PREVENTION OF HEPATITIS A AND B

BY IMMUNISATION

A Thesis submitted for the degree of Doctor of Medicine

by

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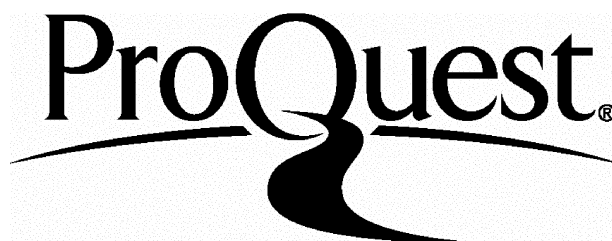
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ABSTRACT

1.   **Hepatitis A**

Prevention of hepatitis A by immunisation is a high priority in many
countries. Improvement in socio-economic conditions has resulted in a shift
from asymptomatic infection in early life to clinically significant illness with
increasing age. The immunogenicity, reactogenicity and safety of two
inactivated vaccines were studied by three clinical trials: 1) A randomised,
double blind, placebo-controlled study in 286 healthy volunteers of a
vaccine containing 720 ELU antigen prepared from the HM 175 hepatitis A
strain; 2) A study designed to protect susceptible patients with congenital
coagulation disorders by subcutaneous administration of vaccine using a
rapid immunisation schedule. 97 patients were enrolled; 3) A controlled,
randomised, comparative open trial of the 720 ELU vaccine with a new
vaccine prepared from the GBM strain of HAV in 210 volunteers, and
629 subjects enrolled in three other centres in France and Germany.

All three vaccine preparations were found to be highly immunogenic and
well-tolerated. A statistically significant effect of interaction between time
and vaccine was observed indicating that the kinetics of antibody responses
were different. The rapid immunisation schedule was found to be safe and
immunogenic in children and adults with congenital coagulation disorders.
CD4 counts indicated that patients with low counts did not seroconvert,
although there was no absolute correlation. There were, as expected, lower
seroconversion rates in patients infected with HIV in association with low
CD4 counts.

Serological testing 18 months after primary immunisation showed that all healthy subjects who received the 720 ELU antigen HM 175 vaccine and the 160 antigen units GBM vaccine had significant HAV antibody titres with a projected estimate of antibody persistence for 10 years or longer. The development of immunisation strategies is discussed.

2.   **Hepatitis B**

Between 5-15% of immunocompetent subjects do not produce protective surface antibody (anti-HBs) after immunisation with currently available S antigen vaccines. Non-responders remain susceptible to infection with HBV. A novel recombinant hepatitis B vaccine produced by transfection of mammalian cells and containing pre-S1, pre-S2 and S components of the viral coat protein of subtypes adw and ayw was evaluated in 86 true non-responder health care workers. 55/86 (64%) serconverted following a single dose of the vaccine. A single dose of 20 μg was as effective as two doses of 20 μg or 40 μg in terms of seroconversion, seroprotection or geometric mean titres using two different assays. As part of a separate collaborative study (which is not submitted with this thesis), a high frequency of HLA Class II allele DRB1*0701 and the phenotype B44;DRB1*0701;DQB1*0201 was found in non-responders compared to controls. All the initial non-responders expressing the phenotype B7;DRB1*1501;DQB1*0602 responded to the new vaccine. The majority of those who failed to mount an antibody response expressed two other phenotypes. Immunogenetic analysis thus confirmed that an initially distinct group of non-responders was indeed included in the vaccine study. The issues of repeated revaccination of non-responders and silent HBV infection are discussed.

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SECTION 1:

**A placebo-controlled study of an inactivated hepatitis A
vaccine prepared from the HM 175 strain of virus**

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**Accelerated Immunisation with high doses of
inactivated Hepatitis A vaccine of adults and children
with congenital coagulation disorders**

SECTION 3:

A multicentre clinical trial of two hepatitis A vaccines prepared from the HM 175 and the GBM strains of HAV

SECTION 4:

**Overcoming non-responsiveness to hepatitis B
immunisation in health care workers using a
novel recombinant vaccine**

INTRODUCTION AND SCOPE OF THE STUDY

Hepatitis A

Although infection with hepatitis A virus (HAV) is self-limited, with fulminant hepatitis and death occurring in only a small proportion of patients, it is a significant cause of morbidity (and consequently economic losses) in many parts of the world. Rates of infection are associated inversely with levels of sanitation and personal hygiene. Improvements in sanitation and hygiene can reduce the transmission of hepatitis A virus, but such improvements in countries with lower socio-economic conditions result paradoxically in an increase in the burden of significant clinical disease, because the peak rates of infection shift from early childhood, when infection is largely asymptomatic, to older age groups with symptomatic illness. Passive immunoprophylaxis using immune globulin can prevent disease in individuals who are exposed to HAV, but the protective effect is temporary (4-6 months) and immune globulin is not effective for controlling hepatitis A on a population level. The development of vaccines which provide active immunity represents a major advance in the ability to control HAV infection and reduce the burden of disease.

Two randomised controlled studies were undertaken in 1992-93 at the Royal Free Hospital School of Medicine to assess the reactogenicity, safety and immunogenicity of two inactivated hepatitis A vaccines prepared from two different strains of HAV, using two different immunisation schedules and vaccines with different antigen contents per dose in susceptible healthy adult volunteers.

A third study was undertaken in 1993 in susceptible adults and children with congenital coagulation defects attending the Haemophilia Centre and Haemostasis Unit of the Royal Free Hospital, using an accelerated immunisation schedule with reduced number of injections and administration by the subcutaneous route. The results established clearly the safety and high immunogenicity of the inactivated HAV vaccines which were studied.

Hepatitis B

Immunisation against hepatitis B is recognised as a high priority in preventive medicine in all countries. Strategies for immunisation against hepatitis B are being revised and universal vaccination of infants and adolescents is under examination as a possible strategy to control the transmission of this important infection. However, between 5-15% or more of healthy immunocompetent persons do not mount an antibody response to currently available plasma-derived and recombinant vaccines containing the purified S component of the hepatitis B surface antigen. Such individuals remain susceptible to infection, a factor of major importance to health care personnel at risk of parenteral exposure to hepatitis B virus.

A single centre study of 86 health care personnel who failed to respond to immunisation against hepatitis B with currently available vaccines was undertaken using a novel recombinant vaccine containing the pre-S1, pre-S2 and S antigens of the surface protein of hepatitis B virus expressed in a continuous mammalian cell line after transfection with recombinant HBsAg DNA using a bovine papillomavirus vector.

100 volunteers were recruited from various hospitals in London and from other regions of the country and 86 true non-responders participated in the study which was conducted between October and December 1994.

The overall rate of seroconversion in terms of hepatitis B surface antibody (anti-HBs) with a titre of >10 IU/l was 66% of the 86 non-responders, with rates ranging from 55-76% across different antigen dose groups. The kinetics of antibody response revealed another important finding namely that a single dose of the new vaccine was as effective as two doses. These results constitute a significant advance in protection against hepatitis B by immunisation.

VIRAL HEPATITIS. A BRIEF REVIEW

The last two decades have witnessed an explosion in knowledge of viral

hepatitis, a major public health problem throughout the world affecting

several hundreds of millions of people. Viral hepatitis is a cause of

considerable morbidity and mortality in the human population both from

acute infection and chronic sequelae which include, with hepatitis B and

hepatitis C infection, chronic active hepatitis, cirrhosis and primary liver

cancer.

The hepatitis viruses include a range of unrelated and often unusual human

pathogens:

Hepatitis A virus (HAV), a small unenveloped symmetrical RNA virus which

shares many of the characteristics of the picornavirus family. This virus has

been classified as hepatovirus within the heparnavirus genus and is the

cause of infectious or epidemic hepatitis transmitted by the faecal-oral route.

Hepatitis B virus (HBV), a member of the hepadnavirus group, double-

stranded DNA viruses which replicate by reverse transcription. Hepatitis B

virus is endemic in the human population and hyperendemic in many parts

of the world. Natural hepadnavirus infections also occur in other mammals

including woodchucks, beechy ground squirrels and ducks.

Hepatitis C virus (HCV), an enveloped single-stranded RNA virus which appears to be distantly related (possibly in its evolution) to flaviviruses, although hepatitis C is not transmitted by arthropod vectors. Several genotypes have been identified. Infection with this virus is common in many countries, and it is associated with chronic liver disease and also with primary liver cancer at least in some countries.

Hepatitis D virus (HDV) is an unusual single-stranded circular RNA virus with a number of similarities to certain plant viral satellites and viroids. This virus requires hepadnavirus helper functions for propagation in hepatocytes, and is an important cause of acute and severe chronic liver damage in some regions of the world.

Hepatitis E virus (HEV), is an enterically-transmitted non-enveloped, single-stranded RNA virus, which shares many biophysical and biochemical features with caliciviruses. Hepatitis E virus is an important cause of large epidemics of acute hepatitis in the subcontinent of India, Central and South-East Asia, the Middle East, parts of Africa and elsewhere; and this virus is responsible for high mortality during the third trimester of pregnancy.

The GB hepatitis viruses (GBV-A, GBV-B and GBV-C:HGV). The GB hepatitis viruses were cloned recently and preliminary genomic characterisation shows that they are related to other positive-stranded RNA viruses with local regions of sequence identity with various flaviviruses.

Phylogenetic analysis of genomic sequences showed that these viruses are not genotypes of hepatitis C virus. The hepatitis G virus (HGV) which was cloned independently, is believed to be very similar to if not identical with GBV-C.

HEPATITIS A

Outbreaks of jaundice have been described frequently for many centuries and the term infectious hepatitis was coined in 1912 to describe the epidemic form of the disease. Hepatitis A virus (HAV) is spread by the faecal-oral route and continues to be endemic throughout the world and hyperendemic in areas with poor standards of sanitation and hygiene. The seroprevalence of antibodies to HAV has declined since World War II in many countries, but large epidemics do occur. For example, an outbreak of hepatitis associated with the consumption of clams in Shanghai in 1988 resulted in almost 300,000 cases.

The incubation period of hepatitis A is about four weeks. The virus replicates in the liver. Very large quantities of virus are shed in the faeces during the incubation period before the onset of clinical symptoms. A brief period of viraemia occurs. The severity of illness ranges from the asymptomatic to anicteric or icteric hepatitis and rarely fulminant hepatitis. The virus is non-cytopathic when grown in cell culture. Its pathogenicity in vivo, which involves necrosis of parenchymal cells and histiocytic periportal inflammation, may be mediated via the cellular immune response.

By the time of onset of symptoms, excretion of virus in the faeces has declined and may have ceased and anti-HAV IgM, which is diagnostic of acute infection, increases in titre. Anti-HAV IgG may be detected one to two weeks later and persists for years.

Classification

Examination by electron microscopy of concentrates of filtered faecal extracts from patients in the early stages of infection reveals 27 nm particles typical of the <u>Picornaviridae</u>. HAV was classified in 1983 in the genus Enterovirus (as enterovirus 72) of the family <u>Picornaviridae</u>, on the basis of its biophysical and biochemical characteristics, including stability at low pH. However, this classification pre-empted the isolation and analysis of complementary DNA (cDNA) clones leading to the determination of the entire nucleotide sequence of the viral genome (Cohen et al. 1987). Comparison with other picornavirus sequences revealed limited homology to the enteroviruses or, indeed, the rhinoviruses; although the structure and genome organisation is typical of the <u>Picornaviridae</u>. HAV has now been placed as hepatovirus within the heparnavirus genus.

Organisation of the HAV Genome

The HAV genome comprises about 7,500 nucleotides (nt) of positive sense RNA which is polyadenylated at the 3' end and has a polypeptide (VPg) attached to the 5' end. A single, large open reading frame (ORF) occupies most of the genome and encodes a polyprotein with a theoretical molecular

mass of M$_r$ 252,000. An untranslated region of around 735 nt precedes the ORF. Secondary structure within this region of the genome may be important for efficient translation of the RNA. There is also a short untranslated region at the 3' end of the HAV genome.

The viral polyprotein is processed to yield the structural (located at the amino-terminal end) and non-structural viral polypeptides. Many of the features of replication of the picornaviruses have been deduced from studies of prototype Enteroviruses and Rhinoviruses, in particular Poliovirus Type 1.

The three dimensional structures of a number of picornaviruses have been solved by high resolution crystallography: polypeptides VP1 (1D), VP2 (1B) and VP3 (1C) are exposed on the surface of the virion whilst VP4 (1A) is located internally. Following release of the structural domain from the polyprotein, the 3C protease cleaves the 1B/1C and 1C/1D junctions to yield VP0 (VP4 plus VP2), VP3 and VP1. The three polypeptides remain associated as a protomer and five protomers assemble to form a pentamer, so that the five copies of VP1 form the apex. Finally, twelve pentamers assemble around a molecule of viral RNA to form the icosahedral capsid. As the structure locks into place, most copies of VP0 cleave (presumably autocatalytically) to yield VP2 and VP4. However, in the case of HAV, it has not been possible to demonstrate VP4, which is predicted to comprise only 23 amino acids.

The functions of some of the other cleavage products of the polyprotein, such as 2B, 2C and 3A are less well understood. Product 3B corresponds to the genome-linked polypeptide VPg, which in other picornaviruses is the primer for the synthesis of both genomic sense RNA and the negative sense RNA found in replicative intermediates. Polypeptide 3AB may be the precursor of VPg. Finally, the 3D product seems to be the viral replicase and contains the gly-asp-asp motif common to viral RNA-dependent RNA polymerases.

Prevention and Control of Hepatitis A

In areas of high prevalence, most children are infected early in life and such infections are generally asymptomatic. Infections acquired later in life are of increasing clinical severity. Less than 10% of cases of acute hepatitis A in children up to the age of six are icteric but this increases to 40-50% in the 6-14 age group and to 70-80% in adults. Of 115,551 cases of hepatitis A in the USA between 1983 and 1987, only 9% of the cases, but more than 70% of the fatalities, were in those aged over 49. It is important, therefore, to protect those susceptible to this risk because of personal contact with infected individuals or because of travel to a highly endemic area.

Other groups at risk of hepatitis A infection include staff and residents of institutions for the mentally-handicapped, day care centres for children, sexually active male homosexuals, intravenous drug abusers, sewage workers, certain groups of health care workers such as medical students on elective studies in countries where hepatitis A is common, military personnel, and certain low socio-economic groups in defined community settings.

