, is a desirable outcome following immunization. However there is evidence that the increase of antibody concentration following immunization does not always correlate with the functional activities of the immune response. Amir et al. (1990) described sera from three adults that lacked bactericidal activity, although their anti-PRP concentrations were >8.6 μg/ml and showed that the impaired functional activities of the antibodies were attributed to their lower avidity. These anti-PRP antibodies were markedly deficient in their ability to activate complement-mediated killing of Hib organisms *in vitro*, suggesting that vaccine-related differences in avidity may have biologic importance with respect to protection against disease. This further emphasizes the importance of investigating the qualitative as well as the quantitative aspects of immune responses.

Although antibody affinity in response to vaccination, is likely to be affected by a number of host related factors including diet, hormonal factors and genetic background

(Steward M. 1981), an important variable is probably the immunocompetence of the vaccinee. In HIV infection the immune system impairment caused by the virus, could affect the maturation of immune responses and minimize the benefits from immunization. Recent evidence for the deleterious effect of the virus on FDCs with progression of the disease, could result in a defective antigen presentation with subsequent impaired B cell selection for the establishment of high affinity plasma and memory B cell clones. In addition the T-cell dependent B cell activation and differentiation could be affected by the profound abnormalities of all T cell subsets observed in HIV infected individuals. Finally the abnormalities of the cytokine network with the impaired intracellular signaling could also had an effect on the maturation of immune responses in seropositive individuals. Furthermore, avidity studies could possibly have some predictive value for the progression of the disease in HIV infected individuals. Interestingly two recent reports demonstrated an association between high-avidity immune responses to p24 and p17 antigens (Chargelegue D. et al. 1995) and V3 loop of the virus (Brostrom et al. 1995) and slow progression of the disease. The production of high avidity antibodies not only could result in a prolonged asymptomatic period but could also be an indication that the infected individual could respond effectively to different pathogenic microorganisms. However, the qualitative aspects of immune responses to immunizations in HIV infected populations have not been investigated. Paediatric HIV infected populations are particularly suitable for studies on the maturation of immune responses and the establishment of immunologic memory since they may encounter antigens for the first time whereas most of the infected adults have already established high avidity memory B cell clones before they become seropositive.

Immune responses to polysaccharide antigens are not the ideal candidates for avidity studies because of their TI nature. However there is recent evidence that B cells triggered by TI antigens, can be activated in follicles, and isolated FDC can synergize with TI signals *in vitro,* whereas studies in congenitally athymic rats have shown that they do develop GC (see chapter 1). Furthermore studies on the immune responses to the Hib polysaccharide vaccine in humans, have shown that the avidity of the produced antibodies increased post immunization indicating that even TI antigens can trigger somatic mutation for the emergence of high affinity B cell clones (Griswold et al. 1989). To this end, we decided to evaluate the integrity of immune responses to pneumococcal vaccine described in chapter 3, in terms of functional activity of the produced antibodies.

The immunogenicy study of the 23 valent pneumococcal vaccine described in chapter 3, revealed that in the majority of HIV infected children, the actual antibody levels for PS19F and 23F obtained post immunization, were significantly lower in comparison with the U. Since it is possible that avidity becomes biologically more important in low antibody concentrations (Schlesinger and Granoff, 1992) impaired functional activities of the produced antibodies could easily be associated with the observed susceptibility of seropositive children to *S pneumoniae*.

A wide variety of techniques for measuring the avidity-dependent kinetics of antigen-antibody interactions, has been described [(Friguet et al. 1985), (Pullen et al. 1986), (Macdonald et al. 1988), (Rath et al. 1988)]. The traditional method of equilibrium dialysis, has allowed the determination of equilibrium association constant K which expresses the ratio between association and dissociation rate constant in the

antigen-antibody interaction and can be calculated from the following equation:

$$K = \frac{Ka}{Kd} = \frac{[AgAb]}{[Ag] \times [Ab]}$$

where Ag represents free antigen, Ab represents free antibody, and AgAb the antigen-antibody complex. However K value can be interpreted reliably only when measuring monovalent binding in purified hapten- pure antibody solutions.

The development of modified solid phase ELISAs has allowed the determination of avidity for polyclonal heterogeneous sera not amenable to equilibrium dialysis. By employing simple and convenient ELISA methodology, using appropriate antibody concentrations, an estimation of the functional activity of large and complex molecules like polysaccharides may be attempted. Conformational changes induced in the antigen following it's binding to the solid phase in addition to the serum antibody heterogeneity and non Fab antigen-antibody binding, may alter the characteristics of the antigen-antibody interaction and subsequently affect avidity measurements. Despite these theoretical problems, solid phase assays appear to correlate well with equilibrium dialysis.

Two different techniques for the measurement of antibody avidity have been developed, the competitive inhibition ELISA which employs free antigen to competitively inhibit the antibody binding (Rath et al. 1988), and the elution assay which employs a chaotropic agent or a protein denaturant, to disrupt the antigen-antibody binding or to inhibit the antibody binding (Pullen et al. 1986). Elution assays do not fulfill the strict criteria required for measuring equilibrium constants, however they permit the ranking of avidity of various antibodies by producing a shift on the dose-response curve, which is inversely correlated to antibody avidity, and have been

proved useful in following the avidity maturation of antibodies in polyclonal sera following immunization (Devey et al. 1990). However these assays require validation and careful consideration of the effect of chaotrope /denaturant on the integrity of the reagents and the appropriate epitope density for the test antigen in order to give a measure of the average antibody avidity in a mixture of high and low avidity antibodies.

This chapter deals with the validation of an elution ELISA specific for pneumococcal antibodies and its application for the qualitative assessment of immune responses to the 23-valent pneumococcal vaccine, given by the study population described in chapter 3.

6.2. Methods

6.2.1. The effect of Ammonium Thiocyanate on the solid phase

For our study, we employed the elution ELISA described in detail in chapter 2, using NH_4SCN as a chaotropic agent. The direct effect of NH_4SCN on the binding of the antigen to the solid phase was investigated by comparing dose-response curves obtained with and without pretreatment of the ELISA coated plates with 5M NH_4SCN. Briefly, ELISA plates precoated with PS3, 19F and 23F, were incubated with NH_4SCN at RT for 15, 30, 40 and 50 min. After washing with PBS-Tw, a normal type specific assay as described in chapter 2, was carried out. Results showed in figure 6.1 revealed that preincubation with NH_4SCN had no effect on the subsequent dose-response curve for PS19F and PS23F, but affected the binding of PS3.

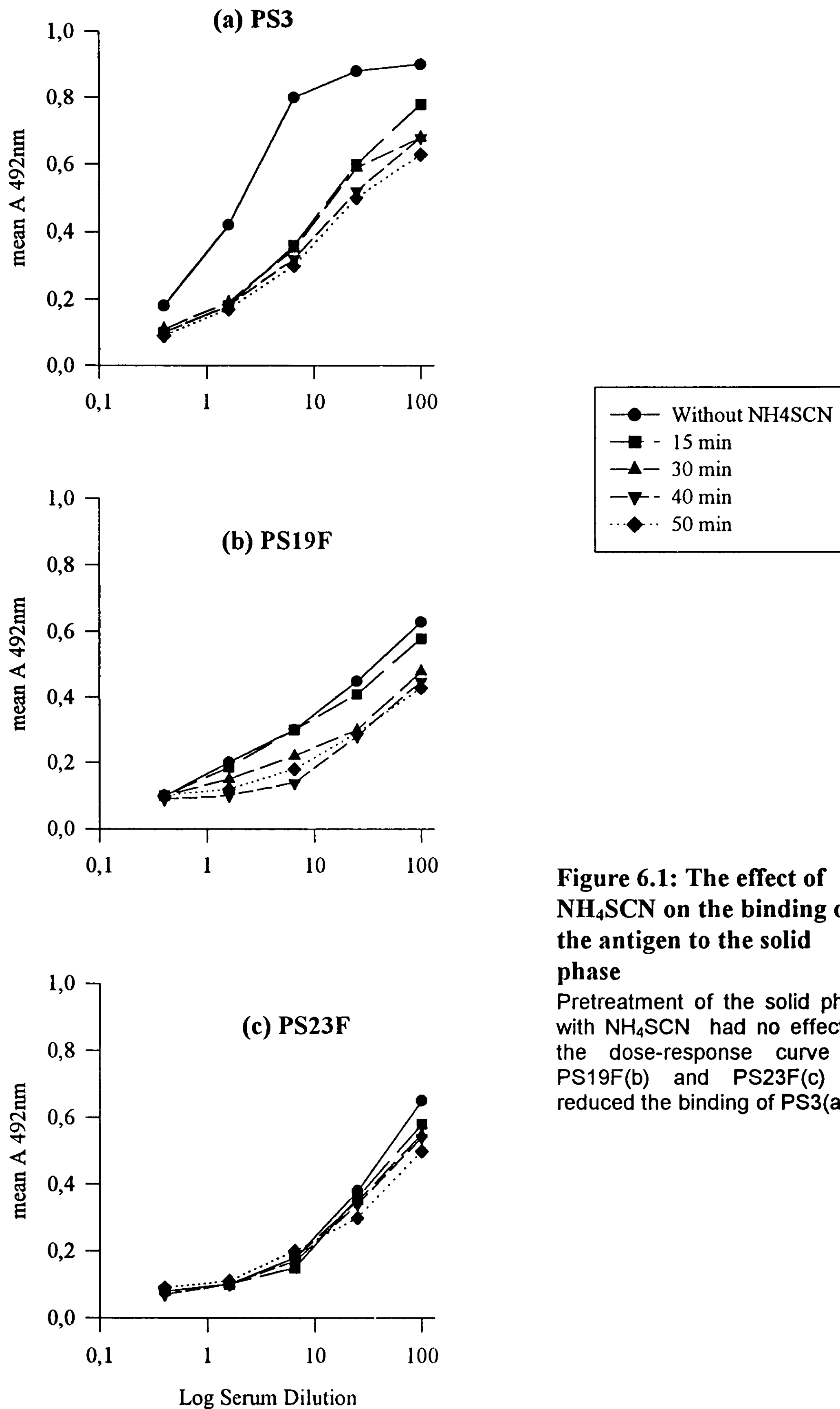

Figure 6.1: The effect of NH_4SCN on the binding of the antigen to the solid phase

Pretreatment of the solid phase with NH_4SCN had no effect on the dose-response curve for PS19F(b) and PS23F(c) but reduced the binding of PS3(a).

6.2.2. Validation of the assay

We validated the ranking of antibody avidity obtained by the NH_4SCN-ELISA with a competitive inhibition ELISA described by Devey et al (1988) in which a range of different concentrations of free antigen added to a constant dilution of the tested serum competitively inhibits the binding of antibodies of decreasing avidities. ELISA assays using serotypes 19F and 23F as free antigens in a concentration range between 0.02 μg/ml and 10μg/ml, were performed as described in chapter 2. The amount of free antigen required to inhibit the antibody binding was inversely correlated to the antibody avidity. Comparison of the ranking order for 3 clinical sera with high affinity and 3 with low affinity antibodies against PS19F (Figure 6.2) and PS23F defined by NH_4SCN-ELISA, revealed good correlation between the two assays.

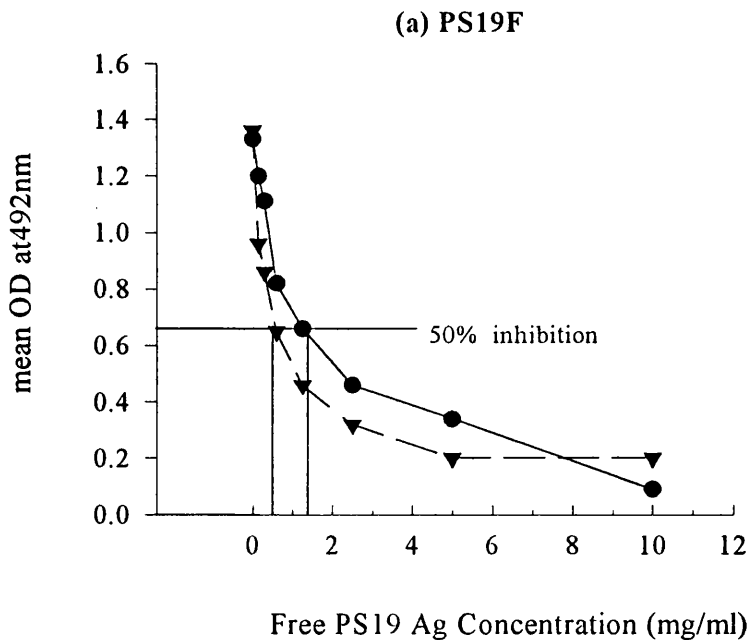

Figure 6.2: Competitive inhibition ELISA for pneumococcal antibody avidity
Increasing concentrations of free PS19F(antigen were added in a constant dilution 1:40 of two clinical sera ranked as higher (■) and lower (•) avidity sera by NH_4SCN -ELISA. Less free antigen was required to inhibit the binding of antibodies of higher avidity.

6.3. Statistics

Differences between geometric mean AI were analyzed by Student's *t* test. Correlations were calculated by Pearson's correlation coefficient (r). Differences in the mean antibody avidity between VI and U children were compared by unpaired *t* test.

6.4. Results

Sera from 21 (14U and 7VI) vaccinated children who responded to PS19F and from 34 (14U and 20VI) responders to PS23F, were assayed for the evaluation of their average functional affinity at 1 and 12 months post vaccination,. The calculation of the average AI at each time point, permitted the ranking of PS19F and 23F IgGT pneumococcal antibodies in the two groups.

6.4.1. Avidity index and antibody concentration

To establish whether antibody avidity could be measured independently of titer as measured by the immunogenicity study, AI of all 21 vaccinated children who responded to PS19F and the 34 responders to PS23F were plotted against the corresponding antibody concentrations measured in the 12 month samples (figure 6.3). There was no significant correlation between antibody titer and avidity (r= 0.03 and -0.2 for PS19F and PS23F respectively) indicating that these assays were able to determine avidity independently of titer.

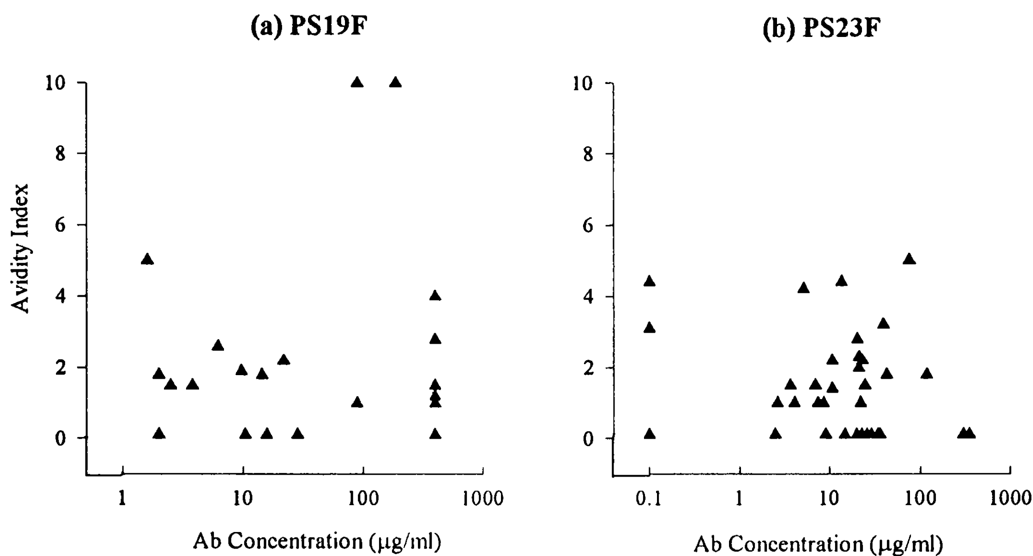

Figure 6.3: Correlation between avidity index and antibody concentration
AI measured in all responders to PS 19F (n=21) and PS23F (n=34), were plotted against the corresponding antibody levels. Correlation coefficient r=0.03 and -0.2 for PS 19F and PS23F respectively, revealed that avidity measurement was independent of antibody concentration.

6.4.2. Antibody avidity and age

It is known that immune responses to polysaccharides are age dependent. To exclude the possibility that differences in avidity, between VI and U children were age-related, we investigated the relationship between AI and age in the group of U children. The distribution of AI to PS19F (n=14) and 23F (n=14) as measured at 12 month, according to age is shown in figure 6.4. There was no significant correlation between AI and antibody concentration by Pearson's correlation coefficient (r = 0.1 and -0.5, for PS19F and 23F respectively) indicating the independence of the two variables.

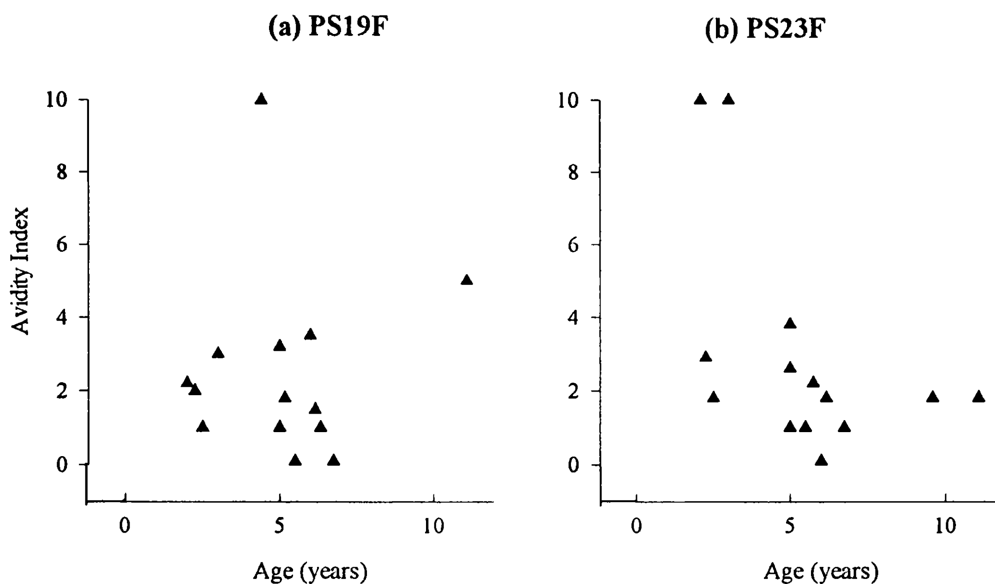

Figure 6.4: Correlation between avidity index and age.
AI for serotypes 19F (n=14) and 23F (n=14) were measured in controls, 12 months post vaccination. Correlation coefficient of 0.1 for PF19F and -0.5 for PS23F indicates no significant association between AI and age.

6.4.3. The effect of cell wall polysaccharide adsorption

Since the ELISA we employed gives the average functional affinity of polyclonal heterogeneous populations, we investigated the effect of removal of cps antibodies on the measurement of mean antibody avidity. For that purpose we compared the mean AI amongst all responders to PS19F (n=21) and 23F (n=34), at 1 and 12 months before and after serum adsorption (figure 6.5). Removal of cps antibodies reduced the mean AI at both time points. For PS19F antibodies, the mean AI was 1.8 vs 1.45 (p=0.2) at 1 month and 3.95 vs 2.9 (p=0.01) at 12 months. Similarly values for PS23F were 2.75 vs 1.42 (p<0.001) at 1 month, and 3.89 vs 1.47 (p < 0.001) at 12 months.

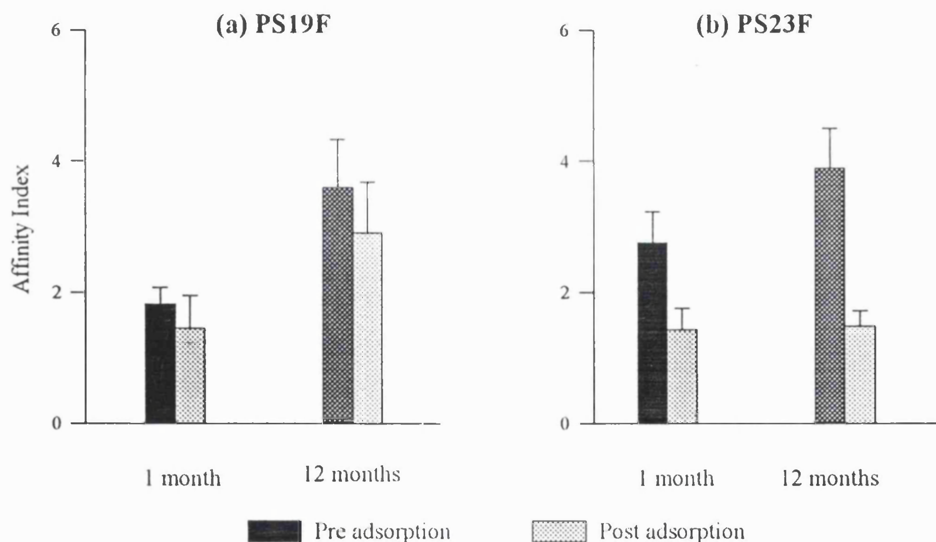

Figure 6.5: The effect of cell wall polysaccharide adsorption on avidity index
Mean AI to PS19F (n=21) and PS23F (n=34) measured in clinical sera taken at 1 and 12 months post vaccination, before and after adsoprtion with cps. Removal of cps antibodies reduced the mean AI for both serotypes significantly at both time points (for PS 19F p=0.2 and 0.01, for PS23F p<0.001 at 1 and 12 months).