Patients with chronic liver disease, especially if visiting an endemic area, should be immunised against hepatitis A. In some developing countries, the incidence of clinical hepatitis A is increasing as improvements in socio-economic conditions result in infection later in life and strategies for immunisation are yet to be developed and agreed.

Passive immunisation

Control of hepatitis A infection is difficult. Since faecal shedding of the virus is at its highest during the late incubation period and the prodromal phase of the illness, strict isolation of cases is not a useful control measure. Spread of hepatitis A is reduced by simple hygienic measures and the sanitary disposal of excreta.

Normal human immunoglobulin, containing at least 100 international units (IU)/ml of anti-hepatitis A antibody, given intramuscularly before exposure to the virus or early during the incubation period will prevent or attenuate a clinical illness. The dosage should be at least 2 IU of anti-hepatitis A antibody/kg body weight, but in special cases such as pregnancy or in patients with liver disease that dosage may be doubled (Table 1). Immuno-globulin does not always prevent infection and excretion of hepatitis A virus, and inapparent or subclinical hepatitis may develop. The efficacy of passive immunisation is based on the presence of hepatitis A antibody in the immunoglobulin, and the minimum titre of antibody required for protection is believed to be about 10 IU/l.

HAV antibody titres vary among batches of pooled normal human immuno-
globulin and are decreasing in batches obtained from pooled plasma of
donors in industrialised countries, resulting in clinical cases despite
prophylaxis with immunoglobulin (Behrens and Doherty 1993).
Immunoglobulin is used most commonly for close personal contacts of
patients with hepatitis A and for those exposed to contaminated food.
Immunoglobulin has also been used effectively for controlling outbreaks in
institutions such as homes for the mentally handicapped and in nursery
schools. Prophylaxis with immunoglobulin is recommended for persons
without hepatitis A antibody visiting highly endemic areas. After a period of
six months the administration of immunoglobulin for travellers needs to be
repeated, unless it has been demonstrated that the recipient had developed
hepatitis A antibodies.

**Table 1: Passive immunisation with normal immunoglobulin
for travellers to highly endemic areas**

Person's Body Weight	Period of stay < 3 months	Period of stay > 3 months
< 25 kg	50 IU anti-HAV (0.5 ml)	100 IU anti-HAV (1.0 ml)
25-30 kg	100 IU anti-HAV (1.0 ml)	250 IU anti-HAV (2.5 ml)
< 50 kg	200 IU anti-HAV (2.0 ml)	500 IU anti-HAV (5.0 ml)

Active immunisation against hepatitis A

In areas of high prevalence, most children have antibodies to hepatitis A
virus by the age of three years and such infections are generally
asymptomatic. Infections acquired later in life are of increasing clinical
severity. It is important, therefore, to protect those at risk because
of personal contact or because of travel to highly endemic areas.

Other groups at risk of hepatitis A infection include staff and residents of institutions for the mentally-handicapped, day care centres for children, sexually active male homosexuals, intravenous narcotic drug abusers, food handlers, sewage workers, health care workers, military personnel, and certain low socio-economic groups in defined community settings. In some developing countries, the incidence of clinical hepatitis A is increasing as improvements in socio-economic conditions result in infection later in life and protection by immunisation would be prudent, but strategies are yet to be agreed.

Killed hepatitis A vaccines

The foundations for a hepatitis A vaccine were laid in 1975 by the demonstration that formalin-inactivated virus extracted from the liver of infected marmosets induced protective antibodies in susceptible marmosets on challenge with live virus (Provost and Hilleman 1975). Subsequently hepatitis A virus was cultivated, after serial passage in marmosets, in a cloned line of fetal rhesus monkey kidney cells (FRhK6), thereby opening the way to the production of hepatitis A vaccines (Provost and Hilleman 1979). Later it was demonstrated that prior adaptation in marmosets was not a prerequisite to growth of the virus in cell cultures and various strains of virus have been isolated directly from clinical material using several cell lines, including human diploid fibroblasts. Various techniques have been employed to increase the yield of virus in cell culture (reviewed by Ellis and Provost 1989). Safety and immunogenicity studies of two formalin-inactivated hepatitis A vaccines are described in this thesis. One of the vaccines has been licensed in several countries at the end of 1992. Other preparations are under clinical trial.

Live attenuated hepatitis A vaccines

The major advantages of live attenuated vaccines (viz the Sabin type of oral poliomyelitis vaccines) include the ease of administration on a large scale by the oral route, relatively low cost (since the virus vaccine strain replicates in the gut) the production of both local immunity in the gut and humoral immunity, thereby mimicking natural infection, and also longer term protection. Disadvantages include the potential of reversion towards virulence, interference with the vaccine strain by other viruses in the gut, relative instability of the vaccine and shedding of the virus strain in the faeces for prolonged periods.

The most extensively studied live attenuated hepatitis A vaccines are based on the CR 326 and HM 175 strains of the virus attenuated by prolonged passage in cell culture.

Two variants of the CR 326 strain have been investigated after passage in marmoset liver in FRhK6, MRC5 and WI-38 cells. Inoculation of susceptible marmosets demonstrated seroconversion, and protection on challenge. Biochemical evidence of liver damage did not occur in susceptible chimpanzees, although a number had histological evidence of mild hepatitis with the F variant and the vaccine virus was shed in the faeces for about 12 weeks prior to seroconversion. There was no evidence of reversion towards virulence. Studies in human volunteers indicated incomplete attenuation of the F variant, but better results were obtained with the F^1 variant without elevation of liver enzymes.

Studies with the HM 175 strain, which was isolated and passaged in African green monkey kidney cells, showed that this strain was not fully attenuated for marmosets, although it did not induce liver damage on challenge. Further passages and adaptation of HM 175 revealed some evidence of virus replication in the liver of chimpanzees and minimal shedding of the virus into faeces. Other studies are in progress in non-human primates.

As with vaccine strains of polioviruses, attenuation may be associated with mutations in the 5' non-coding region of the genome which affect secondary structure. There is also evidence that mutations in the region of the genome encoding the non-structural polypeptides may be important for adaptation to cell culture and attenuation. However, markers of attenuation of HAV have not been identified and reversion to virulence may also be a problem. On the other hand, there is also concern that 'over-attenuated' viruses may not be sufficiently immunogenic.

Current candidate live attenuated hepatitis A vaccines require administration by injection. Preparations which may be suitable for oral administration are not available so far.

HEPATITIS E

Retrospective testing of serum samples from patients involved in various epidemics of hepatitis associated with contamination of water supplies with human faeces indicated that an agent other than HAV (or hepatitis B) was involved. Epidemics of enterically transmitted non-A, non-B hepatitis in the Indian subcontinent were first reported in 1980, but outbreaks involving tens

of thousands of cases have also been documented in the USSR, South-East Asia, Northern Africa, Mexico and previously in India. The average incubation period is slightly longer than for hepatitis A, with a mean of six weeks. The highest attack rates are found in young adults and high mortality rates of up to 20% have been reported in women during pregnancy.

Virus-like particles have been detected in the faeces of infected individuals by immune electron microscopy using convalescent serum. However, such studies have often proved inconclusive and a large proportion of the excreted virus may be degraded during passage through the gut. The particles have a mean diameter of 32-34 nm. Cross reaction studies between sera and virus in faeces associated with a variety of epidemics in several different countries suggests that a single serotype of virus is involved.

Studies on hepatitis E virus (HEV), have progressed following transmission to susceptible non-human primates. HEV was first transmitted to cynomolgous macaques and a number of other species of monkeys including chimpanzees also have been infected. Attempts to amplify the virus by replication in cell culture have been unsuccessful.

Hepatitis E virus was cloned in 1991 and the entire 7.5 kb sequence is known. The organisation of the genome is distinct from the Picornaviridae and the non-structural and structural polypeptides are encoded respectively at the 5' and 3' ends. HEV resembles the Caliciviruses in the size and organisation of its genome as well as the size and morphology of the virion (reviewed by Krawczynski 1993).

Sequencing of the HEV genome has allowed the development of a number of specific diagnostic tests. For example, HEV RNA was detected, using the polymerase chain reaction (PCR), in faecal samples obtained during a recent epidemic in Kanpur (North India). An enzyme immunoadsorbent assay, which detects both IgG and IgM anti-HEV, has been developed using a recombinant HEV-glutathione-S-transferase fusion protein and used to detect antibodies in sporadic cases of enterically transmitted non-A, non-B hepatitis in children in Egypt.

Preliminary but significant progress has been reported (Purdy et al. 1993) towards the development of hepatitis E vaccine, using the trpE-C2 fusion protein. In limited experiments, 3 doses of the fusion protein, which represents the carboxyl two-thirds of the putative capsid protein, prevented the development of biochemical evidence of hepatitis after challenge with wild-type virus.

HEPATITIS B

Hepatitis B virus was originally recognised as the agent responsible for "serum hepatitis", the most common form of parenterally transmitted viral hepatitis, and an important cause of acute and chronic infection of the liver. The incubation period of hepatitis B is variable, with a range of 1 to 6 months. The clinical features of acute infection resemble those of the other viral hepatitides. Acute hepatitis B is frequently anicteric and asymptomatic although a severe illness with jaundice can occur and occasionally acute liver failure may develop.

Distinctive properties

The virus persists in approximately 1-10% of immunocompetent adults, and in as many as 90% of infants infected perinatally. Persistent carriage of hepatitis B defined by the presence of hepatitis B surface antigen (HBsAg) in the serum for more than six months, has been estimated to affect about 350 million people worldwide. The pathology is mediated by the responses of the cellular immune response of the host to the infected hepatocytes. Long term continuing virus replication may lead to progression to chronic liver disease, cirrhosis and hepatocellular carcinoma.

In the first phase of chronicity, virus replication continues in the liver and replicative intermediates of the viral genome may be detected in DNA extracted from liver biopsies. Markers of virus replication in serum include HBV DNA, the surface proteins (HBsAg) and a soluble antigen, hepatitis B e antigen (HBeAg) which is secreted by infected hepatocytes. In those infected at a very young age this phase may persist for life but, more usually, virus levels decline over time. Eventually, in many individuals, there is immune targetting of infected hepatocytes associated with seroconversion from HBeAg to anti-HBe.

During the period of replication, the viral genome may integrate into the chromosomal DNA of some hepatocytes and these cells may persist and expand clonally. Rarely, seroconversion to anti-HBs follows clearance of virus replication but, more frequently, HBsAg persists during a second phase of chronicity as a result of the expression of integrated viral DNA.

Structure of the Virus

The hepatitis B virion is a 42 nm particle comprising an electron-dense core (nucleocapsid) 27 nm in diameter surrounded by an outer envelope of the surface protein (HBsAg) embedded in membraneous lipid derived from the host cell (Figure 1). The surface antigen is produced in excess by the infected hepatocytes and is secreted in the form of 22 nm particles and tubular structures of the same diameter (initially referred to as Australia antigen).

The 22 nm particles are composed of the major surface protein in both non-glycosylated (p 24) and glycosylated (gp 27) form in approximately equimolar amounts, together with a minority component of the so-called middle proteins (gp 33 and gp 36) which contain the pre-S2 domain, a glycosylated 55 amino acid N-terminal extension. The surface of the virion has a similar composition but also contains the large surface proteins (gp 39 and gp 42) which include both the pre-S1 and pre-S2 regions. These large surface proteins are not found in the 22 nm spherical particles (but may be present in the tubular forms in highly viraemic individuals) and their detection in serum correlates with viraemia. The domain which binds to the specific HBV receptor on the hepatocyte is believed to reside within the pre-S1 region.

The nucleocapsid of the virion consists of the viral genome surrounded by the core antigen (HBcAg). The genome, which is approximately 3.2 kilobases in length, has an unusual structure and is composed of two linear strands of DNA held in a circular configuration by base-pairing at the 5' ends.
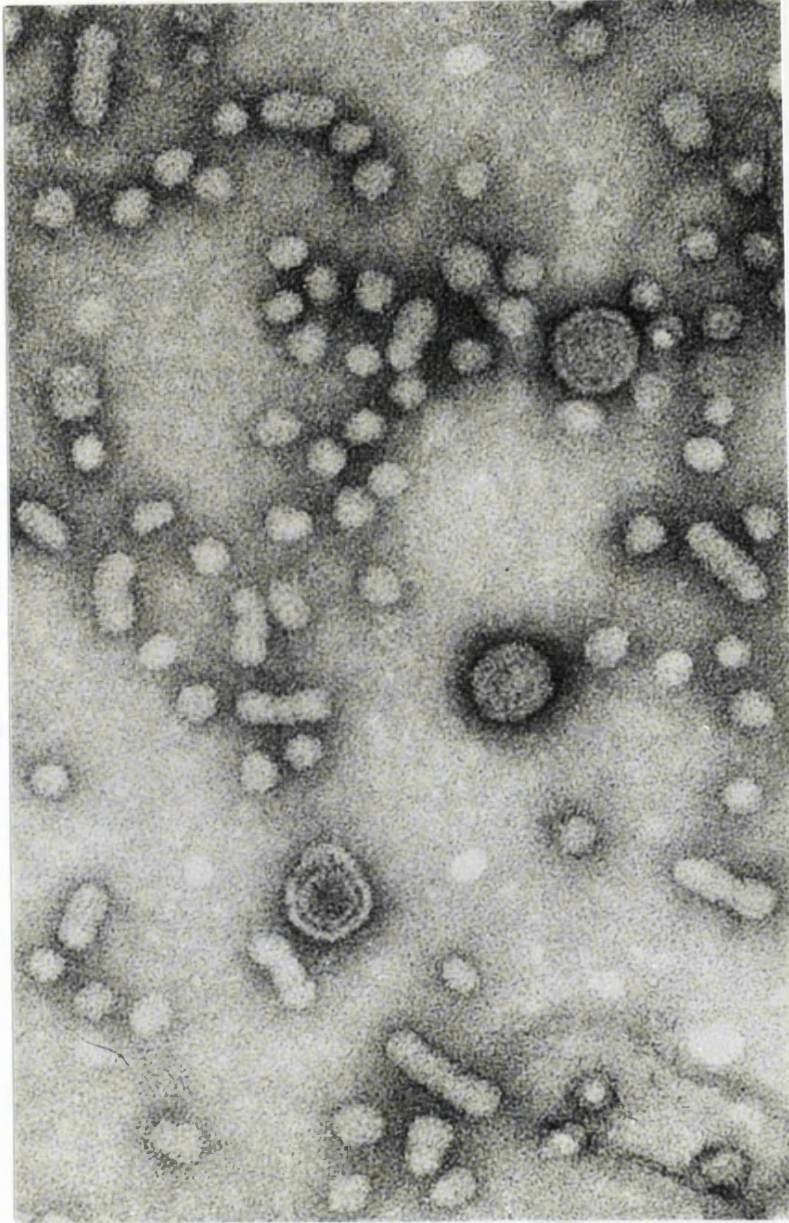
Figure 1: Electron micrograph of whole serum showing the three

morphological forms of hepatitis B: small pleomorphic

surface antigen particles; tubular forms of varying length;

and the complete double-shelled hepatitis B virions

x 252 000

One of the strands is incomplete and the 3' end is associated with a DNA polymerase molecule which is able to complete that strand in the presence of deoxynucleoside triphosphates.

Organisation of the HBV Genome

The genomes of more than a dozen isolates of hepatitis B virus have been cloned and the complete nucleotide sequences determined. Analysis of the coding potential of the genome reveals four open reading frames (ORFs) which are conserved between all of these isolates (Figure 2).

The first ORF encodes the various forms of the surface protein and contains three in-frame methionine codons which are used for initiation of translation. A second promoter is located upstream of the pre-S1 initiation codon. This directs the synthesis of a 2.4 kb mRNA which is co-terminal with the other surface messages and is translated to yield the large (pre-S1) surface proteins.

The core open reading frame also has two in-phase initiation codons. The "pre-core" region is highly conserved, has the properties of a signal sequence and is responsible for the secretion of HBeAg.

The third ORF, which is the largest and overlaps the other three, encodes the viral polymerase. This protein appears to be another translation product of the 3.5 kb RNA, and is synthesised apparently following internal initiation of the ribosome. The amino terminal domain is believed to be the protein

Figure 2: The genome of hepatitis B virus
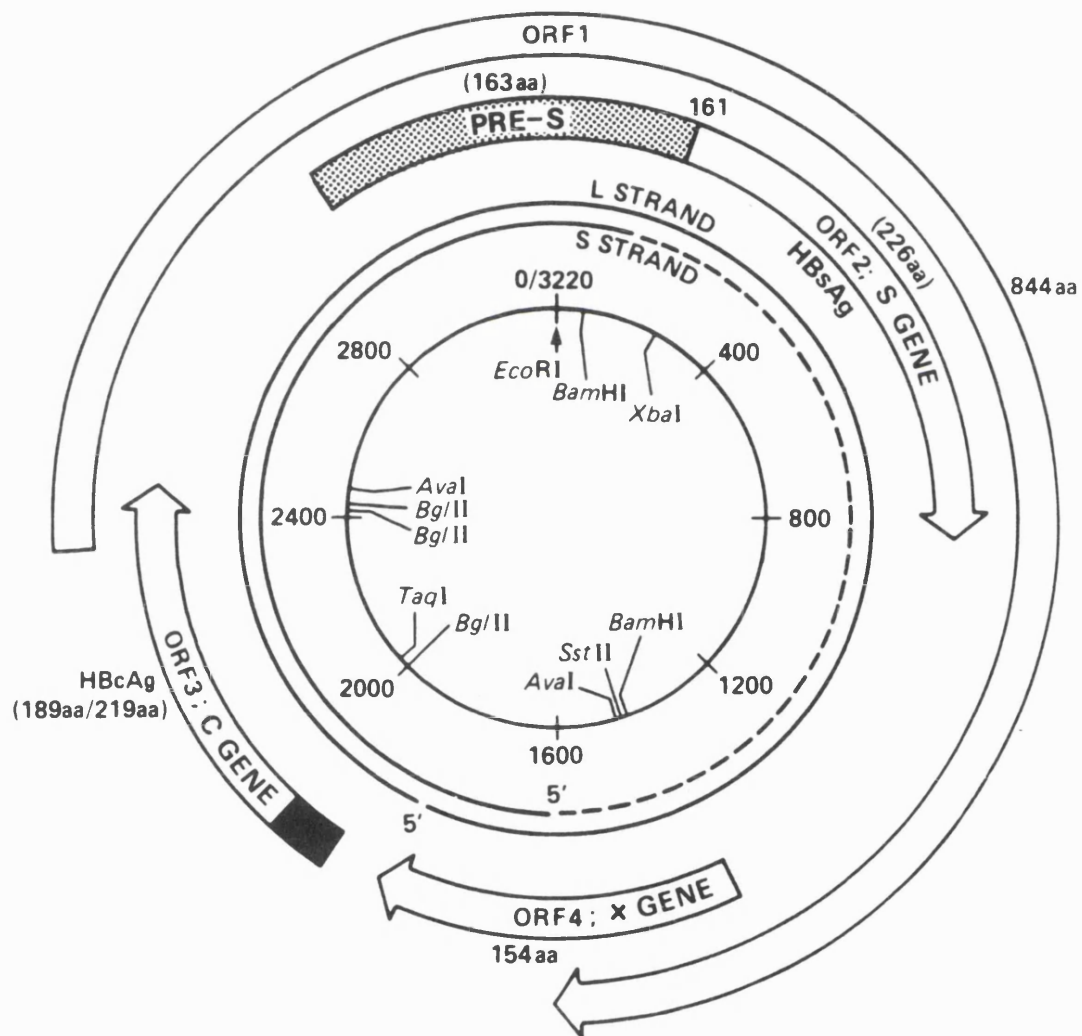
primer for minus strand synthesis. There is then a spacer region followed by the (RNA and DNA-dependent) DNA polymerase.

The fourth ORF was designated "x" because the function of its small gene product was not known. However, "x" has now been demonstrated to be a transcriptional transactivator.

Host Defences

Antibody and cell-mediated immune responses to various types of antigens are induced during the infection; however, these do not always seem to be protective and, in some instances, may cause autoimmune phenomena that contribute to disease pathogenesis. The immune response to infection with hepatitis B virus is directed toward at least three antigens: hepatitis B surface antigen, the core antigen, and the e antigen. The view that hepatitis B exerts its damaging effect on hepatocytes by direct cytopathic changes is inconsistent with the persistence of large quantities of surface antigen in liver cells of many apparently healthy persons who are carriers. Additional evidence suggests that the pathogenesis of liver damage in the course of hepatitis B infection is related to the immune response by the host.

The surface antigen appears in the sera of most patients during the incubation period, 2-8 weeks before biochemical evidence of liver damage or onset of jaundice. The antigen persists during the acute illness and usually clears from the circulation during convalescence. Next to appear in the circulation is the virus-associated DNA polymerase activity, which

correlates in time with damage to liver cells as indicated by elevated serum transaminases. The polymerase activity persists for days or weeks in acute cases and for months or years in some persistent carriers. Antibody to the core antigen is found in the serum 2-10 weeks after the surface antigen appears, and it is frequently detectable for many years after recovery. The titre of core antibody appears to correlate with the amount and duration of virus replication. Finally, antibody to the surface antigen component appears.

During the incubation period and during the acute phase of the illness, surface antigen-antibody complexes may be found in the sera of some patients. Immune complexes have been found by electron microscopy in the sera of all patients with fulminant hepatitis, but are seen only infrequently in non-fulminant infection. Immune complexes also are important in the pathogenesis of other disease syndromes characterised by severe damage of blood vessels (for example, polyarteritis nodosa, some forms of chronic glomerulonephritis, and infantile papular acrodermatitis).

Immune complexes have been identified in variable proportions of patients with virtually all the recognized chronic sequelae of acute hepatitis. Deposits of such immune complexes have also been demonstrated in the cytoplasm and plasma membrane of hepatocytes and on or in the nuclei; why only a small proportion of patients with circulating complexes develop vasculitis or polyarteritis is, however, not clear. Perhaps complexes are critical pathogenic factors only if they are of a particular size and of a certain antigen-to-antibody ratio.

Cellular immune responses are known to be particularly important in determining the clinical features and course of viral infections. The occurrence of cell-mediated immunity to hepatitis B antigens has been demonstrated in most patients during the acute phase of hepatitis B and in a significant proportion of patients with surface-antigen-positive chronic active hepatitis, but not in asymptomatic persistent hepatitis B carriers. These observations suggest that cell-mediated immunity may be important in terminating the infection and, under certain circumstances, in promoting liver damage and in the genesis of autoimmunity. Also, evidence suggests that progressive liver damage may result from an autoimmune reaction directed against hepatocyte membrane antigens, initiated in many cases by infection with hepatitis B virus. Although exogenous interferon may be effective in treating some patients with chronic hepatitis, as yet endogenous interferon production has not been detected during the natural infection. More studies to define the role of interferon are needed.

Epidemiology

Although various body fluids (blood, saliva, menstrual and vaginal discharges, serous exudates, seminal fluid, and breast milk) have been implicated in the spread of infection, infectivity appears to be especially related to blood. The epidemiological propensities of this infection are therefore wide; they include infection by inadequately sterilized syringes and instruments, transmission by unscreened blood transfusion and blood products, by close contact, and by sexual contact. Antenatal (rarely) and perinatal (frequently) transmission of hepatitis B infection from mother to child may take place; in some parts of the world (South-East Asia and Japan), perinatal transmission is very common.

Diagnosis

Direct demonstration of virus in serum samples is feasible by visualizing the virus particles by electron microscopy, by detecting virus-associated DNA polymerase, and by assay of viral DNA. All these direct techniques are impractical under general diagnostic laboratory conditions, and specific diagnosis must therefore rely on serological tests.

Hepatitis B surface antigen first appears during the late stages of the incubation period and is easily detectable by radioimmunoassay or enzyme immunoassay. The antigen persists during the acute phase of the disease and sharply decreases when antibody to the surface antigen becomes detectable. Antibody of the IgM class to the core antigen is found in the serum after the onset of the clinical symptoms and slowly declines after recovery. Its persistence at high titre suggests continuation of the infection. Core antibody of the IgG class persists for many years and provides evidence of past infection.

Protection against hepatitis B

The discovery of variation in the epitopes presented on the surface of the virions and subviral particles identified several subtypes of HBV which differ in their geographical distribution. All isolates of the virus share a common epitope, a, which is a domain of the major surface protein which is believed to protrude as a double loop from the surface of the particle. Two other pairs of mutually exclusive antigenic determinants, d or y and w or r, are also present on the major surface protein. These variations have been correlated with

single nucleotide changes in the surface ORF which lead to variation in single amino acids in the protein. Four principal subtypes of HBV are recognised: adw, adr, ayw and ayr. Subtype adw predominates in Northern Europe, the Americas and Australasia and also is found in Africa and Asia. Subtype ayw is found in the Mediterranean region, Eastern Europe, Northern and Western Africa, the near East and the Indian subcontinent. In the Far East, adr predominates but the rarer ayr occasionally may be found in Japan and Papua New Guinea.

Passive immunisation

Hepatitis B immunoglobulin (HBIG) is prepared from pooled plasma with high titre of hepatitis B surface antibody and may confer temporary passive immunity under certain defined conditions. The major indication for the administration of hepatitis B immunoglobulin is a single acute exposure to hepatitis B virus, such as occurs when blood containing surface antigen is inoculated, ingested or splashed onto mucous membranes and the conjunctiva. The optimal dose has not been established but doses in the range of 250-500 IU have been used effectively. It should be administered as early as possible after exposure and preferably within 48 hours, usually 3 ml (containing 200 IU of anti-HBs per ml) in adults. It should not be administered seven days following exposure. It is generally recommended that two doses of hepatitis B immunoglobulin should be given 30 days apart.

Results with the use of hepatitis B immunoglobulin for prophylaxis in babies at risk of infection with hepatitis B virus are encouraging if the immunoglobulin is given as soon as possible after birth or within 12 hours of birth. Hepatitis B immunoglobulin reduces the chance of the baby developing the persistent carrier state by about 70%. More recent studies using combined passive and active immunisation indicate an efficacy approaching 90%. The dose of hepatitis B immunoglobulin recommended in the newborn is 1-2 ml (200 IU of anti-HBs per ml).

Active immunisation

The major response of recipients of hepatitis B vaccine is to the common a epitope with consequent protection against all subtypes of the virus. First generation vaccines were prepared from 22 nm HBsAg particles purified from plasma donations from chronic carriers. These preparations are safe and immunogenic but have been superseded in some countries by recombinant vaccines produced by the expression of HBsAg in yeast cells. The expression plasmid contains only the 3' portion of the HBV surface ORF and only the major surface protein, without pre-S epitopes, is produced. Vaccines containing pre-S2 and pre-S1 as well as the major surface proteins expressed by recombinant DNA technology are undergoing clinical trial, and one such preparation has been evaluated as part of the work embodied in this thesis (Section 4).

In many areas of the world with a high prevalence of HBsAg carriage, such as China and South-East Asia, the predominant route of transmission is perinatal. Although HBV does not usually cross the placenta, the infants of viraemic mothers have a very high risk of infection at the time of birth.

Administration of a course of vaccine with the first dose immediately after birth is effective in preventing transmission from an HBeAg-positive mother in approximately 70% of cases and this protective efficacy rate may be increased to greater than 90% if the vaccine is accompanied by the simultaneous administration of hepatitis B immune globulin (HBIG).

Immunisation against hepatitis B is now recognised as a high priority in preventive medicine in all countries and strategies for immunisation are being revised. Universal vaccination of infants and adolescents is under examination as a possible strategy to control the transmission of this infection. About 75 countries now offer hepatitis B vaccine to all children, including the United States.

However, immunisation against hepatitis B is at present recommended in a number of countries with a low prevalence of hepatitis B only to groups which are at an increased risk of acquiring this infection. These groups include individuals requiring repeated transfusions of blood or blood products, prolonged in-patient treatment, patients who require frequent tissue penetration or need repeated circulatory access, patients with natural or acquired immune deficiency and patients with malignant diseases. Viral hepatitis is an occupational hazard among health care personnel and the staff of institutions for the mentally-retarded, and in some semi-closed institutions. High rates of infection with hepatitis B occur in narcotic drug addicts and intravenous drug abusers, sexually active male homosexuals and prostitutes. Individuals working in high endemic areas are, however, at an increased risk of infections and should be immunised.