6.4.4. Avidity of type-specific pneumococcal antibodies

Antibody avidities of the 21 vaccinated children who responded to PS19F and the 34 responders to PS23F were normally distributed when data were log transformed. The geometric mean AI in VI and U children are shown in table 6.1. Although mean AI increased with time post vaccination, in both groups of children, differences were not statistically significant, with exception the responses to PS19F in the group of infected children when avidity was measured in unadsorbed sera (p= 0.01). The comparison of the mean AI between infected and U children revealed no statistically significant differences between the two groups for both serotypes with exception the avidity to PS19F at 12 months (p=0.03). Removal of cps antibodies resulted in reduction of the mean AI in all children at both time points.

Table 6.1: Geometric mean avidity index of IgGT antibodies to serotype 19F and 23F

(a) PS19F

	No	Before Adsorption		After Adsorption	
		1 Month post	12 Months post	1 Month post	12 Months post
Uninfected	14	1.03 [a]	1.45[b]	**0.5**	**0.85**
HIV infected	7	1.36[c]	4.5[d]	**0.66**	**1.95**

(c *vs* d: p=0.01, b *vs* d: p=0.03,

(b) PS23F

	No	Before Adsorption		After Adsorption	
		1 Month post	12 Months post	1 Month post	12 Months post
Uninfected	**14**	**1.3**	1.9	**0.7**	**0.8**
HIV infected	**20**	**1.5**	3.3	**0.5**	**0.6**

6.5. Discussion

A solid phase elution assay was employed, for the qualitative assessment of immune responses to pneumococcal vaccination in a group of HIV infected children and controls born to HIV infected mothers. ELISA based methodology is convenient for the measurement of avidity to biological sera, but is not suitable for all antigen-antibody systems. The choice of the chaotropic agent requires careful consideration on its effect on the antigen binding to the solid phase and the immunological properties of the reacting substances. Chaotropic agents act by unfolding and dissociating macromolecules particularly through the breaking of hydrophobic, ionic and hydrogen bonds which are the major forces responsible for stabilizing the antigen-antibody interaction. Different chaotropic agents including urea, guanidine hydrochloride, thiocyanate and diethylamine (DEA) have been used in elution ELISAs. The validation of the assays accomplished by the comparison with results obtained from other techniques, revealed that not all of the chaotropic agents fulfill the above requirements. Urea has become popular by clinical laboratories for its use in avidity assays distinguishing between primary infection and reinfection; however it may affect the stability of bound antigen on the solid phase. DEA had been shown to correlate well with competition-inhibition assays (Devey et al. 1988) but it has recently reported that it exerts its effect by pH changes which could subsequently affect the integrity of the antibody molecule (Goldblatt et al. 1993). In contrast, thiocyanate elution assay has been shown to be pH-independent dissociating the antigen-antibody complex through the breaking the existing hydrophobic bonds without producing irreversible gross structural changes (Hilleman et al. 1981). Furthermore MacDonald et al. (1988) have compared the thiocyanate elution assay to equilibrium dialysis using 6 monoclonal antibodies with different affinities and showed that both techniques rank similarly

antibody affinity. Despite some methodological considerations for the effects of NH_4SCN on the antigen-antibody binding [(Hall and Heckel, 1988), (Gray and Shaw, 1993)] in general, it is considered particularly useful in monitoring avidity maturation following immunization.

We have shown that the method we used for avidity estimation, was independent of antibody concentration, an important prerequisite for a reliable evaluation of the average functional affinity in polyclonal heterogeneous populations. A further validation of the assay by comparing it with a competitive inhibition ELISA revealed similar ranking for high and low avidity antibodies. However the effects of NH_4SCN on PS3 coated plate, led us to apply the method to PS19F and PS23F antibodies only. Although the biological interpretation of high avidity antibodies in terms of protective efficacy, was not possible, since the assay did not measure absolute *K* values, our data provided a useful means of comparing VI and U children using the same methodology.

We could not establish any age related differences in the avidity of pneumococcal antibodies in response to vaccination in the group of U children. Impaired immune responses to carbohydrates have been characterized by unresponsiveness to polysaccharide antigens in the first 2 years of life, followed by isotype inappropriate immune responses in older children. As already discussed in chapter 1, children aged 18-23 months respond to polysaccharide vaccines predominantly with IgG1 antibodies whereas the maturation of immune responses had been associated with the acquisition of IgG2 antibodies which have been reported to be of high avidity in adults (Persson et al. 1988). Several studies have shown that different antibody subclasses have differing affinities (Devey et al. 1990), indicating that age-related IgG subclass differences in association with subclass-related avidity differences could result in an overall

qualitatively different immune response to the vaccine in younger children. In our study we investigated the functional avidity of the IgG antibodies and we found that this was not age related. Although conclusions for the biological significance of this finding in terms of clinical protection are difficult to make, it may indicate that the heterogeneous IgG1 and IgG2 responses given by younger children may overall have similar functional activities with the high avidity IgG2 responses observed in adults. This hypothesis is in accordance with findings from ongoing research in our laboratory where, although IgG1 pneumococcal antibodies antibodies were of lower avidity in children younger than 2 years of age in comparison with the older children, the overall avidity of IgGT antibodies did not demonstrate any significant differences at all ages between younger and older children. Interestingly Weinberg and Granoff (1988), have shown that IgG1 and IgG2 anti-PRP antibodies have comparable *in vitro* complement-mediated bactericidal activity and protective activity against experimental Hib bacteremia in rats. However studies on the average avidity for each IgG subclass separately, that could provide a more detailed inside on the qualitative aspects of immune responses to polysaccharide vaccines, have not been published.

The removal of cps antibodies resulted in significant alterations in the average avidity of the tested sera, indicating that they are probably of relatively high avidity. As already discussed in chapter 5, pneumococcal antibodies found in the prevaccination sera usually induced by the natural colonization of the URT by pneumococcus, were mainly against cps. The high avidity of cps antibodies may indicate that even for the TI polysaccharide antigens there is a shift towards higher avidity B cell clones over time.

We could not establish any statistically significant qualitative differences of the immune responses between VI children and controls although there was a trend for

higher AI in both groups, at 12 months. The only statistically significant increase, was observed in the group of infected children for one serotype (PS19F) when AI were measured in unadsorbed serum. However the number of infected children was quite small and the average AI could easily be raised by the presence of a small amount of high -avidity antibodies. Furthermore, differences were diminished when avidity was measured in adsorbed sera. Our findings could be explained by the TI nature of pneumococcal antigens and are in accordance with a recently published study showing no avidity maturation of immune responses to pneumococcal vaccine in elderly individuals (Konradsen, 1995). This does not rule out the possibility that our method was not sensitive enough to detect less prominent differences and recently developed surface sensitive optical techniques (biomolecular interaction analysis using surface plasmon resonance) may be necessary to confirm these results. Studies performed in our laboratory, comparing different solid phase avidity assays with a bio-sensor equipment (Biacore, Pharmacia), revealed the reduced sensitivity of the modified ELISAs in comparison with the surface plasmon resonance technology but good correlation with NH_4SCN elution assay (McCloskey et al. 1997).

Our findings indicate that HIV infected children are able to mount immune responses qualitatively similar to controls and provide a further justification for the administration of the pneumococcal vaccine in infected children. However the introduction of the conjugate polysaccharide vaccines will probably provide an interesting area for further research on the qualitative aspects of immune responses in HIV infection .Results from our laboratory indicate an increase in antibody avidity overtime following Hib conjugate vaccine avidity (Goldblatt et al. 1998). The establishment of high avidity B cell clones in infected individuals could be particularly

important for their protection with the progression of the disease resulting in attrition of antibody levels; in addition memory B cell clones if established and maintained, could offer long term protection. Janoff et al (1991) have shown that high avidity responses to recall antigens, are well preserved in HIV infected adults. Similar results if found in children, in combination with data from immunogenicity studies, could provide a further evidence that early immunizations with polysaccharide conjugate vaccines can be justified as a cost effective approach in paediatric HIV infection.

CHAPTER 7

General Discussion

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7

7.1. Aspects on immunity to polysaccharides in HIV infected children

Infections secondary to encapsulated bacteria have emerged as a major clinical problem in the HIV infected populations. This increase is almost certainly related to dysfunctional host defenses related to the HIV infection rather than an increased exposure to the pathogenic microorganisms (Janoff et al.1993). However the immunologic basis of this susceptibility seems to be multifactorial and its nature has not been fully elucidated at present. As already discussed in detail, the interaction between the microorganism and the immune system is a complex phenomenon that requires intact humoral immunity, with adequate antibody production and effective complement-mediated opsonization and phagocytosis in the bloodstream, liver and spleen.

Aspects of humoral immunity that were investigated in this thesis, revealed that seropositive children had lower naturally acquired IgG and IgG2 subclass pneumococcal antibody levels than the controls. The finding of low baseline pneumococcal antibody levels in paediatric populations has also been reported by King et al. (1996) and could be attributed to impaired development of natural immunity to *S pneumoniae* induced by colonization of the URT or by cross reacting bacteria,

rendering seropositive children more susceptible to pneumococcal infections. Furthermore, the relative unresponsiveness of HIV infected children to polysaccharide antigens, indicated in the immunogenicity study by the reduced number of responded serotypes after pneumococcal vaccination, implies that a similar defective immune response could occur after natural infection with encapsulated bacteria. There was no indication that this defect could be related to impaired immunoglobulin class switching because the pattern of IgG2 immune responses to vaccination was similar in HIV infected children and controls. Similar findings have been reported by Carson et al. (1995) who demonstrated that seropositive adults had normal numbers of specific IgG2 producing antigen secreting cells, after vaccination.

The elucidation of the pathogenesis of pneumococcal disease in paediatric HIV infection requires the investigation of the effect of HIV infection on all aspects of pneumococcal immunity. There is evidence from studies in adults that baseline functional activities of antipneumococcal antibodies are impaired in HIV infection. Janoff et al. (1997) reported impaired functional activity of anti PS14 naturally occurring antibodies to HIV infected Kenyan women while in a previous study he had shown lower complement-mediated serum killing activity in convalescent sera from HIV-infected adults recovering from invasive pneumococcal disease than in controls (Janoff et al. 1993). Other studies have shown that HIV-infected patients have abnormal complement activation (Tausk et al. 1986), impaired polymorphonuclear-mediated clearance of opsonized pneumococci in the bloodstream (Murphy et al. 1988) and defective macrophage-dependent phagocytosis in the liver and spleen (Bender et al. 1987). Age-related different immune responses to polysaccharides in association

with the distinct immunological profile of paediatric HIV infection, indicate that similar studies should be performed in HIV infected paediatric populations.

7.2. Polysaccharide vaccines in HIV infected children: Future perspectives

Until more is known about the pathogenesis of bacterial infections in patients with HIV infection, an important mechanism for their protection is prophylactic immunization with polysaccharide vaccines. Although efficacy trials that could be conclusive on the utility of vaccination strategies have not been performed, the increased morbidity and mortality amongst HIV infected populations caused by bacteria with polysaccharide capsule in association with the emergence of multi drug resistant strains of *S pneumoniae* worldwide [(Frankel et al. 1996), (Raz et al. 1997), (Syrogiannopoulos et al. 1997)] justify the policy of vaccinating seropositive populations with polysaccharide vaccines. Data presented in this thesis, where most of the HIV infected children older than 18 months of age, gave adequate immune responses to both vaccines, not only during the period of relative immunocompetence but sometimes even at the symptomatic stage of the disease, provide further evidence to support this strategy.

In the past few years, the observation that pneumococcal vaccine was associated with increased HIV viral load (Brichacek et al. 1996) raised concerns that immunizations could accelerate the clinical progression of HIV disease. However a recent study by Katzenstein et al (1996) who reported no detectable increase in HIV replication 6 weeks after immunization with pneumococcal vaccine indicates that this is a transient phenomenon and has no impact on the clinical progression of the disease.

Results presented in this thesis have highlighted the reduced immunogenicity of both vaccines in children with advanced immunodeficiency indicating that these vaccines are more effective when they are given as soon as the diagnosis of HIV infection has been established. A further evidence for this, is provided by a study by Gebo et al. (1996) who found that administration of pneumococcal vaccine was associated with a reduced risk of pneumococcal disease only in patients with CD4 cell counts over 200 cells/mm^3. In vertically infected children the administration of Hib conjugate vaccine soon after birth will probably have the most beneficial effects. However the unresponsiveness of very young children to pure polysaccharides indicates that similar policy cannot be justified for the currently used pneumococcal vaccine. Investigators trying to enhance the pneumococcal vaccine immunogenicity suggested increase in the amount of polysaccharide contained in the vaccine (Robbins et al. 1983), or multiple-dose regimens. However there is little evidence from relevant studies in adults that these strategies could be successful. Rodriguez-Barradas et al. (1996) have shown that among HIV-infected low responders to pneumococcal vaccine, revaccination with a double dose of pneumococcal polysaccharide did not stimulate IgG responses.

The development of the new conjugate pneumococcal vaccine preparations will be a major step in reducing the incidence of pneumococcal disease, if these vaccines will be proved to be safe and immunogenic in HIV infected children. The first study on immunogenicity of a conjugate pneumococcal vaccine preparation in HIV infected individuals by Ahmed et al. (1996), revealed no differences in the immunogenicity of a 5-valent pneumococcal conjugate vaccine preparation, (composed of PS6B, 14, 18C, 19F, and 23F, linked to CRM_{197}) in comparison with the 23-valent polysaccharide

vaccine. The observed hyporesponsiveness could be attributed to the HIV infection associated T cell impairment. However in a latter study, King et al.(1997) have shown that 3 doses of the same vaccine were equally immunogenic in HIV infected children and controls, although only asymptomatic seropositive children were more likely to have protective anti pneumococcal antibody titers after the first and second dose. This finding suggests that more doses of the vaccine are required to establish protective immunity in infected individuals and underscores the importance of controlled clinical evaluations of new vaccines in immunocompromised as well in healthy populations.

Conjugation of polysaccharides to protein carriers, by inducing T cell dependent responses, could result not only in improved immunogenicity but also in the immunological priming of the vaccinee. The establishment of memory B cell clones may be more important for long term protection of seropositive children on a subsequent exposure to polysaccharide antigens than the actual antibody concentration (Kurikka et al. 1995). Quantitative as well as qualitative studies on immune responses to booster doses given by seropositive populations, are necessary to confirm the establishment of immunologic memory, which in association with data on the duration of post immunization antibody levels induced by conjugate vaccines, will indicate the most appropriate time for revaccination.

It is also important to evaluate the protective efficacy of the conjugate polysaccharide vaccines in immunocompromised populations. Protection from bacteremic infection is a complex phenomenon balancing bacterial multiplication against specific and non specific bacterial clearance mechanisms which in immunocompromised populations, may have different dynamics than in healthy individuals. The establishment of minimum protective antipneumococcal antibody

levels, could be a useful tool for the prediction of the protective efficacy of immunizations. While protective anti-pneumococcal antibody titers are not currently known, anti-PRP antibody levels >1μg/ml have been associated with long term protection. However the minimum protective antibody requirements induced by conjugate vaccines may differ than those induced by the polysaccharide vaccines. Furthermore studies in asplenic rats have shown that they require significantly higher concentrations of anti PRP antibodies than normal rats for protection against invasive disease (Rubin L. 1988), indicating that host related factors other than antibody, eg complement and intact spleen function are also associated with the protective activities of the immune response and immunocompromised populations may have higher antibody requirements for protection against invasive disease. Reestablishment of the minimum protective antibody levels induced by conjugate vaccines in immunocompromised populations is probably necessary for the more accurate evaluation of these vaccines in HIV infected populations.

While quantitatively adequate immune responses, are not always indicative of effector antibody production (Hetherington and Rutkowski, 1990), the investigation of qualitative aspects of immune responses is important for the evaluation of new conjugate vaccines. Antibody avidity is an indicator of antibody activity and modest avidity differences can profoundly affect the *in vitro* bactericidal activities of immune responses (Lucas and Granoff 1995). Avidity studies on immune responses induced by different conjugate vaccines will aid clinicians in the choice of the vaccine formulation that induces high avidity immune responses in immunocompromised populations. This is particularly important because suboptimal vaccine immunogenicity could be compensated by the production of antibodies of higher functional activity.

Since there is evidence that HIV infected individuals show impaired serum functional activity to infection with polysaccharide antigens, it is possible that they may respond to vaccination with less active antibody in complement-mediated bacterial killing or opsonization assays, than healthy controls. Jannoff et al. (1997) have shown increased serum bactericidal activity of PS14 antibodies in HIV infected Kenyan women after vaccination with a 5-valent conjugate pneumococcal vaccine. However in children, the pattern of IgG sublass immune responses to conjugate polysaccharide vaccines is mainly restricted to IgG1 isotype while adults respond with IgG2 subclass (Schlesinger et al. 1992). The effect that these differences could have on protective immunity remains to be elucidated.

The identification of *in vitro* functional assays that can be translated directly into vaccine-induced clinical protection is not clear at present. Amir et al. (1990), reported a positive correlation between antibody avidity and serum bactericidal activity, while Hetherington and Lepow (1992) have showed that the avidity of anti PRP-D antibodies was the only parameter significantly correlated with serum bactericidal activity. Animal studies are necessary to elucidate the association between functional activities of the immune responses as measured in bactericidal and avidity assays, with the *in vivo* protection. The elucidation of such associations could be of both theoretical and practical importance. Not only could they provide a quick and inexpensive way for prediction of the clinical efficacy of the new conjugate vaccines in healthy and immunocompromised individuals, but also could provide a useful model for a more sophisticated analysis of immune responses in individuals with recurrent bacterial infections.

7.3. Concluding remarks

Over the next several years the life span of HIV infected populations will increase significantly as a result of the introduction of highy effective antiretroviral combination therapies. In addition the development of new vaccines will definitely have a significant contribution on the effective prophylaxis of these patients from secondary infections.

The increasing sophistication of new technologies using different vaccine compositions and structures, has resulted in the manufacturing of more antigenic new generation vaccine formulations. However for polysaccharide vaccines, much is still to be discovered related to the confirmation of the saccharide moiety, availability of antigenic structural components and sites of saccharide attachment of protein carriers that could enhance the vaccine immunogenicity. The improvement on the qualitative aspects of immune responses is necessary for the optimization of the new vaccines. It has been suggested that different vaccine adjuvants could result in variances in the relative contribution of TD and TI mechanisms which subsequently could influence the avidity of the produced antibodies. While it has been suggested that a greater T cell dependency may favor greater affinity maturation of the antibody, the induction of somatic mutation could result in differences in the fine structure of the antigen-combining sites and subsequently affect the antigen-antibody binding. Further studies are necessary to analyze the association between polysaccharide vaccine form and the functional activities of the immune responses.

In addition the elucidation of the contribution of different parts of the immune system on the generation of effective antibody responses will facilitate the use of immunomodulatory factors that could selectively enhance humoral B-cell responses or

cytotoxic T cell responses following an antigenic stimulation. Immunomodulatory factors could also influence the selection of high affinity antibodies either directly by induction of somatic mutation of immunoglobulin variable region genes or by preventing B cell apoptosis which may allow time for formation of germinal centers.

The elucidation of such regulatory mechanisms, not only will improve our understanding on the requirements to establish, maintain and trigger immunologic memory but also will facilitate our ability to stimulate key elements of the immune system in order to achieve optimal protection. Especially for immunocompromised patients the stimulation of the immune system in a more efficient and specific way could be of critical importance for the establishment of high specificity and long lasting immunity.

Appendix

Affinity indices of anti-pneumococcal antibodies to serotypes 19F and 23F

Table A: Avidity indices (AI) of **PS19F** specific antipneumococcal antibodies measured in clinical sera taken 1 and 12 months post vaccination, before and after removal of cps antibodies.

Patient	AI at 1 month		AI at 12 months	
	cps(-)	cps(+)	cps(-)	cps(+)
1	1.8	10	3	2
2	3.6	1	5	1
3	3	2.2	10	10
4	1.5	10	3.5	10
5	2	0.1	2.5	1.5
6	1	1	1	2.6
7	2.4	1.6	10	1.8
8	2	0.1	10	0.1
9	1	0.1	2.2	1
10	2.5	1	3.5	2.8
11	2.8	0.1	2.2	1.5
12	1	0.1	1	1.8
13	0.1	0.1	0.1	0.1
14	1.5	0.1	2	1.9
15	0.1	0.1	1.9	0.1
16	1	0.8	1	1.5
17	1	0.1	1.8	0.1
18	1.8	1.8	1.5	1.2
19	3.5	5	3.2	2.2
20	2.2	2.5	3	5
21	0.1	1.1	0.1	0.1

Table B: Avidity indices (AI) of **PS23F** specific antipneumococcal antibodies measured in clinical sera taken 1 and 12 months post vaccination, before and after removal of cps antibodies.