Young infants, children and susceptible persons (including travellers) living in certain tropical and sub-tropical areas where present socio-economic conditions are poor and the prevalence of hepatitis B is high should also be immunised.

Site of injection for vaccination and antibody response

Hepatitis B vaccination should be given in the upper arm or the anterolateral aspect of the thigh and not in the buttock. There are over 100 reports of unexpectedly low antibody seroconversion rates after hepatitis B vaccination using injection into the buttock. In one centre in the United States a low antibody response was noted in 54% of healthy adult health care personnel. Many studies have since shown that the antibody response rate was significantly higher in centres using deltoid injection than centres using the buttock. On the basis of antibody tests after vaccination, the Advisory Committee on Immunization Practices of the Centers of Disease Control, USA recommended that the arm be used as the site for hepatitis B vaccination in adults, as have the Departments of Health in the United Kingdom.

A comprehensive study in the United States showed that participants who received the vaccine in the deltoid had antibody titres that were up to 17 times higher than those of subjects who received the injections into the buttock (Shaw et al. 1989). Furthermore, those who were injected in the buttock were two to four times more likely to fail to reach a minimum antibody level of 10 IU/l after vaccination. Recent reports have also implicated buttock injection as a possible factor in a failure of rabies post-exposure prophylaxis using a human diploid cell rabies vaccine.

The injection of vaccine into deep fat in the buttocks is likely with needles shorter than 5 cm, and there is a lack of phagocytic or antigen presenting cells in layers of fat. Another factor may involve the rapidity of which antigen becomes available to the circulation from deposition in fat, leading to delay in processing by macrophages and eventually presentation to T and B cells. An additional factor may be denaturation by enzymes of antigen which has remained in fat for hours or days. The importance of these factors is supported by the finding at the Royal Free and elsewhere that thicker skin fold was associated with a lowered antibody response (Cockcroft et al. 1990).

These observations have important public health implications, well illustrated by the estimate that about 20% of subjects immunised against hepatitis B via the buttock in the United States by March 1985 (about 60,000 people) failed to attain a minimum level of antibody of 10 IU/l and were therefore not protected.

Hepatitis B surface antibody titres should be measured in all individuals who have been immunised against hepatitis B by injection into the buttocks, and when this is not possible a complete course of three injections of vaccine should be administered into the deltoid muscle or the anterolateral aspect of the thigh, the only acceptable sites for hepatitis B immunisation (Zuckerman et al. 1992).

Apart from the site of injection there are several other factors which are associated with a poor or no antibody response to currently licensed

vaccines reviewed in Section 4. Indeed all studies of antibody response to plasma-derived hepatitis B vaccines and hepatitis B vaccines prepared by recombinant DNA technology have shown that between 5% and 10% or more of healthy immunocompetent subjects do not mount an antibody response (anti-HBs) to the surface antigen component (HBsAg) present in these preparations (non-responders) or that they respond poorly (hypo-responders). The exact proportion depends partly on the definition of non-responsiveness or hypo-responsiveness, generally less than 10 IU/l or 100 IU/l respectively against an international antibody standard.

A clinical study of a novel hepatitis B vaccine in true persistent non-responders to immunisation was undertaken as part of this thesis and is described in Section 4.

Hepatitis B Antibody Escape Mutants

Production of antibodies to the group antigenic determinant a mediates cross-protection against all sub-types, as has been demonstrated by challenge with a second subtype of the virus following recovery from an initial experimental infection. The epitope a is located in the region of amino acids 124-148 of the major surface protein, and appears to have a double-loop conformation. A monoclonal antibody which recognises a region within this a epitope is capable of neutralising the infectivity of hepatitis B virus for chimpanzees, and competitive inhibition assays using the same monoclonal antibody demonstrate that equivalent antibodies are present in the sera of subjects immunised with either plasma-derived or recombinant hepatitis B vaccine.

During a study of the immunogenicity and efficacy of hepatitis B vaccines in Italy, a number of individuals who had apparently mounted a successful immune response and became anti-surface antibody (anti-HBs)-positive, later became infected with HBV. These cases were characterised by the co-existence of non-complexed anti-HBs and HBsAg, and in 32 of 44 vaccinated subjects there were other markers of hepatitis B infection.

Furthermore, analysis of the antigen using monoclonal antibodies suggested that the a epitope was either absent or masked by antibody. Subsequent sequence analysis of the virus from one of these cases revealed a mutation in the nucleotide sequence encoding the a epitope, the consequence of which was a substitution of arginine for glycine at amino acid position 145 (Carman et al. 1990).

There is now considerable evidence for a wide geographical distribution of the point mutation in hepatitis B virus from guanosine to adenosine at position 587, resulting in an amino acid substitution at position 145 from glycine to arginine in the highly antigenic group determinant a of the surface antigen. This stable mutation has been found in viral isolates from children several years later and it has been described in Italy, Singapore, Japan, and Brunei, and from liver transplant recipients with hepatitis B in the USA, Germany, and the UK who had been treated with specific hepatitis B immunoglobulin or humanised hepatitis B monoclonal antibody.

The region in which this mutation occurs is an important virus epitope to which vaccine-induced neutralising antibody binds as discussed above,

and the mutant virus is not neutralised by antibody to this specificity. It can replicate as a competent virus, implying that the amino acid substitution does not alter the attachment of the virus to the liver cell. Variants of HBV with altered antigenicity of the envelope protein show that HBV is not as antigenically singular as previously believed and that humoral escape mutation can occur in vivo. This finding gives rise to two causes for concern: failure to detect HBsAg may lead to transmission through donated blood or organs, and HBV may infect individuals who are anti-HBs positive after immunisation. Variation in the second loop of the a determinant seems especially important. Mutants, variants, altered genotypes, and unusual strains are now being sought in many laboratories (Zuckerman et al. 1994).

HBV Precore mutants

Vaudin et al. (1988) reported the nucleotide sequence of the genome of a strain of HBV cloned from the serum of a naturally infected chimpanzee. A surprising feature was a point mutation in the penultimate codon of the pre-core region which changed the tryptophan codon (TGG) to an amber termination codon (TAG). The nucleotide sequence of the HBV pre-core region from a number of anti-HBe-positive Greek patients was investigated by direct sequencing PCR-amplified HBV DNA from serum (Carman et al. 1991). An identical mutation of the penultimate codon of the pre-core region to a termination codon was found in seven of eight anti-HBe positive patients who were positive for HBV DNA in serum by hybridisation. In most cases there was an additional mutation in the proceeding codon.

Similar variants were found by amplification of HBV DNA from serum from anti-HBe positive patients in Italy and Greece. These variants are not confined to the Mediterranean region; the same nonsense mutation (without a second mutation in the adjacent codon) has been observed in patients from Japan and elsewhere along with rarer examples of defective pre-core regions caused by frameshifts or loss of the initiation codon for the pre-core region.

In many cases, pre-core variants have been described in patients with severe chronic liver disease and who may have failed to respond to therapy with interferon. This observation raises the question of whether they are more pathogenic than the wild-type virus.

HBV and Hepatocellular Carcinoma

When tests for HBsAg became widely available, regions of the world where the chronic carrier state is common were found to be coincident with those where there is a high prevalence of primary liver cancer. Furthermore, in these areas, patients with tumour almost invariably are seropositive for HBsAg. A prospective study in Taiwan revealed that 184 cases of hepatocellular carcinoma (HCC) occurred in 3,454 carriers of HBsAg at the start of the study, but only ten such tumours arose in the 19,253 control males who were HBsAg negative (Beasley and Hwang 1991).

Southern hybridisation of tumour DNA yields evidence of chromosomal integration of viral sequences in at least 80% of HCCs from HBsAg carriers.

There is no similarity in the pattern of integration between different tumours, and variation is seen both in the integration site(s) and in the number of copies or partial copies of the viral genome. Sequence analysis of the integrants reveals that the direct repeats in the viral genome often lie close to the virus/cell junctions, suggesting that sequences around the ends of the viral genome may be involved in recombination with host DNA. Integration seems to involve microdeletion of host sequences and rearrangements and deletions of part of the viral genome also may occur. When an intact surface gene is present, the tumour cells may produce and secrete HBsAg in the form of 22 nm particles. Production of HBcAg by tumours is rare, however, and the core ORF is often incomplete and modifications such as methylation may also modulate its expression. Cytotoxic T cells targeted against core gene products on the hepatocyte surface seem to be the major mechanism of clearance of infected cells from the liver. Thus, there may be immune selection of cells with integrated viral DNA which are incapable of expressing HBcAg.

The mechanisms of oncogenesis by HBV remain obscure. HBV may act non-specifically by stimulating active regeneration and cirrhosis which may be associated with long-term chronicity. However, HBV-associated tumours occasionally arise in the absence of cirrhosis and such hypotheses do not explain the frequent finding of integrated viral DNA in tumours. In rare instances, the viral genome has been found to be integrated into cellular genes such as cyclin A and a retinoic acid receptor. Trans-locations and other chromosomal rearrangements also have been observed. Although insertional mutagenesis of HBV remains an attractive hypothesis

to explain its oncogenicity, there is insufficient supportive evidence.

The x gene may be an important transcriptional activator, and may interact with the p53 gene.

Like many other cancers, development of hepatocellular carcinoma is likely to be a multifactorial process. The clonal expansion of cells with integrated viral DNA seems to be an early stage in this process and such clones may accumulate in the liver throughout the period of active virus replication. In areas where the prevalence of primary liver cancer is high, virus infection usually occurs at an early age and virus replication may be prolonged although the peak incidence of tumour is many years after the initial infection.

HEPATITIS D

Delta hepatitis (reviewed in Gerin et al. 1991) was first recognised following detection of a novel protein, delta antigen (HDAg), by immunofluorescent staining in the nuclei of hepatocytes from patients with chronic active hepatitis B. Hepatitis delta virus (HDV) is now known to require a helper function of HBV for its transmission. HDV is coated with HBsAg which is needed for release from the host hepatocyte and for entry in the next round of infection.

Two forms of delta hepatitis infection are known. In the first, a susceptible individual is co-infected with HBV and HDV, often leading to a more severe form of acute hepatitis caused by HBV. Vaccination against HBV also prevents co-infection. In the second, an individual chronically infected with HBV becomes superinfected with HDV. This may cause a second episode

of clinical hepatitis and accelerate the course of the chronic liver disease, or cause overt disease in asymptomatic HBsAg carriers. HDV itself seems to be cytopathic and HDAg may be directly cytotoxic.

Delta hepatitis is common in some areas of the world with a high prevalence of HBV infection, particularly the Mediterreranean region, parts of Eastern Europe, the Middle East, Africa and South America. It has been estimated that 5% of HBsAg carriers worldwide (approximately 18 million people) are infected with HDV. In areas of low prevalence of HBV, those at risk of hepatitis B, particularly intravenous drug abusers, are also at risk of HDV infection.

Distinctive Properties of HDV

The HDV particle is approximately 36 nm in diameter and composed of an RNA genome associated with HDAg, surrounded by an envelope of HBsAg. The HDV genome is a closed circular RNA molecule of 1679 nucleotides and resembles those of the satellite viroids and virusoids of plants and similarly seems to be replicated by the host RNA polymerase II with autocatalytic cleavage and circularisation of the progeny genomes via trans-esterification reactions (ribozyme activity). Consensus sequences of viroids which are believed to be involved in these processes also are conserved in HDV.

Unlike the plant viroids, HDV codes for a protein, HDAg. This is encoded in an open reading frame in the antigenomic RNA but four other open reading frames which are also present in the genome do not appear to be used.

The antigen, which contains a nuclear localisation signal, was detected originally in the nuclei of infected hepatocytes and may be detected in serum only after stripping off the HBsAg outer envelope of the virus with detergent.

HEPATITIS C

Transmission studies in chimpanzees established that the main agent of parenterally acquired non-A, non-B hepatitis was likely to be an enveloped virus some 30 to 60 nm in diameter. These studies made available a pool of plasma which contained a relatively high titre of the agent. In order to clone the genome, the virus was pelleted from the plasma. Because it was not known whether the genome was DNA or RNA, a denaturation step was included prior to the synthesis of complementary DNA so that either DNA or RNA could serve as a template. The resultant cDNA was then inserted into the bacteriophage expression vector lambda gt 11 and the libraries screened using serum from a patient with chronic non-A, non-B hepatitis. This approach led to the detection of a clone (designated 5-1-1) which was found to bind to antibodies present in the sera of several individuals infected with non-A, non-B hepatitis. This clone was used as a probe to detect a larger, overlapping clone in the same library. It was possible to demonstrate that these sequences hybridised to a positive-sense RNA molecule of around 10,000 nt which was present in the livers of infected chimpanzees but not in uninfected controls. No homologous sequences could be detected in the chimpanzee or human genomes. By employing a "walking" technique, it was possible to use newly detected overlapping clones as hybridisation probes in turn to detect further virus-specific

clones in the library. Thus, clones covering the entire viral genome were assembled and the complete nucleotide sequence determined (reviewed in Houghton et al. 1993).

Diagnosis of HCV Infection

Successful cloning of portions of the viral genome permitted the development of new diagnostic tests for infection by the virus. Since the 5-1-1 antigen was originally detected by antibodies in the serum of an infected patient it was an obvious candidate as the basis of an ELISA to detect anti-HCV antibodies. A larger clone, C100, was assembled from a number of overlapping clones and expressed in yeast as a fusion protein using human superoxide dismutase sequences to facilitate expression. This fusion protein formed the basis of first generation tests for HCV infection. The 5-1-1 antigen comprises amino acid sequences from the non-structural, NS4, region of the genome and C100 contains both NS3 and NS4 sequences.

It is now known that antibodies to C100 are detected relatively late following an acute infection. Furthermore, the first generation ELISAs were associated with a high rate of false positive reactions when applied to low incidence populations and there were further problems with some retrospective studies on stored sera.