Patient	AI at 1 month		AI at 12 months	
	cps(-)	cps(+)	cps(-)	cps(+)
1	4.5	2.5	10	1.48
2	1	1.5	1.8	0.1
3	0.1	0.1	1.2	0.1
4	1.4	0.1	4	0.1
5	3	2.2	2	1.5
6	1	1.2	2.2	1
7	2	1	2	1
8	1	1.8	4.5	1
9	1.5	0.1	3	2.2
10	3.6	0.1	1.5	0.1
11	5	0.1	1.5	0.1
12	1.1	1	10	0.1
13	1	1.5	10	2.3
14	1.5	4	4	2
15	2	0.1	0.1	0.1
16	0.1	1.3	2.2	2.2
17	1.6	0.1	10	0.1
18	10	0.1	10	1.5
19	4.6	0.1	3.8	0.1
20	10	0.1	1.8	0.1
21	2.2	0.1	2.6	4.4
22	10	10	2	4.4
23	0.1	0.1	1.8	1.4
24	5	5	10	4.2
25	1	0.1	1	0.1
26	0.1	0.8	1.8	1
27	1.5	1	2.9	3.2
28	0.1	0.1	1.2	1.8
29	0.1	1.1	1	2.8
30	1.5	1	0.1	10
31	1.8	2	1.8	1.8
32	4	1.8	3.8	0.1
33	5	3.1	10	5
34	2.5	1	1	1

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1. Gibb D, Spoulou V, Giacomelli A, Griffiths H, Masters J, Misbah S, Nokes L, Pagliaro A, Giaquinto C, Kroll S, et al. Antibody responses to Haemophilus influenzae type b and Streptococcus pneumoniae vaccines in children with human immunodeficiency virus infection. *Pediatr. Infect. Dis. J.* 1995; **14:** 129-135

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Antibody responses to *Haemophilus influenzae* type b and *Streptococcus pneumoniae* vaccines in children with human immunodeficiency virus infection

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Antibody responses to *Haemophilus influenzae* type b (Hib) conjugate (ActHIB®; Pasteur Merieux) and pneumococcal (Pneumovax II®; Morson) vaccines were measured in 56 infected children (VI) and 44 uninfected children (U) older than 18 months of age, born to human immunodeficiency virus-positive mothers. Preimmunization, 21% U and 20% VI had protective concentrations of anti-Hib polysaccharide antibodies. Postimmunization, 100% U and 86% VI achieved protective titers (*P* = 0.008). The geometric mean increase in anti-Hib polysaccharide antibody was 7.6 (95% confidence interval, 3.5 to 16.3; *P* = 0.0001) times higher in U than in VI children after adjusting for age and ethnicity. Sixty-one percent U compared to 54% VI showed a 2-fold increase in antibody levels to at least one of the four pneumococcal vaccine serotypes (3, 6, 19, 23) measured (*P* = 0.4). For both vaccines there was a significant trend toward poorer responses in children with acquired immunodeficiency syndrome but no correlation with age adjusted CD4 counts. These data suggest that human immunodeficiency virus-infected children should be immunized with these polysaccharide vaccines early in the course of their disease.

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INTRODUCTION

Severe or recurrent bacterial infections are common in individuals with human immunodeficiency virus (HIV) infection.[1–7] This is particularly true for children and is reflected by the inclusion of severe recurrent bacterial sepsis (defined as 2 episodes within a 2-year period) in the 1987 Centers for Disease Control surveillance definition of pediatric acquired immunodeficiency syndrome (AIDS).[8] Between 1989 and 1992 recurrent bacterial sepsis was the first AIDS indicator disease in 16 to 23% of annual pediatric AIDS reports in the United States (Centers for Disease Control, unpublished data, 1993) and 24% of reports up to 1991 in Europe.[9] Data from prospective trials of placebo-controlled immunoglobulin therapy suggest that approximately 20% of HIV-infected children with low CD4 counts have a serious bacterial infection during a 2-year period.[10] Otitis media has also been reported to be 3 times more frequent in HIV-infected than in uninfected children.[11] Organisms responsible for infections in children with HIV infection are similar to those affecting HIV-uninfected children and include *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib). These organisms contain polysaccharide capsules which are virulence factors and targets for protective antibodies.

The immunologic basis for the increased susceptibility to bacterial infections in HIV-infected children remains unclear. HIV infection is associated with various immunologic abnormalities in addition to destruction of helper T cells (identified by the surface marker CD4). These include B lymphocyte dysregulation with polycloncal hypergammaglobulinemia and reduced responses to mitogenic stimuli.[12–16] The absence of anticapsular antibodies before acquisition of HIV infection in the perinatal period, in addition to poor immunogenicity of carbohydrates in very young children, may in part account for the increased prevalence of bacterial infections in vertically HIV-infected children compared with HIV-infected adults.

Strategies aimed at prevention of frequent bacterial infections in children with HIV infection include prophylactic antibiotics, immunoglobulin infusions and immunization. Immunization against Hib and *S. pneumoniae* have been recommended for children with HIV infection.[17] However, data have suggested that antibody responses to vaccines containing carbohydrate or protein antigens are reduced in HIV-infected adults and, furthermore, no immunogenicity studies of sufficient size to address this issue have been published for HIV-infected children.[18] The aims of this study were to compare the antibody responses and adverse reactions to Hib and *S. pneumoniae* vaccines in vertically HIV-infected children at different stages of disease with uninfected children born to HIV-positive women.

MATERIALS AND METHODS

Subjects. Children HIV-infected (VI) and uninfected (U) born to HIV-infected mothers and attending the Hospital for Sick Children, Great Ormond Street and the Instituto di Clinica Pediatrica, Padua were eligible for the study. When the study started Hib vaccine was not routinely given to infants or children in either country. Children attending the Instituto di Clinica Pediatrica were participating in the European Collaborative Study, an ongoing European prospective natural history study of children born to HIV-positive women.[12]

Classification of HIV-infected children. VI children were classified at enrollment into 3 groups: AIDS; symptomatic; and asymptomatic. The Centers for Disease Control definition of AIDS in vertically HIV-infected children was used[8] but children with mild lymphocytic interstitial pneumonitis were excluded from the AIDS group and placed in the category of symptomatic HIV infection. Children with lymphadenopathy, hepatomegaly and/or HIV-related thrombocytopenia only were assigned to the asymptomatic group.

Schedule of immunization and follow-up. Children older than 18 months were immunized with a single dose of Hib-tetanus toxoid conjugate vaccine (ActHIB®; Pasteur Merieux); infants younger than 6 months of age received 3 doses of ActHIB® at monthly intervals coinciding when possible with other routine immunizations; children ages 6 to 18 months at the time of first dose received 2 doses 1 month apart and a third dose at 18 months. All children more than 6 months of age were offered a 23-valent pneumococcal vaccine (Pneumovax II®; Morson): those 6 to 18 months of age at recruitment received 2 doses 6 months apart and those older than 18 months received a single dose.

Information on HIV infection status, clinical disease status and history of previous bacterial infections was recorded at recruitment. Blood was taken for measurement of T lymphocyte cell subsets and baseline antibody titers against Hib and *S. pneumoniae*. ActHIB® (0.5 ml subcutaneously) and Pneumovax II® (0.5 ml subcutaneously) were given (one injection in each arm) and parents were asked to record local or systemic adverse reactions in the 72 hours after immunization. Blood for assay of antibody concentration was taken 4 weeks after completiong of the immunization schedule in both VI and U children. In VI children receiving monthly intravenous immunoglobulin therapy (IVIG) as prophylaxis against bacterial infections, immunizations were given 4 weeks after the last IVIG and a dose of IVIG was omitted. Postimmunization blood samples were taken 4 weeks later and before the next infusion of IVIG.

Ethical approval was obtained from both participating centers and written informed consent was obtained from all parents, and children where appropriate, before participation.

Laboratory methods. T lymphocyte cell subsets were measured by flow cytometry. Antibodies against Hib capsular polysaccharide (PRP), and pneumococcal serotypes 3, 6, 19 and 23 were measured by enzyme-linked immunosorbent assay as described previously.[19,20] For the pneumococcal assay antibodies were measured after adsorption of sera with cell wall polysaccharide.

Definition of response. A concentration of anti-PRP antibody of 1 μg/ml was taken as indicating long term protection.[21] Overall response to Pneumovax II® was defined as a 2-fold or greater increase in total IgG to at least one of the four vaccine serotypes (3, 6, 19 and 23) 4 weeks after completion of the vaccination schedule. In addition responses within the IgG2 subclass were defined as a 2-fold rise to at least one of the four serotypes.

Statistical power and analysis. Estimation of sample size with the use of the variance in antibody response in normal children[19] suggested that a total of 50 VI and 50 U children would allow the detection of at least a 2.5-fold difference in mean anti-PRP antibody titer with an alpha error of 0.05 and a beta error of 0.20.

Assuming that approximately 70% of immunocompetent children respond to Pneumovax II®,[20] a reduction to 40% in the VI children would be detected with 48 children in each group with the same alpha and beta error.

Statistical analyses were performed using the statistical packages Statistical Analysis System (SAS). Egret was used for logistic regression. Because T cell subsets in children vary with age, CD4 counts and percentages were expressed as standard deviation scores based on age-related values from uninfected children born to HIV-positive mothers.[22]

Antibody titers and increase in antibody titers to Hib (defined as the postimmunization titer divided by the

preimmunization titer) were positively skewed and therefore log-transformed before analysis. The proportions of children with >1 μg/ml Hib antibody levels postimmunization were compared in VI and U children using the chi square test. The increase in antibody titers was compared in VI and U children with the use of a regression model allowing for age and ethnicity and, in VI children, whether or not they had also received IVIG and azidothymidine.

The number of children responding to one or more of the pneumococcal serotypes was determined in VI and U children. Overall response (as defined above) was also compared using a chi square test and a logistic regression model, allowing for age and ethnicity, and for IVIG and azidothymidine therapy in VI children.

RESULTS

A total of 67 VI and 57 U children born to HIV-positive mothers were immunized, of whom all but 8 VI and 4 U children were more than 18 months of age. Adequate pre- and postimmunization serum samples were obtained from all but 24 children (11 VI, 13 U). This included infants who had interruptions of their schedule because of either illness (in 5 of the 8 VI infants) or missed appointments (4 U infants). The immunogenicity analysis was therefore restricted to VI and U children more than 18 months of age from whom there were adequate serum samples (56 VI, 44 U children).

Baseline characteristics. The age distribution in VI and U children was similar. This together with numbers of VI children in each disease stage is shown in Table 1. More VI than U children were of black African origin. In the 18 VI children receiving regular IVIG therapy, the median time between their last IVIG infusion and immunization was 32 days (range, 23 to 68 days). The median interval between immunization and measurement of postimmunization antibodies was 35 days (range, 23 to 98) in VI and 37 days (range, 21 to 91) in U children.

The adverse reaction rate to ActHIB® vaccine was low, with parents of only one VI and no U child reporting a local reaction to the immunization. Local adverse reactions to Pneumovax II® were reported in 4 (6%) of VI and 3 (5%) of U children. A further 5 (7%) VI and 2(4%) U children had a mild fever in the 24 hours after receiving the immunizations.

Response to Hib immunization. Before immunization 21% U and 20% VI children had anti-PRP antibody titers >1 μg/ml, indicating long term protection, whereas all but 1 VI and 5 U children had titers above 0.15 μg/ml, a concentratiom associated with short term protection.[19] Among the VI children those with long term protective titers were evenly distributed among children with AIDS, those with symptomatic disease and those who were asymptomatic. The proportion with long term protective titers was not significantly different in infected children who had received IVIG a month or more previously (7 of 37, 18%) compared with those who had not (5 of 18, 27%) ($P = 0.5$).

HIV was associated with a reduced response to ActHIB®. Whereas 100% of U children acquired long term protection (>1 μg/ml) postimmunization, this was achieved in only 86% of VI children ($P = 0.008$). The geometric mean increase in anti-PRP antibody was 7.6 (95% confidence interval, 3.5 to 16.3; $P = 0.0001$) times higher in U than in VI children after adjusting for age and ethnicity (Table 2). There was a significant trend toward increased response among the asymptomatic children compared with those with AIDS; this was not significantly affected after controlling for IVIG therapy (Table 3). No relationship was observed between age-adjusted CD4 counts and response to ActHIB® when either all children ($P = 0.9$) or only VI children ($P = 0.5$) were included in the regression model (Fig. 1). No relationship was observed between response and zidovudine therapy after adjusting for disease stage ($P = 0.9$).

Response to Pneumovax II®. Forty-one percent of U and 59% of VI children had no detectable antibody to 1 or more of the 4 pneumococcal serotypes before immunization ($P = 0.07$) and 1 of 44 (2%) U compared with 8 of 56 (14%) VI children had no antibodies to any of the serotypes ($P = 0.04$).

Postvaccination 61% of U and 54% of VI children responded to 1 or more of the 4 pneumococcal serotypes measured ($P = 0.4$). However, 45% of U compared with only 20% of VI children responded to 2 or more serotypes (Table 4). Among the U children, serotype 19 resulted in the most and serotype 6 in the fewest

TABLE 1. Characteristics of HIV-infected and uninfected children

Clinical Status	No. of Children	Age		CD4 Z score		Ethnicity Non-Caucasian*	IVIG	Zidovudine
		Median	Range	Median	Range			
AIDS	13	4.2	2.5–13.6	−5.8	<−7 to −1.7	7 (54)†	8 (62)	12 (92)
Symptomatic	24	4.1	1.7–8.1	−3.0	<−7 to +0.7	12 (50)	6 (25)	7 (29)
Asymptomatic	19	5.9	1.8–10.1	−2.4	<−7 to +0.3	4 (21)	4 (21)	4 (21)
Total HIV-infected	56	4.4	1.7–13.6			23 (41)	18 (32)	23 (41)
Uninfected	44	4.9	1.7–9.5	+0.24	−3.0 to +1.8	2 (5)	0	0

* All but one of the non-Caucasian children were Black African.

† Numbers in parentheses, percent.

TABLE 2. Geometric mean fold increase in anti-PRP antibody titers 1 month after Hib immunization, adjusted for age and ethnicity

	Mean Fold Increase*	Relative Response		P
		Crude	Adjusted	
Infection status				
HIV-infected	7.6	1.0	1.0	
HIV-uninfected	73.3	9.6	7.6 (3.5–16.3)†	0.0001
Age (per year)		0.99	1.03 (0.9–1.2)	0.7
Ethnicity				
Black	5.9	1.0	1.0	
White	31.2	5.6	2.0 (0.8–4.9)	0.11

* Geometric mean (post/preimmunization levels).
† Numbers in parentheses, range.

TABLE 3. Geometric mean fold increase in anti-PRP antibody titers in HIV-infected children by clinical disease status, adjusted for age, ethnicity and immunoglobulin therapy

	Mean Fold Increase*	Relative Response		P
		Crude	Adjusted	
Disease status				
AIDS	3.0	1.0	1.0	
Symptomatic	5.6	1.9	3.0 (1.2–8.1)†	
Asymptomatic	20.4	6.8	3.8 (1.1–13.4)	0.05
Age (per year)		0.99	1.03 (0.88–1.2)	0.7
Ethnicity				
Black	9.3	1.0	1.0	
White	5.7	0.6	0.7 (0.27–1.7)	0.4
Immunoglobulin Therapy				
Yes	3.6	1.0	1.0	
No	11.1	3.1	2.7 (0.98–7.4)	0.06

* Geometric mean (post-/preimmunization levels).
† Numbers in parentheses, range.

antibody responses (Table 5). Among VI children, the pattern was similar but responses to serotypes 3 and 19 were fewer (Table 5). In a logistic regression model allowing for age and ethnicity, the U children had a 1.5-fold (95% confidence interval, 0.6 to 3.8) adjusted relative response compared with VI children. Relative response allowing for age, ethnicity and IVIG therapy in children with AIDS compared with those who were asymptomatic was lower but the trend was not significant (data not shown). There was no association between response and zidovudine therapy.

IgG2-specific responses to individual serotypes followed a pattern similar to that pf total IgG responses (data not shown). IgG2 responses did not vary significantly with age.

DISCUSSION

Antibody responses to polysaccharide vaccines are impaired in adults with HIV infection and there has been debate about the possible advantages of immunizing them against Hib and *S. pneumoniae*.[23–25] Despite the paucity of immunogenicity or efficacy studies, these immunizations are presently recommended for children with HIV infection.[17]

ActHIB® and Pneumovax II® vaccines contain T cell-dependent and -independent antigens, respectively. In our study responses to ActHIB® in HIV-infected children and controls were similar to those observed in a study of Hib immunization in adults by Steinhoff et al.[24] That was a randomized trial of unconjugated and conjugated Hib vaccines given to HIV-infected men and controls. Although the percentage of men achieving long term protective titers >1 μg/ml after immunization is not stated in the paper, the fold (post/pre) responses were significantly lower in HIV-infected than in uninfected individuals and those with AIDS had significantly lower responses than those with asymptomatic disease.

Pediatric studies in this area are few and small, and the majority have been performed in infants. A study by Indacochea et al.[26] reported an Hib antibody response of >1 μg/ml in 4 of 5 infants immunized at 2, 4 and 6 months of age with an Hib conjugate vaccine. A further study by Walter et al.[27] reported significantly lower geometric mean titers of anti-PRP after 2 doses of a different Hib conjugate vaccine in 9 HIV-infected infants (geometric mean titer, 0.46 μg/ml) compared with 12 uninfected infants (geometric mean titer, 4.06 μg/ml). Only 2 of 9 HIV-infected and 7 of 12 uninfected infants achieved titers >1 μg/ml. In our study the children were older and although similar differences in response between HIV-infected and uninfected children were observed, 84% of infected and 100% uninfected children achieved titers >1 μg/ml. This contrasts with results from the only other study[28] of single dose Hib immunization in older children. In that study by Peters et al.[28] only 7 of 19 (37%) HIV-infected children older than 15 months of age had titers >0.15 μg/ml. However, titers were measured at a considerably longer interval after immunization (4 to 85 months) than in our study, and it is unknown whether the children failed to mount an adequate response after immunization or whether attrition with time was responsible for the lower levels. Our results would suggest the latter.

Although differences in anti-PRP responses between HIV-infected and uninfected children might be explained on the basis of impaired cellular immunity, we did not find antibody responses to the Hib conjugate vaccine correlated with age-adjusted CD4 count in infected children. This is in keeping with the results of Peters et al.[28] but contrasts with the findings of Steinhoff et al.[24] who observed a linear correlation between CD4 count and antibody responses to conjugated Hib vaccine in HIV-infected men, although not between CD4 count and responses to the unconjugated Hib vaccine. These differences between adults and children may be explained by the wide variance in CD4 counts in normal young children[22] and the relative lack of a clearcut association between an isolated CD4 count and disease progression in children compared with

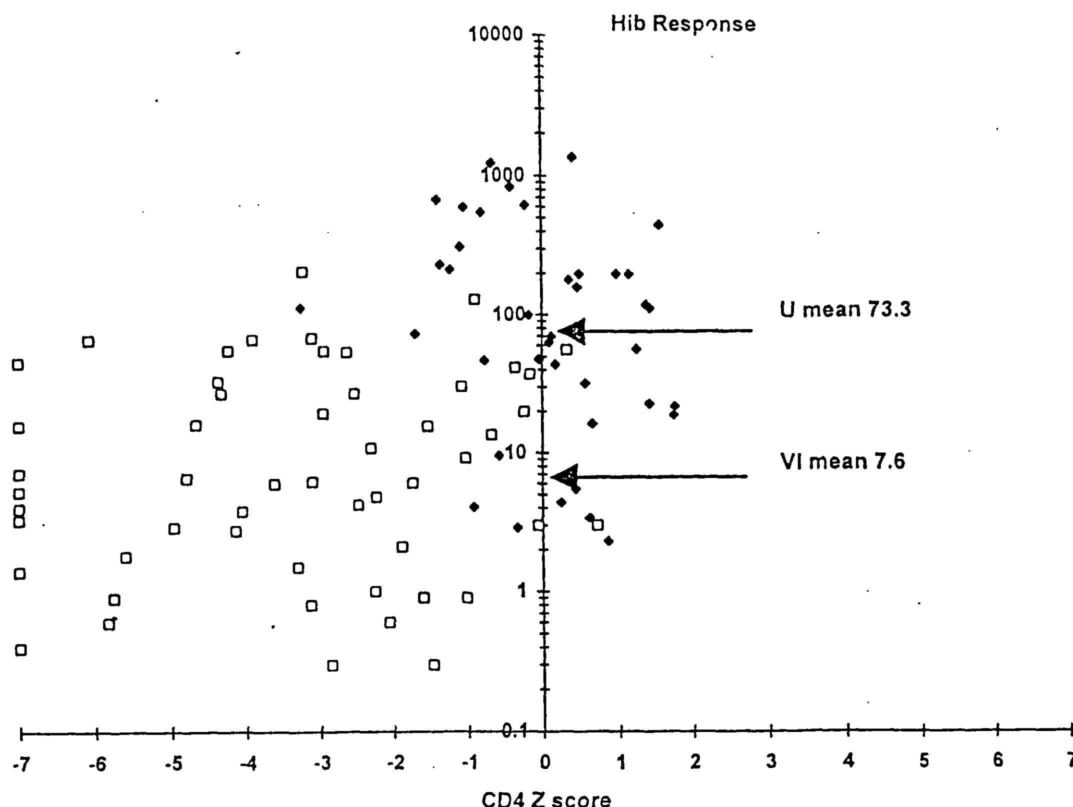

FIG. 1. Hib response (post-/preimmunization levels) on a log scale in HIV-infected () and uninfected (—) children, plotted against CD4 count expressed as an age-adjusted standard deviation score (z score) based on CD4 counts in uninfected children born to HIV-positive women.[22]

TABLE 4. IgG responses* to pneumococcal vaccine serotypes

No. of Serotypes Responded to	Infected	Uninfected
None	26 (46)†	17 (39)
1 or more	29 (54)	27 (61)
Serotype 1	19 (34)	7 (16)
Serotype 2	4 (7)	14 (32)
Serotype 3	3 (7)	5 (11)
Serotype 4	3 (6)	1 (2)
Total	56 (100)	44 (100)

* Defined as a 2-fold increase or more in titer.
† Numbers in parentheses, percent.