Second generation tests include antigens from the nucleocapsid and further non-structural regions of the genome. The former antigen (C22) is particularly useful and antibodies to the HCV core protein appear relatively

early in infection. These second generation tests confirm that HCV is the major cause of parenterally transmitted non-A, non-B hepatitis. Routine testing of blood donations is now in place in many countries and prevalence rates vary from 0.2-0.5% in northern Europe to 1.2-1.5% in southern Europe and Japan. Most of those with antibody have a history of parenteral risk such as a history of transfusion or administration of blood products or of intravenous drug abuse. Sexual or perinatal transmission are not major factors in the epidemiology of HCV and it is not clear what are the natural routes of transmission.

The availability of the nucleotide sequence of HCV made possible the use of the polymerase chain reaction (PCR) as a direct test for the genome of the virus. There is considerable variation in nucleotide sequences among different isolates of HCV and the 5' non-coding region, which seems to be highly conserved, is the preferred target for the PCR.

Current data suggest that about 80% of infections with HCV progress to chronicity. Histological examination of liver biopsies from asymptomatic PCR positive HCV-carriers (blood donors) reveals that none has normal histology and that up to 70% have chronic active hepatitis and/or cirrhosis. Whether the virus is cytopathic or whether there is an immunopathological element remains unclear. HCV infection is also associated with progression to primary liver cancer. For example, in Japan, where the incidence of hepatocellular carcinoma has been increasing despite a decrease in the prevalence of HBsAg, HCV is now the major risk factor.

There is no DNA intermediate in the replication of the HCV genome or integration of viral nucleic acid and viral pathology may contribute to oncogenesis through cirrhosis and regeneration of liver cells. HCV rarely seems to cause fulminant hepatitis.

Distinctive Properties of HCV

The genome of HCV resembles those of the pestiviruses and flaviviruses in that it comprises around 10,000 nt of positive sense RNA, lacks a 3' polyA tract and has a similar gene organisation. It has been proposed that HCV should be the prototype of a third genus in the family Flaviviridae. All of these genomes contain a single large open reading frame which is translated to yield a polyprotein (of around 3000 amino acids in the case of HCV) from which the viral proteins are derived by post-translational cleavage and other modifications.

The amino acid sequence of the nucleocapsid protein seems to be highly conserved among different isolates of HCV. The next domain in the polyprotein also has a signal sequence at its carboxyl-terminus and may be processed in a similar fashion. The product is a glycoprotein which is probably found in the viral envelope and is variably termed E1/S or gp35. The third domain may be cleaved by a protease within the viral polyprotein to yield what is probably a second surface glycoprotein, E2/NS1 or gp70. These glycoproteins have not been found in vivo and the molecular sizes are estimated from sequence data and expression studies in vitro.

Other post-translational modifications, including further proteolytic cleavages, are possible. These proteins are the focus of considerable interest because of their potential use in tests for the direct detection of viral proteins and for HCV vaccines. Nucleotide sequencing studies reveal that both domains contain hypervariable regions. It is possible that this divergence has been driven by antibody selection pressure and that these regions specify important immunogenic epitopes.

The non-structural region of the HCV genome is divided into regions NS2 to NS5. In the flaviviruses, NS3 has two functional domains, a protease which is involved in cleavage of the non-structural region of the polyprotein and a helicase which is presumably involved in RNA replication. Motifs within this region of the HCV genome have homology to the appropriate consensus sequences, suggesting similar functions. NS5 seems to be the replicase and contains the gly-asp-asp motif common to viral RNA-dependent RNA polymerases (Figure 3).

Hepatitis C virus consists of a family of highly related but nevertheless distinct genotypes, numbering at present 6 genotypes and various subtypes with differing geographical distribution, and with a complex nomenclature. The C, NS3 and NS4 domains are the most highly conserved regions of the genome, and therefore these proteins are the most suitable for use as capture antigens for broadly reactive tests for antibodies to HCV. The sequence differences observed between HCV groups suggest that virus-host interactions may be different, which could result in differences in pathogenicity and in response to antiviral therapy.

Hepatitis C Viral Genome
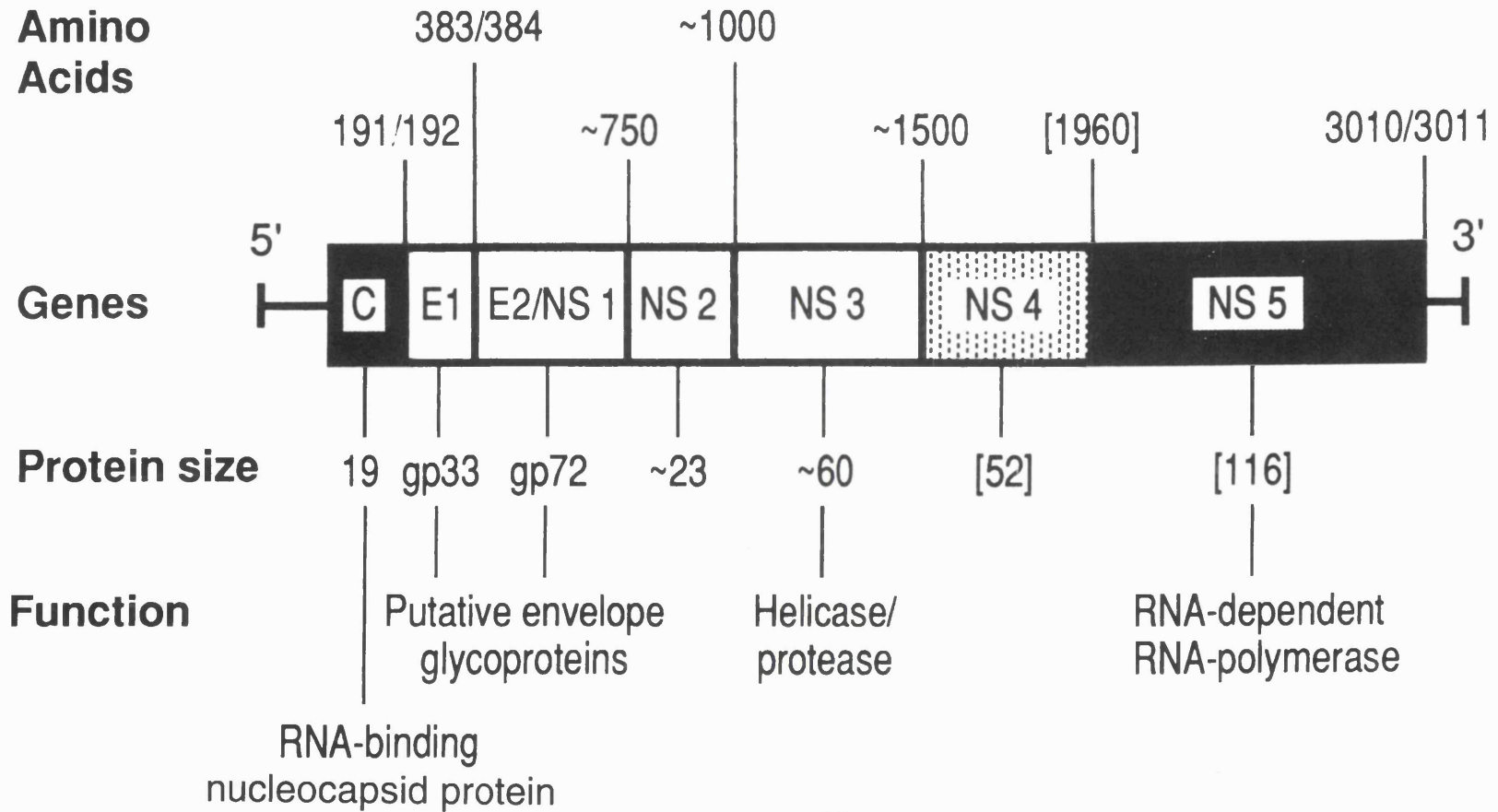
It is important, therefore, to develop group- and virus-specific tests.

The degree of divergence apparent within the viral envelope proteins implies the absence of a broad cross-neutralising antibody response to infection by viruses of different groups.

In addition to the sequence diversity observed between HCV groups, there is considerable sequence heterogeneity among almost all HCV isolates in the N-terminal region of E2/NS1, implying that this region may be under strong immune selection. Indeed, sequence changes within this region may occur during the evolution of disease in individual patients and may play an important role in progression to chronicity.

Vaccine Development

Problems in vaccine development include the sequence diversity between viral groups and the substantial sequence heterogeneity among isolates in the N-terminal region of E2/NS1. Neutralising antibodies have not been identified so far. The virus has not been cultivated in vitro to permit the development of inactivated or attenuated vaccines (cf. Yellow fever vaccines). Much work is in progress employing recombinant DNA techniques.

THE GB HEPATITIS VIRUSES AND HEPATITIS G VIRUS

About 30 years ago, a series of transmission studies of human viral hepatitis were initiated in small South American tamarins or marmosets, which were chosen because of their very limited contact with man implying that they were unlikely to have been infected with human viruses (Deinhardt et al. 1967).

A serum which was obtained on the third day of jaundice from a young surgeon (GB) with acute hepatitis induced hepatitis in each of four inoculated marmosets and was passaged serially in these animals. These important observations remained controversial until the application recently of modern molecular virological techniques (Schlauder et al. 1995). Preliminary results indicate the identification of two independent viruses, GBV-A and GBV-B, in the infectious plasma of tamarins inoculated with GB (Muerhoff et al. 1995; Simons et al. 1995a).

GBV-A does not replicate in the liver of tamarins whereas GBV-B causes hepatitis. Cross-challenge experiments showed that infection with the original infectious tamarin inoculum conferred protection from reinfection with GBV-B but not GBV-A. A third virus, GBV-C, was isolated subsequently from a human specimen which was immunoreactive with a GBV-B protein. GBV-C RNA was found in several patients with clinical hepatitis, and shown to have substantial sequence identity to GBV-A (Simons et al. 1995b).

A series of studies including phylogenetic analysis of genomic sequences showed that GBV-A, B, and C are not genotypes of hepatitis C virus, and that GBV-A and GBV-C are closely related. GBV-A/C and GBV-B and the hepatitis C viruses are members of distinct viral groups. The organisation of the genes of the GBV-A, B, and C genomes shows that they are related to other positive-strand RNA viruses with local regions of sequence identity with various flaviviruses. The three GB viruses and HCV share only limited overall amino acid sequence identity (Leary et al. 1996).

Serological reagents were prepared with recombinant antigens and limited testing for antibodies and by RT-PCR for specific RNA were carried out in groups of patients, blood donors and other selected individuals, patients with non-A, B, C, D, E hepatitis, multitransfused patients, intravenous drug addicts and other populations with a high incidence of viral hepatitis. Preliminary studies indicated the presence of antibody to each of the GB viruses in 3% to as many as 14%.

The development and availability of specific diagnostic reagents will establish the epidemiology of these newly identified viruses, their pathogenic significance in man and their clinical and public health importance. It should be noted that the virus identified more recently as hepatitis G (HGV) as a new transfusion transmitted agent (Linnen et al. 1996) is now believed to be another isolate of the same virus.

SECTION 1:

A placebo-controlled study of an inactivated hepatitis A vaccine

prepared from the HM 175 strain of virus

INTRODUCTION

The first study was a randomised double-blind, placebo controlled study to compare the immunogenicity and reactogenicity of an inactivated hepatitis A vaccine administered to healthy adult volunteers according to two different vaccination schedules (0, 14 days or 0, 1 month, with a booster at month 12). The vaccine was prepared from the HM 175 strain of hepatitis A virus by SmithKline Beecham Biologicals, Rixensart, Belgium.

The trial was conducted at the Royal Free Hospital School of Medicine in accordance with the 1989 Hong Kong Amendment of the Declaration of Helsinki after approval of the Local Ethical Practices Committee of the Royal Free Hospital and the School of Medicine. Volunteers were enrolled with full informed consent from November 1991 to April 1992.

Study objectives

The objectives of the study were:

- To evaluate the anti-HAV levels induced by the vaccine when administered at 0, 14-days or at 0, 1 month with a booster at month 12

- To compare the geometric mean titres (GMTs) obtained in the 0, 14 days, 12 months schedule groups vs the GMTs obtained in the 0, 1, 12 months schedule groups

- To compare the reactogenicity of the hepatitis A vaccine recipient groups vs the placebo recipient groups and the reactogenicity of the hepatitis A vaccine administered according to two different schedules.

<u>Selection of volunteers</u>

Adult male and female volunteers were sought from among the staff and undergraduate medical students of the Royal Free Hospital and School of Medicine by the distribution of information leaflets and by addressing groups of students. Volunteers were informed about the risks/benefits of the vaccine, the inclusion of a placebo control group, and the design and randomisation of the study. A signed informed consent was obtained from each subject, and subjects were informed that they would be free to withdraw from the study at any time.

<u>Inclusion criteria</u>

The subjects were:

- Between the ages of 18 and 65 years;

- In good health with normal cardiovascular, renal, respiratory and particularly liver function as established by history and physical examination at the time of entry into the study;

- Without clinical signs of acute disease at entry;

- Where appropriate, premenopausal female participants were on a contraceptive programme for at least two months before vaccination and agreed to continue contraception during the study and for a further two months after the last dose of vaccine to avoid becoming pregnant;

- They agreed not to participate simultaneously in any other clinical study;

- They agreed not to eat raw shellfish during the study period;

- They agreed not to travel to areas of medium to high endemicity for hepatitis A for 6 weeks prior to the first vaccination until after the blood sample had been collected following the second vaccine dose.

Exclusion criteria

- Travel to underdeveloped countries within previous 6 weeks;

- History of liver disease;

- History of alcohol abuse (defined as intake of more than 35 units of alcohol per week for men and 21 units of alcohol for women);

- Hepatomegaly, right upper quadrant abdominal pain or tenderness;

- Positive for anti-HAV antibodies;

- Abnormal liver enzymes: alanine aminotransferase (ALT) and aspartate aminotransferase (AST);

- Immunosuppressive illness or treatment with immunosuppressive drugs;

- Any maintenance drug treatment, or illness which in the investigator's opinion, precluded entry to the study.

Study design and randomisation

The volunteers were allocated into two equal groups according to the vaccination schedule, and each group was subdivided under double blind conditions to receive either the vaccine or the placebo.

Monodose vials were coded before delivery to the Centre according to a randomisation list prepared using an algorithm of pseudo random numbers.

Three vials were prepared for each subject, one for each vaccination and labelled with the study number, the subject's number and the vaccination number. A copy of the two randomisation lists (for Group 1 and Group 2 respectively) was provided to the investigator in a sealed envelope to be opened if justified by an adverse event, after consulting the independent medical referee.

Blood samples for screening for total antibodies to HAV and for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were taken within two weeks before entry and at 0, 14 days, 1, 2, 12 and 13 months respectively. A standard physical examination was also carried out on enrollment into the study and at other visits if indicated on clinical grounds. The subjects were allocated randomly to one of 4 groups; either vaccine or placebo control preparation, with vaccination schedules at day 0, month 1 and month 12 (Group 1) or day 0, day 14 and month 12 (Group 2).

Quantitative assessment of total HAV antibodies was carried out at months 1, 2, 12 and 13 (Table 2).

Inactivated hepatitis A vaccine

The vaccine was developed by SmithKline Beecham Biologicals, Rixensart, Belgium from a hepatitis A virus strain HM 175 first isolated from an Australian patient with hepatitis A (Daemer et al. 1982). The virus was propagated in culture on human diploid fibroblast cells. The cells were washed extensively to remove culture medium contaminants before extraction of the virus by lysis of the cells.

Table 2:

Flow Sheet

Group 1   (0, 1 month with a booster at month 12 schedule)

	Day 14-Day 1	Day 0	Day 14	Month 1	Month 2	Month 12	Month 13
Preliminary Visit	●						
Vaccine or Placebo Injection		●		●		●	
ALT	●		●	●	●	●	●
AST	●		●	●	●	●	●
Anti-HAV	●		●	●	●	●	●

Group 2   (0, 14 days with a booster at month 12 schedule)

	Day 14-Day 1	Day 0	Day 14	Month 1	Month 2	Month 12	Month 13
Preliminary Visit	●						
Vaccine or Placebo Injection		●	●			●	
ALT	●		●	●	●	●	●
AST	●		●	●	●	●	●
Anti-HAV	●		●	●	●	●	●

The suspension of viral particles was purified by ultrafiltration and by gel chromatography. The purified viral particles were sonicated and then inactivated with formaldehyde. The vaccine preparation met the Requirements of the World Health Organisation and those of the Bureau of Federal Drug Administration, USA for inactivated polio vaccine, using in vitro and in vivo tests to confirm the inactivation of infectious virus and the absence of other microbial contaminants. The vaccine also completed a series of tests to establish its suitability as a human vaccine (Andre et al. 1990; Wiedermann et al. 1990; Andre et al. 1992; Peetermans 1992).

The vaccine (batch VHA004AV) was supplied in monodose vials containing 1 ml of liquid vaccine with 720 ELISA units/ml of purified hepatitis A antigen protein, adsorbed to 0.5 mg aluminium hydroxide with 0.5% 2-phenoxyethanol as a preservative.

The placebo preparation had equivalent components to the vaccine apart from antigen, and was also supplied in monodose vials.

The vaccine or placebo was injected intramuscularly into the deltoid muscle, and each volunteer was observed for 15 minutes after inoculation.

Laboratory tests for hepatitis A antibodies

All volunteers were pre-screened for the presence of antibodies to HAV using enzyme immunosorbant assay (ELISA) which is a qualitative test (HAVAB EIA, Abbott Laboratories, UK). The cut-off level of this qualitative test is about 100 IU/l. Only volunteers below the cut-off level of this test

were considered as seronegative and were enrolled in the study. Serum

samples collected at months 1, 2, 12 and 13 were tested by ELISA for HAV

total antibodies (Hepanostika HAV antibody, Organon Teknika, the

Netherlands). Quantitation was carried out using the first Reference

Preparation Hepatitis A Immunoglobulin (WHO, 1981; nominal value of

100 IU per ampoule), supplied by the Central Laboratory of the Blood

Transfusion Service, Amsterdam, the Netherlands. Antibody concentrations

are expressed in International Units per litre (IU/l).

This assay is based on a modified 'sandwich inhibition' principle. The IgG

fraction of human anti-HAV positive serum is coated on the solid-phase.

The test sample or the appropriate controls are incubated simultaneously

with a fixed amount of formaldehyde-inactivated HAV antigen of faecal origin

from chimpanzees. After a wash cycle and incubation with anti-HAV

(human origin; $F(ab)_2$ fragment) labelled with horseradish peroxidase (HRP)

as the conjugate, a wash cycle follows. Colour is developed with

tetramethyl-benzidine (TMB)/peroxide substrate, and the reaction is stopped

by the addition of sulphuric acid. Adsorbances are read at 450 nm.

The limit of detection of this test was found to be 50 IU/l when calculated by

reading off the standard curve, the mean adsorbance of twenty samples

without antibodies to HAV, minus 2 ± SD (Figure 4).

An aliquot of each serum was stored frozen at -20°C, and the sera were sent

to the laboratory of SmithKline Beecham Biologicals, Belgium for independent

testing for anti-HAV, by an inhibition ELISA developed in their laboratory.
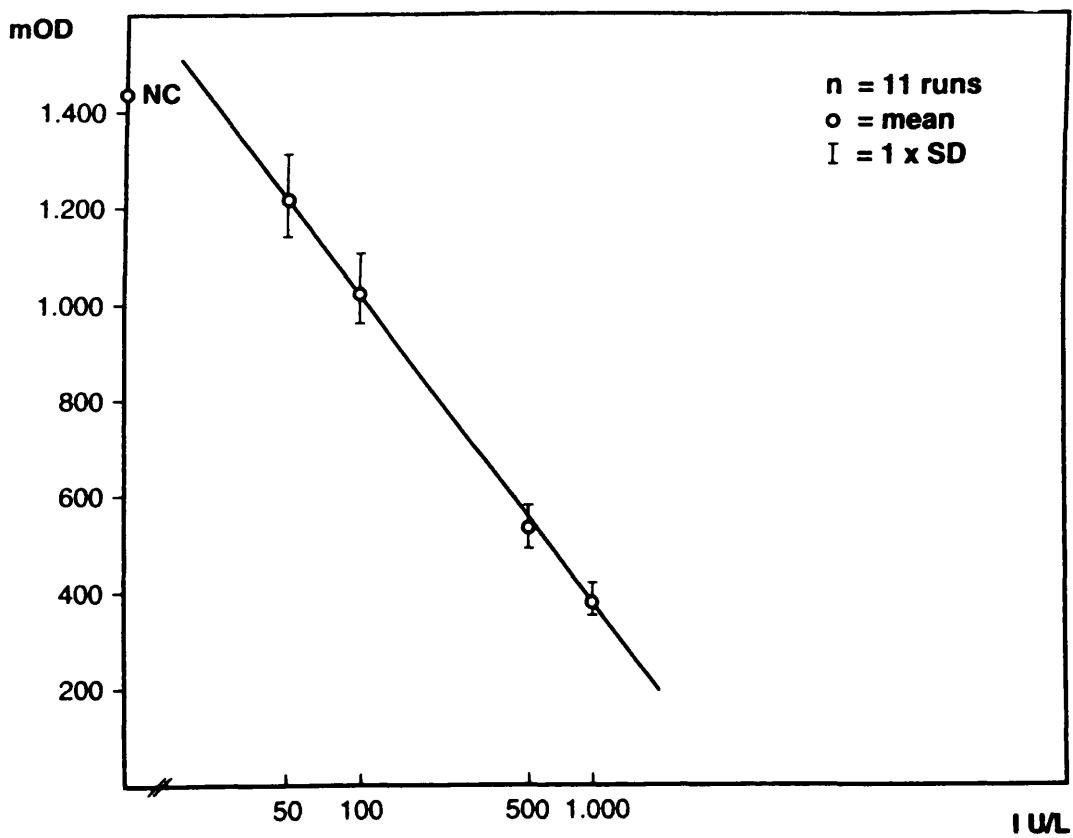
Composite Standard Line

Figure 4: Composite standard line (WHO 1st Reference Preparation, 1981

of Hepatitis A Immunoglobulin); each point of the standard

curve was measured in duplicate; 11 individual runs;

mean ± SD; X-axis, log scale (dose); Y-axis, linear scale

(adsorbance): NC, negative control of test kit.

Microwell plates are coated overnight at room temperature with a purified human IgG anti-HAV. After saturation of the wells with 1% gelatine, purified inactivated HAV is added and the plates incubated for one hour at 37°C. Serum from vaccines is then added in two-fold dilutions and the plates incubated overnight at room temperature.

Human IgG anti-HAV, identical to that used to coat the plates was conjugated with peroxydase. After addition of the conjugate, the plates are further incubated for one hour at 37°C. The optical density (490 nm) of each well is read following addition of the peroxydase substrate (OPDA in citrate buffer containing H_2O_2).

Anti-HAV titres were calculated in IU/l in comparison with an immunoglobulin preparation (WHO standard calibrated at 100 IU/l) using the four parameter method (Kaprinski et al. 1987) with antibody titres $\geq$ 20 IU/l were considered positive.

Biochemical tests of liver function

ALT and AST were determined using the Boehringer Mannheim test kits. Both assays were optimised according to the recommendations of the International Federation of Clinical Chemistry, and were run on a Hitachi 717 analyser. The reference interval for both AST and ALT was 5-40 IU/l.

Reactogenicity: Clinical signs and recording of symptoms

On the day of vaccination and for the following 3 days, follow-up of clinical signs and symptoms were recorded by the vaccinee (subjective evaluation apart from body temperature and size of local reactions).

Clinical signs and symptoms were recorded on appropriate symptoms sheets. The following symptoms were recorded:

General: Temperature
Headache
Fatigue/tiredness
Loss of appetite
Nausea
Vomiting
Local: Soreness
Induration
Redness size
Swelling
Others: Body temperature was recorded 3 and 8 hours after each vaccination and once a day for the three following days

Signs and symptoms were scored as follows:

0 = absent
1 = mild
2 = moderate
3 = severe

The definitions of these terms were as follows:

1. MILD - the adverse reaction does not interfere in a significant manner with the subject's normal functioning level. It may be an annoyance.

2. MODERATE - The adverse reaction produces some impairment of functioning but is not hazardous to health. It is uncomfortable and/or an embarrassment.

3. SEVERE - The adverse reaction produces significant impairment of functioning or incapacitation and is a definite hazard to the subject's health.

The volunteers were instructed to return the complete chart of symptoms and signs at their next visit. The forms were checked individually by the clinical investigator.

Statistical analysis

Preliminary analysis included a verification of the distribution of ages and sexes of the participants.

At each time point of the study, the geometric mean antibody titres (GMTs) were calculated using the log transformation of non-zero titres and taking the anti-log of the mean of these transformed values.

The non-parametric Wilcoxon signed rank test was used to establish statistically significant differences amongst the means and within the groups. The Wilcoxon rank sum test was used to compare the means between groups.

RESULTS

Demographic features

The median ages for subjects in each treatment group were 22, 23, 24 and 25 years for Group 1/vaccine, placebo and the Group 2/vaccine, placebo subjects respectively. The age ranges were 18-50 years for the Group 1/vaccine subjects, 18-54 for the Group 1/placebo subjects and 18-55 for both the Group 2/vaccine and placebo subjects. Overall, the median age was 23 years.

More female subjects entered the study compared to male subjects. The proportion of male and female subjects were approximately the same across the groups with the exception of Group 1/vaccine where a slightly higher proportion of male subjects were recruited. Overall, 119 (42%) subjects were male and 167 (58%) subjects were female.

The mean heights for subjects in each treatment group were 171.6 (range 132-193), 171.2 (range 155-197), 170.7 (range 142-196) and 169.9 (range 150-192) cm for the Group 1/vaccine, placebo and the Group 2/vaccine, placebo subjects respectively. Overall, the mean height was 170.9 cm.

The mean weights for subjects in each treatment group were 67.0 (range 44.0-95.2), 67.7 (range 50.8-95.2), 66.9 (range 43.1-103.0) and 66.4 (range 42.6-111.1) kg for the Group 1/vaccine, placebo and the Group 2/vaccine, placebo subjects respectively. Overall, the mean weight was 67.0 kg.

The four treatment groups were well balanced for age, height and weight and sex. Two hundred and twenty (77%) subjects had been immunised previously with hepatitis B vaccine.

Physical examination

The mean systolic blood pressure for subjects in each treatment group were 113.9 (range 70-150), 114.1 (range 85-140), 114.6 (range 80-150) and 115.3 (range 90-150) mmHg for the Group 1/vaccine, placebo and the Group 2/vaccine, placebo subjects respectively. Overall, the mean systolic blood pressure was 114.4 mmHg.

The mean diastolic blood pressure for subjects in each treatment group were 73.8 (range 50-95), 74.3 (range 60-90), 73.8 (range 55-90) and 73.9 (range 54-90) mmHg for the Group 1/vaccine, placebo and the Group 2/vaccine, placebo subjects respectively. Overall, the mean diastolic blood pressure was 73.9 mmHg.

The mean pulse rate for subjects in each treatment group were 73.0 (range 59-100), 72.8 (range 60-96), 72.1 (range 58-88) and 72.3 (range 60-88) beats/min for the Group 1 vaccine, placebo and the Group 2/vaccine, placebo subjects respectively. Overall, the mean pulse rate was 72.5 beats/min.

The mean oral temperature for subjects in each treatment group were 36.4 (range 35.1-37.3), 36.4 (range 35.1-37.1), 36.4 (range 35.4-37.3) and 36.6 (range 35.5-38.4)°C for the Group 1/vaccine, placebo and the Group 2/vaccine, placebo subjects respectively. Overall, the mean oral temperature was 36.5°C.

Very few abnormalities were identified during the physical examination and no more than 1% of subjects had any single form of abnormality overall.

Vaccination Record

Most of the subjects received all three vaccinations (252 out of the original 286 subjects (88%)).

For the Group 1 subjects the median duration between vaccinations 1 and 2 was 29 days for both the vaccine and placebo sub-groups (range 21-64 and 15-49 days respectively). For the Group 2 subjects the median duration between vaccinations 1 and 2 was 15 days for both the vaccine and placebo sub-groups (range 8-49 and 12-29 days respectively).