TABLE 5. IgG responses* to pneumococcal vaccine serotypes 3, 6, 19 and 23†

Pneumococcal Serotype	Infected Children (n = 56)	Uninfected Children (n = 44)
3	9/54 (17)‡	11/42 (26)
6	9/55 (16)	7/42 (17)
19	14/55 (25)	22/42 (52)
23	19/54 (35)	14/42 (33)
None	26 (46)	17 (39)

* Defined as a 2-fold increase or more in titer.
† Note: some children responded to more than 1 serotype.
‡ Numbers in parentheses, percent.

HIV-infected adults.[29] Furthermore the conjugate vaccine used in the study of Steinhoff et al. was the PRP oligosaccharide-mutant diphtheria conjugate and it is known that antibody responses to the different Hib conjugates vary in titer and affinity.[30]

Currently there is no "gold standard" for the measurement of pneumococcal antibodies and many studies have reported responses to whole pneumococcal vaccine without prior adsorption of the sera with pneumococcal cell wall polysaccharide. This technique has been shown to overestimate antibody responses; hence we chose to measure type-specific anti-pneumococcal responses to 4 clinically relevant serotypes after cell wall polysaccharide adsorption of the sera.[20]

In several studies symptomatic[31] as well as asymptomatic[32, 33] HIV-infected adults showed decreased responses to pneumococcal vaccine in total IgG[31, 32] and IgG2[34] antibody responses compared with HIV-negative controls. In these small studies the relation between T cell immunodeficiency, as measured by CD4 count or clinical disease stage, and antibody response was not established. Arpadi et al.[35] reported no difference in antibody titers to 7 common pneumococcal capsular polysaccharides assayed 1 to 16 months postimmunization in 11 HIV-infected children compared with age-matched unvaccinated HIV-infected and control children. The antibody titers achieved in this study were low in all groups of children.

In our study differences in antibody responses to the

pneumococcal serotypes did not reach statistical significance in the HIV-infected compared with uninfected groups when analyzed according to the number of individuals responding to more than one serotype. However, more detailed analysis showed differences in the numbers responding to 1, 2, 3 or 4 serotypes. The fact that fewer HIV-infected than uninfected children responded to more than one serotype suggests that antibody responses to T cell-independent antigens are impaired in HIV-infected children. The mechanism for this is not clear. IgG2 responses to the four pneumococcal serotypes were greater in uninfected than HIV-infected children, although differences were not significant. It may be that the mechanisms for both the poor immunogenicity of the unconjugated polysaccharide vaccines and the susceptibility of children to infection with encapsulated organisms do not lie solely in their inability to produce specific IgG2. Parkin et al.[34] suggested that an IgG2 deficiency might increase susceptibility to pyogenic infections in HIV patients. Although total serum IgG2 values were not measured in our study, they are a poor marker of the overall ability to produce antigen-specific IgG subclass responses, a more sensitive marker being the production of appropriate antibody subclass responses to immunization or infection.[36]

Children who were receiving IVIG as prophylaxis against bacterial infections had decreased responses to both vaccines. This did not reach statistical significance in the Hib multivariate model (P = 0.06) after allowing for disease stage, age and ethnicity. Children were vaccinated on the average of nearly 5 weeks after their last IVIG infusion and received no further infusion until after the blood sample had been taken for antibody assay 1 month later. Despite the lack of statistical significance it may be that IVIG interferes with the vaccine response and that children receiving therapy might benefit from a longer interval between the last IVIG and vaccine administration. An alternative explanation that the preimmunization Hib levels were elevated by contamination with antibody from the previous month's IVIG infusion is unlikely, inasmuch as the majority of children receiving IVIG had preimmunization Hib levels <1 μg/ml. We observed no difference in vaccine response in children receiving the antiretroviral drug, zidovudine, after controlling for disease status. This contrasts with a small study by Glaser et al.[37] suggesting that zidovudine might increase antibody responses to vaccines.

In conclusion our data support a policy of immunization with both vaccines in all HIV-infected children older than 18 months of age. Even children with AIDS may mount adequate antibody responses and hence the stage of disease should not be a contraindication to immunization. Although we have not reported data on children younger than 18 months of age the efficacy of conjugate Hib vaccines in immunocompetent infants has been well-documented and early immunization of HIV-infected children with Hib may well be appropriate. Although the current formulation of pneumococcal vaccines is not recommended in children younger than 18 months of age because of poor immunogenicity, it is possible that pneumococcal conjugate vaccines might be useful for younger HIV-infected children in the future.

Further research is required to evaluate the clinical efficacy of polysaccharide immunizations in HIV-infected children. In addition more research on the duration of anti-PRP and pneumococcal antibody titers in HIV-infected children may clarify the need for and optimal timing of reimmunization.

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Persistence of antibody responses to *Haemophilus influenzae* type b polysaccharide conjugate vaccine in children with vertically acquired human immunodeficiency virus infection

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Background. Recurrent bacterial sepsis is common in pediatric HIV infection and immunization against *Haemophilus influenzae* type b (Hib) is recommended. Long term persistence of anti-Hib antibody and the need for, or timing of, a booster dose has not been adequately studied.

Methods. Immunogenicity during a 12-month period following immunization with Hib-tetanus conjugate vaccine (ACT-HIB®; Merieux) was evaluated in 48 vertically HIV-infected children and 36 uninfected children, born to HIV-positive mothers. A titer of anti-Hib polysaccharide antibody of ≥0.15 μg/ml was considered to indicate short term and ≥1 μg/ml long term protection.

Results. At 1 month postvaccination 36 (100%) uninfected and 42 (88%) HIV-infected children achieved titers of ≥1 μg/ml. However, by 1 year titers had dropped below this value in 18 (43%) infected compared with only 4 (11%) uninfected children (chi square, 9.7; $P = 0.002$). Although the rate of fall of antibody titer was greater in uninfected than in infected children, this was no longer the case after adjustment for the 1-month postimmunization titer. The rate of antibody titer decline was not significantly related to HIV disease status or to either the age-related CD4 count at the time of immunization or the change in age-adjusted CD4 count during the 12 months after immunization.

Conclusions. Not only was the initial antibody response to Hib conjugate vaccine decreased in children with HIV infection and AIDS but also 1 year later only 57% of the initial responders had persisting titers above the level associated with long term protection. The need for reimmunization of children with HIV infection against Hib requires further evaluation.

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INTRODUCTION

Severe or recurrent bacterial infections are commonly observed in children with HIV infection and recurrent severe bacterial sepsis is therefore included in the 1987 Centers for Disease Control surveillance definition of pediatric AIDS. The organisms responsible for infections in children with HIV infection are similar to those affecting HIV-uninfected children and include *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib). The precise reason for the increased susceptibility to infection with encapsulated bacteria is unclear, and immunization against both Hib and *S. pneumoniae* is recommended.

We previously reported the results of a study designed to evaluate the immunogenicity of the Hib conjugate vaccine in children with vertically acquired HIV infection.[1] Although the vaccine was immunogenic in symptomatic and asymptomatic HIV-infected children, the magnitude of the Hib response was 7.6-fold less than in uninfected children, raising concerns about the longevity of protection. A circulating antibody titer of ≥1 μg/ml is associated with long term protection against invasive Hib infection, and antibody persistence is thought to be critical for long term protection. Little is known, however, about the persistence of Hib antibodies in HIV-infected children, and this information would be important for deciding on the need for and timing of a booster dose(s) of vaccine.

In this study we report the results of anti-Hib polysaccharide antibody persistence in relation to postimmunization antibody titers 12 months after Hib conjugate vaccine administration to a cohort of HIV-infected children at various stages of disease and a control group of uninfected children born to HIV-positive women.

METHODS

Subjects. The original group of 56 vertically HIV-infected and 44 uninfected children, born to HIV-infected women, who received Hib capsular polysaccharide (PRP) tetanus toxoid conjugate (ACT-HIB®, Merieux) between 1992 and 1994 has been described previously.[1] Of this group 48 HIV-infected and 36 uninfected children were available for follow-up 1 year postimmunization.

Follow-up. All children were >15 months of age and received a single dose of ACT-HIB®. At the time routine immunization against Hib was not available in either the UK or Italy; therefore none of the children had been immunized. Information on HIV clinical disease status in infected children and blood for T lymphocyte subsets on all children was collected at the time of immunization and at 1 and 12 months postimmunization. Infected children were prospectively classified into three groups, AIDS, symptomatic and asymptomatic. Classification was according to the CDC definition of AIDS in vertically HIV-infected children, except that children with mild lymphocytic interstitial pneumonitis were placed in the symptomatic and not the AIDS group. Children with lymphadenopathy, hepatomegaly and/or HIV-related thrombocytopenia only were assigned to the asymptomatic group.

In HIV-infected children receiving monthly intravenous immunoglobulin (IVIG) therapy as prophylaxis against bacterial infections, immunization was given instead of and 4 weeks after the last IVIG, and postimmunization blood samples were taken 4 weeks later and before the next infusion of IVIG. Similarly blood taken at 1 year postimmunization was drawn 4 weeks after the last dose of IVIG.

The protocol received ethical approval from the Hospitals for Sick Children, Great Ormond Street, London, and the Clinica Pediatrica, Padua, Italy. Written informed consent was obtained from all parents, and children where appropriate, before participation.

Laboratory methods. T lymphocyte cell subsets were measured by flow cytometry. Antibodies against Hib PRP were measured by enzyme-linked immunosorbent assay as previously described in detail.[2] The Oxford laboratory takes part in the European quality control scheme for the standardization of measurement of antibodies to Hib. In accordance with established convention, a PRP antibody titer of ≥0.15 μg/ml was taken as indicating short term protection and a titer of ≥1 μg/ml as indicating long term protection.

Statistical analyses. Statistical analyses were performed using the statistical package Statistical Analysis System (SAS, Cary, NC). CD4 counts and percentages were expressed as standard deviation scores based on age-related values from uninfected children born to HIV-positive mothers.[3] Anti-PRP antibody titers and change in titers (defined as the 1-month postimmunization titer divided by the 12-month postimmunization titer) were positively skewed and were therefore log transformed before analysis. The proportions of children with ≥0.15-μg/ml and ≥1-μg/ml antibody titers at the different time points were compared in HIV-infected and uninfected children by the chi square test. The rates of antibody titer decline were compared in infected and uninfected children with a regression model allowing for age, ethnicity and the titer achieved 1 month postimmunization. In infected children rates of antibody decline were compared by disease status, age-adjusted baseline CD4 count and CD4 count change, allowing for IVIG or zidovudine (azidothymidine) therapy received.