The median number of days between vaccinations 1 and 3 were 368 (range 223-428), 367 (range 329-435), 366 (range 281-421) and 368 (range 267-438) days for the Group 1/vaccine, placebo and the Group 2/vaccine, placebo subjects respectively.

ALT and AST estimations

Very few evaluable subjects had levels of alanine aminotransferase (ALT) twice the upper limit of normal; 1, 1, 4, 1, 1, and none of the subjects at times 0, 14 days, 1, 2, 12 and 13 months respectively. Similar observations were recorded for aspartate aminotransferase with levels twice above the upper limit of normal in 0, 1, 2, 2, 1 and 1 of the subjects at times 0, 14 days, 1, 2, 12 and 13 months respectively.

Reactogenicity

The percentage of subjects reporting symptoms after each vaccination were 78%, 67% and 69% for vaccinations 1, 2 and 3 respectively. There were no obvious trends in the data except that the number of subjects with symptoms was lower for the Group 2/vaccine subjects compared to the other three Groups at each vaccination.

The percentage of subjects reporting local symptoms were 72%, 59% and 63% for vaccinations 1, 2 and 3 respectively. There were no obvious trends in the data except that the number of subjects with symptoms was lower for the Group 2/vaccine subjects compared to the other three Groups at each vaccination. The most commonly reported local symptoms were soreness and redness with between 52-63% of all subjects reporting soreness at each vaccination and between 22-30% of all subjects reporting redness at each vaccination.

The percentage of subjects reporting general symptoms were 29%, 27% and 29% for vaccinations 1, 2 and 3 respectively. There were no obvious trends in the data. The most commonly reported general symptoms were tiredness and headache with between 19-20% of all subjects reporting tiredness at each vaccination and between 13-15% of all subjects reporting headache after each vaccination.

37 (13%) of subjects reported 46 adverse events in total. Of these 46 events, 30 (65%), 8 (17%) and 6 (13%) were classified as mild, moderate and severe respectively. Thirteen (28%) events were suspected as being related to the vaccination, only one of which was from a subject randomised to receive the placebo. Overall, five of the events requiring further action were reported by subjects who had been randomised to receive vaccine and four events from subjects randomised to receive placebo.

In summary, subjects suffered a higher incidence of local symptoms rather than general symptoms. There was no evidence of any difference in the

incidence of symptoms between the active and placebo groups over the three vaccinations. However, the number of subjects with symptoms was lower for the Group 2/vaccine subjects compared to the other three groups after each vaccination.

The most commonly reported local symptoms were soreness and redness with between 52-63% of all subjects reporting soreness after each vaccination and between 22-30% of all subjects reporting redness after vaccination.

The most commonly reported general symptoms were tiredness and headache with between 19-20% of all subjects reporting tiredness after each vaccination and between 13-15% of all subjects reporting headache after each vaccination.

Only 13% of subjects reported unsolicited events during the course of the study with no evidence to suggest any difference in the incidence of such events between the four treatment groups. A total of thirteen events were thought to be related to the treatment, twelve of these were almost equally distributed between the two vaccine groups. Six subjects experienced severe adverse events, one each in the two vaccine groups and two in each of the placebo groups.

Dropouts

42 (15%) subjects withdrew from the study for a number of reasons.

36 were lost to follow-up, 2 were withdrawn by the investigator because of an adverse effect, 3 because of lack of compliance and 2 were withdrawn at the request of the subject because of an adverse event and 1 for other reasons. The distribution of dropouts was very similar between the vaccine and placebo groups.

Immunogenicity

Immunisation with the hepatitis A vaccine induced a seroconversion rate in 94% at one month in Group 1 (Regimen I) and of 97% in Group 2 (Regimen II) (Table 3). The quantitative responses (GMTs) over the months 1-13 are shown in Figures 5 and 6.

The GMTs of antibodies to HAV in Group 1 (Regimen I) were 340 IU/l at month 1 decaying to 188 IU/l at month 12 and increasing to 11,300 IU/l at month 13, i.e. one month after administration of the booster dose. In Group 2 (Regimen II), the GMTs were 470 IU/l at month 1 falling to 210 IU/l at month 12 and increasing to 5,130 IU/l one month following the booster dose. There were no statistical differences between GMTs in the two groups using the different regimens.

Sharply increasing antibody concentrations were demonstrated in both groups following the administration of the booster dose. At month 13 all subjects had seroconverted (Table 3).

Antibodies to HAV were not detected in the two groups receiving the placebo preparations.

Table 3:

**Seroconversion rates in volunteers after immunisation
with Hepatitis A vaccine**

	Month 1	Month 2	Month 12	Month 13
Group 1 (Regimen I)	31 (94%)	23 (100%)	28 (79%)	32 (100%)
Group 2 (Regimen II)	33 (97%)	16 (100%)	26 (88%)	33 (100%)

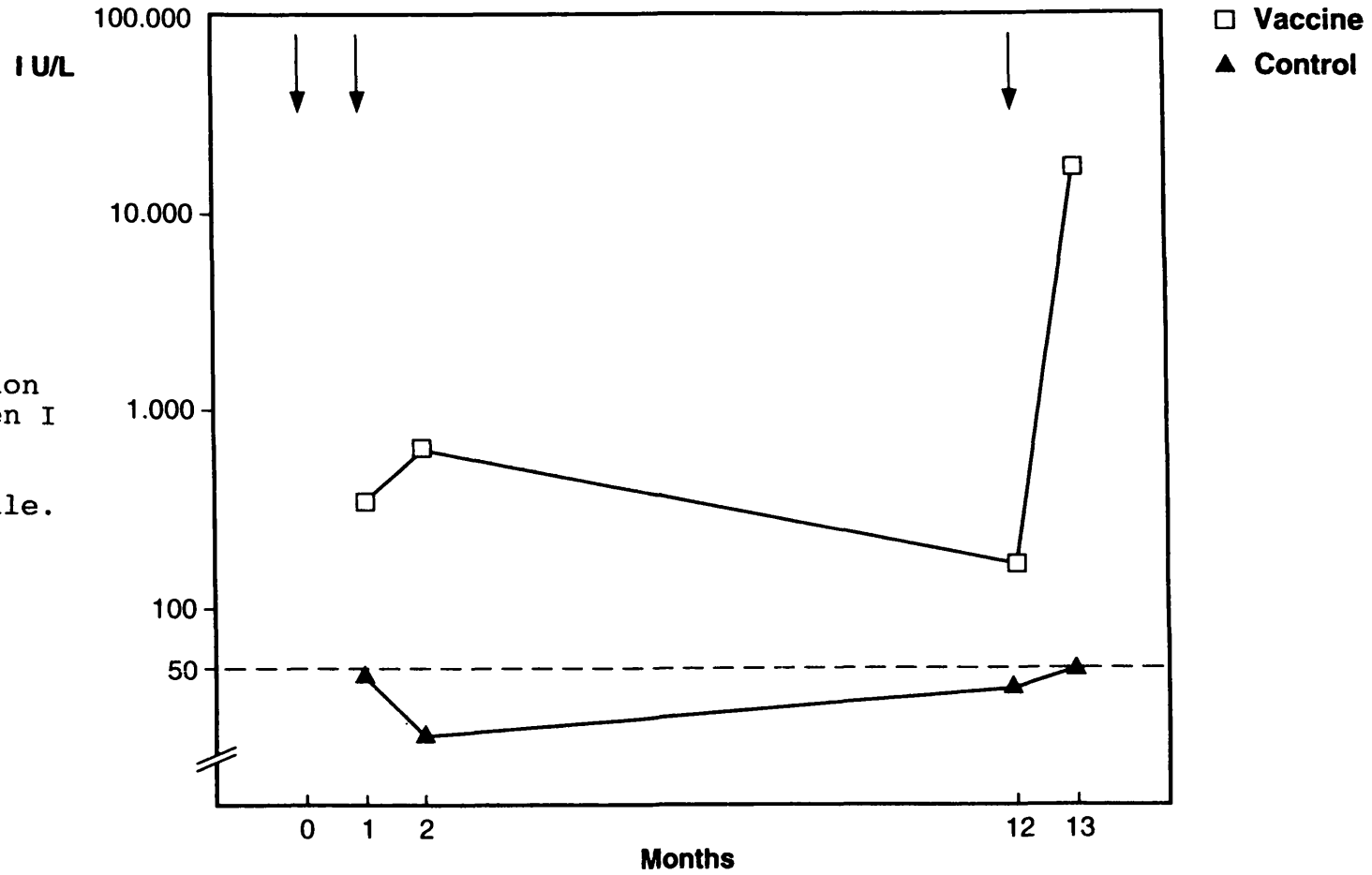Seroconversion: titre of HAV antibodies > 50 IU/l

Regimen I

Figure 5:

Geometric mean
titres in IU/l
using vaccination
schedule Regimen I
in relation to
time (months).
Y-axis, log scale.

Regimen II
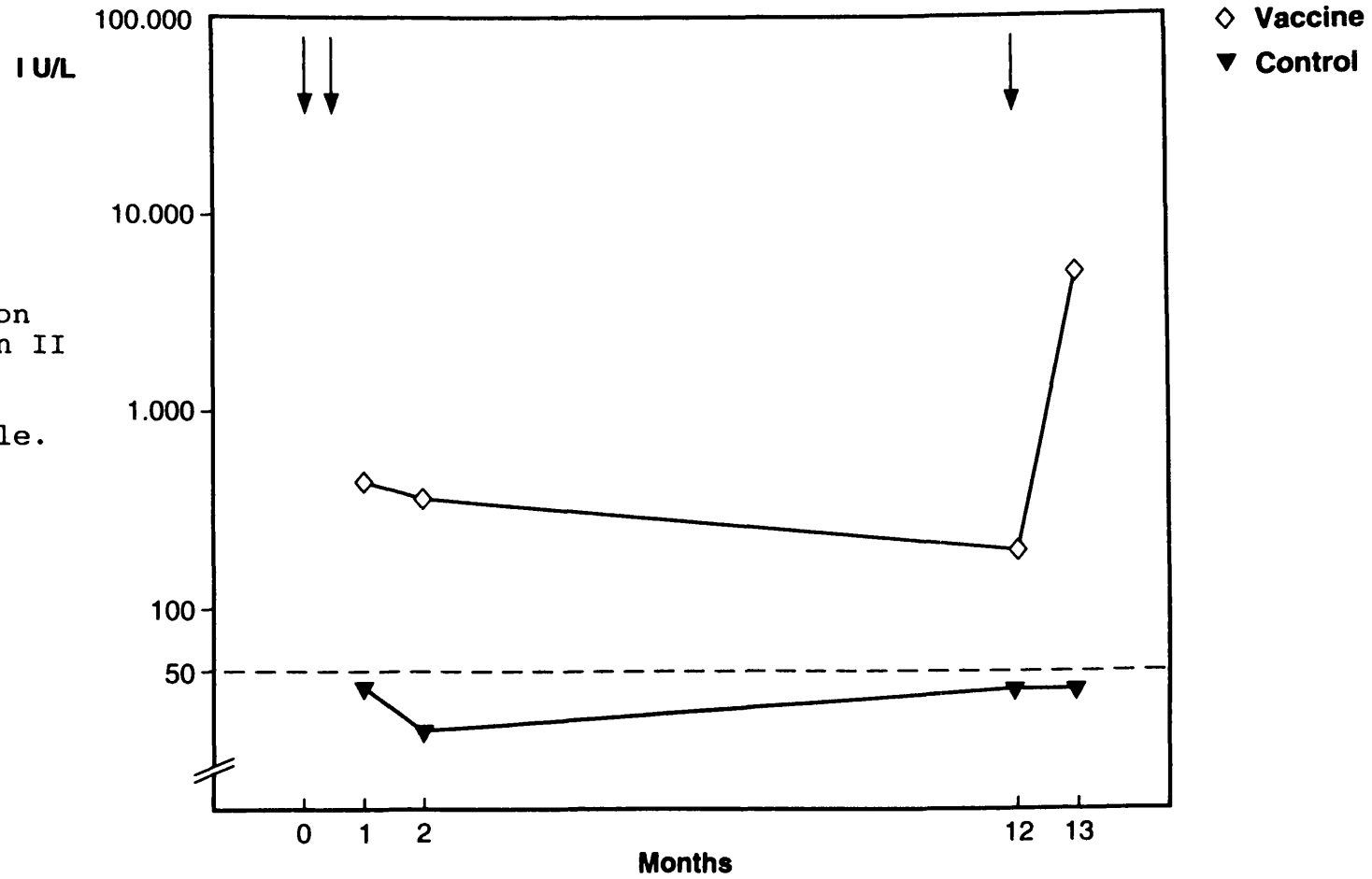
◇ Vaccine
▼ Control

Figure 6:

Geometric mean titres in IU/1 using vaccination schedule Regimen II in relation to time (months). Y-axis, log scale.

Months

Fifty male and 63 female subjects were included in this study. The possible differences in response between the sexes receiving the two different vaccination regimens was assessed by the Wilcoxon rank sum test. This demonstrated a significantly lower GMT in male subjects at month 1 in Group 2 (Regimen II) ($p = 0.01$) and slightly lower concentrations at month 12 in Group 1 (Regimen I) in female subjects ($p = 0.06$). The other GMTs of both regimens did not demonstrate sex differences.

DISCUSSION AND CONCLUSIONS

The formalin-inactivated hepatitis A vaccine (720 ELU) proved to be highly immunogenic with an overall seroconversion rate of 100%. Both vaccination groups mounted a measurable and high response. GMTs of 340-470 IU/l were obtained with both regimens at month 1, well above the presently accepted minimum protective level of 10-20 IU/l. GMTs at month 2 were not significantly different from those at month 1. However, there was trend of decreasing antibody titres by 12 months which increased rapidly by a factor of 10-100 following the administration of the third dose.

Sera from the control groups did not give a quantitative hepatitis A antibody response.

It is noteworthy that in the literature on HAV vaccine trials, control or placebo groups are mentioned only occasionally, and that published measurement data on those samples are difficult to find.

A gender difference in the reponse to hepatitis A vaccine was observed at month 1 (Regimen I) in this study with a lower response occurring in male subjects. Antibody responses were found to be higher in males than in females in a previous study (Tilzey et al. 1992).