RESULTS

Of the original 56 HIV-infected children, 48 had antibody titers measured at a median of 419 (range, 315 to 693) days (13.8 months) after immunization. Five of the 8 remaining children who were immunized

ad died of AIDS and 3 were lost to follow-up. Thirty-ix uninfected children were evaluated a median of 393 range, 280 to 658) days (13.1 months) after immuni-ation; 8 uninfected children had been lost to follow-up r were reluctant to have blood taken. Details of isease status, age distribution and CD4 *Z* scores at he time of immunization as well as changes in CD4 *Z* core during the 12-month period of follow-up are hown in Table 1. More infected compared with unin-ected children were of black African origin. In the 19 nfected children receiving IVIG therapy as prophy-axis against bacterial infections, the median time etween their last IVIG infusion and the final postim-unization evaluation was 32 days (range, 28 to 175 days).

Anti-PRP titers: changes with time. The numbers of HIV-infected and uninfected children with anti-PRP titers >1.0 μg/ml at 1 and 12 months postimmunization are shown in Table 2. All 36 (100%) uninfected compared with 42 (88%) infected children had titers ≥1.0 μg/ml 1 month after immunization. Twelve months postimmunization 32 (89%) of the uninfected children but only 24 (57%) of the infected children who had responded to the initial immunization had anti-PRP titers ≥1.0 μg/ml (chi square, 9.7; $P = 0.002$). All uninfected and all but 1 of the infected children still had titers ≥0.15 μg/ml 12 months postimmunization.

Geometric mean titers at 1 month and 1 year after ACT-Hib® immunization are shown in Table 2. As previously reported the geometric mean increase in anti-PRP antibody after immunization was 7.6 (95% CI 3.5 to 16.3) times higher in uninfected than in infected children after adjusting for age and ethnicity. The rate of fall from these postimmunization values during the subsequent year was not related to age, ethnicity or whether a child was receiving IVIG therapy (Table 3). It was greater by a factor of 1.4 (95% CI 0.7 to 3.0) in the uninfected compared with the infected children after adjusting for these variables. However, there was a significant correlation between the 1-month and 1-year postimmunization titers in both infected and uninfected children (Fig. 1). If the 1-month postimmunization titers were also allowed for in the regression, the rate of fall was greater in infected than in uninfected children, although this did not reach statistical significance (Table 3).

Among infected children the rate of fall in antibody levels was not significantly different in children with AIDS or symptomatic disease compared with those with asymptomatic disease at the time of immunization, after adjusting for age, ethnicity, 1 month postimmunization titers and whether or not a child was receiving IVIG. Similarly neither the age-adjusted CD4 count (CD4 *Z* score) at the time of immunization nor the rate of fall of age-adjusted CD4 count (i.e. difference in CD4 *Z* score) during the year after immunization were significantly related to the rate of fall of anti-PRP antibody titers.

DISCUSSION

Antibody responses to the Hib conjugate vaccine are preserved in HIV-infected children and even the majority of children with AIDS are able to mount an antibody titer above the long term protective level of 1 μg/ml immediately postimmunization. Although the geometric mean titer (GMT) of anti-PRP antibody 1 month postimmunization is significantly higher in uninfected controls (7.6-fold), at 12 months this difference has fallen to just under 2-fold. This narrowing of the difference in GMTs is related to the more rapid decline in antibody titer in those with higher titers immediately postimmunization. When allowing for the GMT values 1 month postimmunization of the two study groups, the rate of decline of specific IgG appears to be similar in both groups and HIV infection does not appear to correlate with an increase in the turnover rate of specific IgG. This is in contrast to the findings and conclusions of Al-Attar et al.[4] who have recently reported the decline of measles antibody titers after the immunization of HIV-infected children. They concluded that a major factor in the previously reported low prevalence of measles antibody in this population was loss of antibody after an initial response. However, in their study no control group was included. They found no significant association between CD4 counts at the time of vaccination and the slope of decline of

TABLE 1. Characteristics of HIV-infected and -uninfected children

Clinical Status at Immunization	No. of Children	Median Age (yr) at Immunization	Median CD4 *Z* Score at Immunization	Median Fall in CD4 *Z* Score* 1 yr Postimmunization	Ethnicity: Non-Caucasian Origin†	IVIG‡	Zidovudine‡
AIDS	8	5.5 (1.7–13.6)§	−20 (−80 to −2.6)	6.8 (−8.5–66.1)	3	7	8
Symptomatic	22	4.6 (1.7–8.1)	−3.8 (−12.7 to 0.38)	−0.93 (−13.9–5.5)	11	7	6
Asymptomatic	18	5.3 (1.8–10.1)	−3.0 (−15.4 to 0.28)	−0.7 (−2.6–1.0)	4	5	3
Total HIV-infected	48	5.0 (1.7–13.6)	−6.0 (−80 to 0.38)	0.54 (−13.9–66.1)	23	19	17
Uninfected	36	4.7 (1.7–9.5)	−0.16 (−3.6 to 1.7)	0.01 (−2.1–2.7)	2		
Total	81				25		

* A negative sign denotes an increase in median CD4 *Z* score.

† All but one of the non-Caucasian children were Black African.

‡ At time of immunization.

§ Numbers in parentheses, range.

TABLE 2. Acquisition and persistence of protective antibody titers ≥1 μg/ml after immunization with ACT-HIB vaccine in HIV-infected and -uninfected children born to HIV-infected mothers

Infection Status	1 mo Postimmunization		1 year Postimmunization	
	GMT (μg/ml)	No. ≥1 μg/ml	GMT (μg/ml)	No. ≥1 μg/ml*
HIV-uninfected (n = 36)	38.3	36 (100)†	4.6	32 (89)
HIV-infected				
Asymptomatic (n = 18)	10.0	18 (100)	1.9	11 (61)
Symptomatic (n = 22)	3.1	17 (77)	0.8	9 (53)
AIDS (n = 8)	3.6	7 (88)	0.9	4 (57)
Total (n = 48)	4.9	42 (88)	1.2	24 (57)

* Data refer only to the children who had titers ≥1 μg/ml 1 month postimmunization.
† Numbers in parentheses, percent.

TABLE 3. Changes in anti-PRP IgG titers over time in HIV-infected and uninfected children born to HIV-infected women

	Mean Fold Fall*	Relative Response		P
		Crude	Adjusted	
Infection status				
HIV-infected	4.3	1.0	1.0	
HIV-uninfected	8.3	1.92	0.6 (0.3–1.1)†	0.07
Age (per yr)		1.03	0.99 (0.95–1.03)	0.8
Ethnicity				
White	6.4	1.7	1.1 (0.6–2.2)	
Black	3.7	1.0	1.0	0.5
Postimmunization titer		3.0	3.4 (2.4–4.9)	0.0001
Immunoglobulin therapy				
Yes	3.6	1.0	1.0	
No	6.5	1.8	1.2 (0.4–3.2)	0.6

* Ratio of geometric means (1 month post: 1 year postimmunization levels).
† Numbers in parentheses, 95% confidence interval.

measles antibody, a finding similar to that reported here.

The persistence of Hib antibody after vaccination has been studied by a number of groups although these studies have predominantly taken place in healthy infants who have received the vaccine in the first year of life.[5-9] In general most of the studies demonstrate that 1 year after immunization with a Hib conjugate vaccine, 100% of healthy infants have maintained an antibody titer above the minimum protective level of 0.15 μg/ml. Figures for those maintaining titers >1 μg/ml at 1 year range from 90 to 100%. Despite the difference in study designs, antibody persistence in our uninfected control group is similar to that in the published literature. Peters and Sunil[10] have studied the immunogenicity of Hib vaccines in HIV-infected children given a single dose of PRP or PRP conjugate. The 19 children described in their study received vaccines 15 to 56 months before analysis. No immediate postimmunization serologic data were presented, making it difficult to ascertain whether the low percentage of children with persisting antibody titers ≥1 μg/ml is the result of loss of antibody over time or an initial failure to seroconvert.

The relevance of the circulating anti-PRP antibody concentration as a surrogate marker of protection against Hib when immunity has been induced by a T-dependent vaccine has recently been questioned.[11] Protective antibody titers against invasive Hib disease were originally deduced from studies of a pure polysaccharide vaccine which, in contrast to the conjugate vaccines, does not induce immunologic memory.[12] In theory even in the absence of circulating antibody, an individual primed with a conjugate vaccine should be protected from infection because of an ability to produce high affinity IgG (anamnestic response) rapidly after subsequent contact with the bacterium or cross-reacting antigen. Although this may be true for vaccinees with a normal immune system, circulating specific antibody titers may be particularly important for the HIV-infected individual in whom the anamnestic response is impaired. This impairment might be caused by a variety of factors including the reported loss of CD45RO-positive memory.[13]

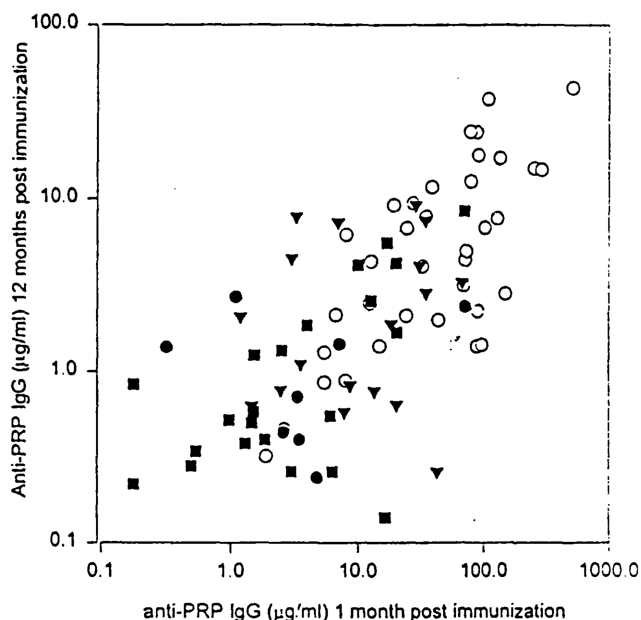

FIG. 1. Correlation between the 1-month and 1-year postimmunization titers in HIV-uninfected children (O) and infected children categorized according to those who at the time of immunization were asymptomatic (▼), were symptomatic (■) or had AIDS (●).

Children with HIV infection are able to mount an immune response to the Hib conjugate vaccine, al-

ough even in asymptomatic children this is reduced mpared with uninfected controls.[1] In addition a eater proportion of infected children who mount itial protective antibody responses have a significant duction in antibody titer 1 year after immunization. here are no clinical data in the literature on failure of ib immunization in HIV infection. These and further ta on the longevity and ability to boost the antibody sponse beyond this period are required to allow the tional timing of booster immunization.

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