Other studies report findings similar to our observations that vaccination with an inactivated hepatitis A vaccine produces antibody concentrations at least 20 times higher than those induced by passive immunisation with human immunoglobulin (Berger et al. 1993). The duration of protection conferred by the vaccine, and therefore the need for a booster as well as the response to exposure to a natural hepatitis A infection after vaccination, have yet to be fully established (Wiedermann et al. 1992) although it has been calculated that according to the vaccine induced antibody kinetics and the magnitude of antibody decrease over time, the predicted duration of persistence of antibody was at least 20 years (van Damme et al. 1994).

SECTION 2:

Accelerated immunisation with high doses of inactivated Hepatitis A vaccine of adults and children with congenital coagulation disorders

INTRODUCTION

An open assessment randomised trial of an inactivated hepatitis A vaccine administered subcutaneously to patients with congenital coagulation disorders was carried out to compare the immunogenicity, reactogenicity and safety of a recently developed single-dose primary course of vaccine (the 1440 ELU hepatitis A antigen). This new preparation has the important advantages of reducing the number of injections and discomfort, and increasing scheduling convenience and patient compliance. The immunisation schedule was 0 and 6 months (or 0 and 12 months) in comparison with the classical schedule of the 720 ELU hepatitis A vaccine of 0, 1, 6 or 0, 1, 12 months. This new vaccine preparation has been shown to be safe and highly immunogenic offering rapid seroconversion after intramuscular administration to healthy adults (Van Damme et al. 1994a; Van Damme et al. 1994b; Briem and Safary 1994).

The rationale for this study was based on two important considerations. Firstly, several reports of hepatitis A amongst patients with haemophilia in Italy, Germany, Ireland, Belgium, South Africa and the USA receiving highly purified Factor VIII concentrate prepared by solvent/detergent fractionation of plasma (Mannuci 1992; Gerritzen et al. 1992; Normann et al. 1992; Shouval and Gerlich 1992; Mannuci et al. 1994; Kedda et al. 1995; Centers for Disease Control 1996), led to a recommendation that as a precautionary measure, all recipients of clotting factors derived from plasma pools should be immunised against hepatitis A (Communicable Disease Report 1992; Centers for Disease Control 1996).

Secondly, individuals with congenital coagulation disorders are at risk from bloodborne viruses such as hepatitis B and hepatitis C, which may result in chronic liver disease, and it would be prudent to protect these against infection with another hepatotropic virus, hepatitis A.

The vaccine

The vaccine was manufactured by SmithKline Beecham Biologicals, Rixensart, Belgium using the HM 175 strain of HAV cultivated by classical cell culture methods, inactivated with formaldehyde and formulated to contain twice the amount of viral antigen protein (1440 ELISA units per 1 ml dose) than used in the vaccine licensed previously.

The study was carried out according to the Declaration of Helsinki with the 1989 Hong Kong Amendment and Good Clinical Practices Guidelines at the Haemophilia Centre and Haemostasis Unit of the Royal Free Hospital with the approval of the Local Ethical Practices Committee in volunteer male patients who gave written informed consent and who were between the age of 0 and 65 years, with mild, moderate or severe haemophilia A or B or von Willebrands disease excluding those with clinical signs of acute disease.

Objectives and design of the study

The primary objectives were to determine the immunogenicity and reactogenicity of a 1440 ELU dose of inactivated hepatitis A vaccine administered as a single dose with a booster at month 6, and to evaluate the safety of the vaccine when administered by the subcutaneous route.

This was an open, within group comparison study of the newly formulated
1440 ELU hepatitis A vaccine conducted at a single centre in the UK
(Haemophilia Centre and Haemostasis Unit of the Royal Free Hospital).
The study population were subjects who were invited to participate and who
fulfilled the inclusion and exclusion criteria set out below:

Inclusion criteria

- Male subjects between the ages of 0 and 65 years inclusive
  with moderate-severe haemophilia A or B

- Absence at entry of clinical signs of significant acute disease other
  than the underlying condition

- Subjects agreeing not to consume raw shellfish nor to travel to areas
  of medium to high hepatitis A endemicity during the study period

- Subjects assessed by a physician as being medically fit for
  vaccination

- Subjects will to give written informed consent to their participation,
  parental or guardians' consent must be obtained for individuals less
  than 18 years of age

- Results available for HIV test during previous 12 months

Exclusion criteria

- Travel to developing countries or medium to high hepatitis A
  endemicity areas within the previous 6 weeks

- History of alcohol abuse (defined as intake > 35 U per week for men)

- Positive anti-HAV antibodies

- Any chronic drug treatment or illness which, in the investigators opinion, precluded entry to the study

- Likelihood of travel to an area of medium to high hepatitis A endemicity during the study period

- Subjects with severely elevated liver enzyme levels (>10 times upper limit of normal)

- History of allergy or hypersensitivity which is likely to be stimulated by a component of the vaccine

- Subjects who are unwilling or unable to comply with the protocol

- Subjects participating in any other drug investigational trial involving anti-HIV drugs and new concentrates will be allowed to participate subject to haematological and biochemical parameters being stable

Outline of Study Procedures

	Day 28 to Day 0	Month 0	Month 1	Month 6	Month 7
Screening	●				
Vaccination		●		●	
Diary card checking			●		●
ALT	●				
AST	●				
Anti-HAV	●		●	●	●
CD4 cell counts	●		●	●	●
Full blood count	●				

The vaccine

The hepatitis A vaccine was prepared from the HM 175 strain of HAV propagated by classical culture methods in human diploid cells and inactivated with formaldehyde (see Section 1). Each 1 ml dose was formulated to contain 1440 ELU of hepatitis A viral antigen protein adsorbed on aluminium hydroxide adjuvant and suspended in isotonic buffered saline containing 0.5% 2-phenoxyethanol as preservative. The paediatric dose was 720 ELU of hepatitis A viral antigen protein in 0.5 ml.

The vaccine was administered subcutaneously into the deltoid area of the upper arm as a single dose of 1 ml (1440 ELU) to those aged over 16 years and a paediatric dose of 0.5 ml (720 ELU) to those aged under 16 years. A booster dose was given at month 6.

Follow-up was essentially as described in Section 1 and as outlined in Table 2. Two analyses were carried out: 'intention-to-treat' including all subject data except those excluded from reactogenicity (i.e. those with no data concerning the vaccination), and secondly, including only subject data corresponding to all the criteria specified in the protocol. Data from those under 16 and over 16 years of age were separated from the overall group and analysed separately.

Study population

Male subjects between the ages of 0 and 65 years with mild, moderate or severe haemophilia A or B or von Willebrands disease meeting the inclusion criteria as defined above were enrolled. Table 4 summarises the demographic details of the volunteers.

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Table 4:

Demographic Details of the 97 Volunteers

Age (years)					
Number	Minimum	Maximum	Median	Mean	SD
Age < 16 years					
30	1.8	14.7	6.7	7.7	4.1
Age > 16 years					
67	16.3	72.8	32.1	34.2	12.7
All ages					
97	1.8	72.2	26.7	26.0	16.4

Height (cm)					
Number	Minimum	Maximum	Median	Mean	SD
Age < 16 years					
30	83	185	121	126	27
Age > 16 years					
67	157	193	175	175	6
All ages					
97	83	193	172	160	28

Weight (kg)					
Number	Minimum	Maximum	Median	Mean	SD
Age < 16 years					
30	11.0	78.0	24.5	30.3	17.0
Age > 16 years					
67	46.5	100.1	71.1	70.9	12.7
All ages					
97	11.0	100.1	62.0	58.3	23.5

The intention-to-treat population included all randomised patients who received the first vaccination at visit 2. The per protocol population consisted of the subjects in the intention-to-treat population who did not violate any of the specified exclusion criteria. Treatment was allocated on the basis of age at entry and the data were analysed by treatment group and then overall.

97 subjects were enrolled into the study of whom 30 were children in Group A and 67 were adults in Group B. At screening, two subjects in Group A and eight in Group B were found to be seropositive for hepatitis A on confirmatory testing, and one adult subject had markedly elevated liver function tests. These subjects were considered to be protocol violators and therefore were not eligible for inclusion in the per protocol analysis.

Serological methods

The seroconversion rate and the geometric mean titres were used to evaluate the immunogenicity of the vaccine in each group for all time points for which blood samples were taken. Seroconversion was defined as the presence of hepatitis A antibody titres > 33 IU/l.

The serological titrations were carried out at SmithKline Beecham Biologicals, Rixensart, Belgium using a commercial ELISA kit, anti-HAV Enzymun-Test (Boehringer Mannheim, Germany). The cut-off level is 33 IU/l. Only subjects below the cut-off level of this test were considered as seronegative and were enrolled in the study.

<u>Statistical analysis</u>

Serological responses between the two age groups were compared using the unpaired Student's t test with logarithmically transformed data. 95% confidence intervals were calculated for the geometric mean titres by age group and month. Descriptive statistics were also used to compare the reactogenicity and safety of the vaccine between the two age groups.

RESULTS

97 volunteer male patients were entered into the study 11 of whom were not eligible for inclusion into the per protocol analysis. Therefore the per protocol population consisted of 28 children and 58 adults with congential coagulation disorders. The median age for the children (Group A) and the adults (Group B) were 6.7 and 32.1 years respectively. Eighty four (87%) of the subjects were Caucasian. Twenty eight (29%) of the patients were HIV positive, 40% of adults and 3% of children.

<u>Reactogenicity</u>

There was a higher incidence of local rather than general symptoms following each inoculation of the vaccine in both the child and adult groups (Table 5). The adult patient group reported the highest incidence of symptoms by dose. Overall 79/117 (68%) of doses led to subjects reporting at least one symptom over the course of the study in this group compared to 32/58 (55%) of doses in the children. Pain was the most commonly reported symptom in the children followed by redness and then swelling with 26/58 (45%), 24/58 (41%) and 14/58 (24%) respectively. The order was different in the adult group with redness the most commonly reported local symptom followed by pain and

then swelling with 62/177 (53%), 48/117 (41%) and 35/177 (30%) of the doses given respectively. Nausea was the most commonly reported general symptom in children with 3/58 (5%) of dose related episodes. Headache, malaise and nausea were the most commonly reported general symptoms in the adult group. Headache was reported with 12/177 (10.2%) of the doses given to adults. There were no severe reactions. It is interesting to note that the local symptoms and signs, particularly redness and swelling, attributable to the 1440 ELU vaccine given subcutaneously were considerably more frequent than the local redness (4.2%) and swelling (4.9%) observed after intramuscular inoculation in healthy adult volunteers (van Damme et al. 1994). The general signs and symptoms were very similar to those observed with the 1440 ELU vaccine and indeed with the earlier 720 ELU hepatitis A vaccine given intramuscularly to healthy adults.

Immunogenicity

One month following a single dose of vaccine, 18/20 (90%) children and 28/38 (73%) adults seroconverted. In total, 79% 46/58 (79%) of individuals seroconverted (95% confidence interval 67%-89%) (Table 6). The geometric mean titre was 258 IU/l for both groups combined (95% confidence interval 194%-342%) of subjects and there was no significant difference in the geometric mean titres between the two groups. There was also no significant difference in the antibody responses in either of the two groups between months 0 and 6.

Table 5 : Incidence of local and general symptoms reported

Month	Age Group (years)	No. of Doses given	Number (%) Symptoms Reported				
			Local Alone	General Alone	Local and General	Local*	General +
0 (after 1st vaccination)	Age < 16	30	14 (47%)	0 (0%)	4 (13%)	18 (60%)	4 (13%)
	Age > 16	67	34 (51%)	1 (1%)	11 (16%)	45 (67%)	12 (18%)
6 (after 2nd vaccination)	Age < 16	28	11 (39%)	1 (4%)	2 (7%)	13 (46%)	3 (11%)
	Age > 16	50	24 (48%)	1 (2%)	8 (16%)	32 (64%)	9 (18%)
Overall (after all vaccinations)	Age < 16	58	25 (43%)	1 (2%)	6 (10%)	31 (53%)	7 (12%)
	Age > 16	117	58 (50%)	2 (2%)	19 (16%)	77 (66%)	21 (18%)
Total	All Ages	175	83 (47%)	3 (2%)	25 (14%)	108 (62%)	28 (16%)

*   Subjects with at least one local syptom
+   Subjects with at least one general symptom

Table 6 : Seroconversion rates and geometric mean antibody titres

Month	Seroconversion Rate	Percentage Seroconverted	Geometric Mean Titre	95% Confidence Interval	Range
Age < 16 Years					
1	18/20	90%	264	167 to 417	45- 980
6	8/10	80%	113	60 to 210	45- 256
7	12/12	100%	1102	523 to 2321	81-3946
Age > 16 years					
1	28/38	74%	254	176 to 366	35-1483
6	19/27	70%	110	73 to 164	34- 887
7	23/27	85%	879	513 to 1506	120-9439
All Ages					
1	46/58	79%	258	194 to 342	35-1483
6	27/37	73%	111	79 to 154	34- 887
7	35/39	90%	950	617 to 1462	81-9439

100% seroconversion occurred in the children compared with 85% in the adult group one month following administration of the booster dose. Overall, 90% of subjects seroconverted (95% confidence interval 76%-97%), one month following the booster (Figure 7).

Statistical comparison of the rate of seroconversion and geometric mean titres with CD4 counts demonstrated that those individuals with low CD4 counts did not appear to seroconvert. However, several adults who did seroconvert also had low CD4 counts and so it is not possible to determine a causal relationship (Figure 8). CD4 counts were also found to be higher in the child group compared with the adult group.

DISCUSSION AND CONCLUSIONS

Inactivated hepatitis A vaccine has been available since 1992, and was shown to be safe and highly immunogenic at a dose of 720 ELU (Andre et al. 1990; Wiedermann et al. 1990; Andre et al. 1992; Tilzey et al. 1992). A higher dose, 1440 ELU of antigen has been used more recently, offering a high degree of rapid protection (Van Damme et al. 1994a and b; Briem and Safary 1994) and it is this preparation which is being used most widely in Europe at present.

Several outbreaks of acute hepatitis A infection have been reported in haemophiliac patients in Italy, Germany, Ireland, Belgium and South Africa associated with the use of a high purity Factor VIII concentrate manufactured using the solvent/detergent fractionation process (Temperley et al. 1992).
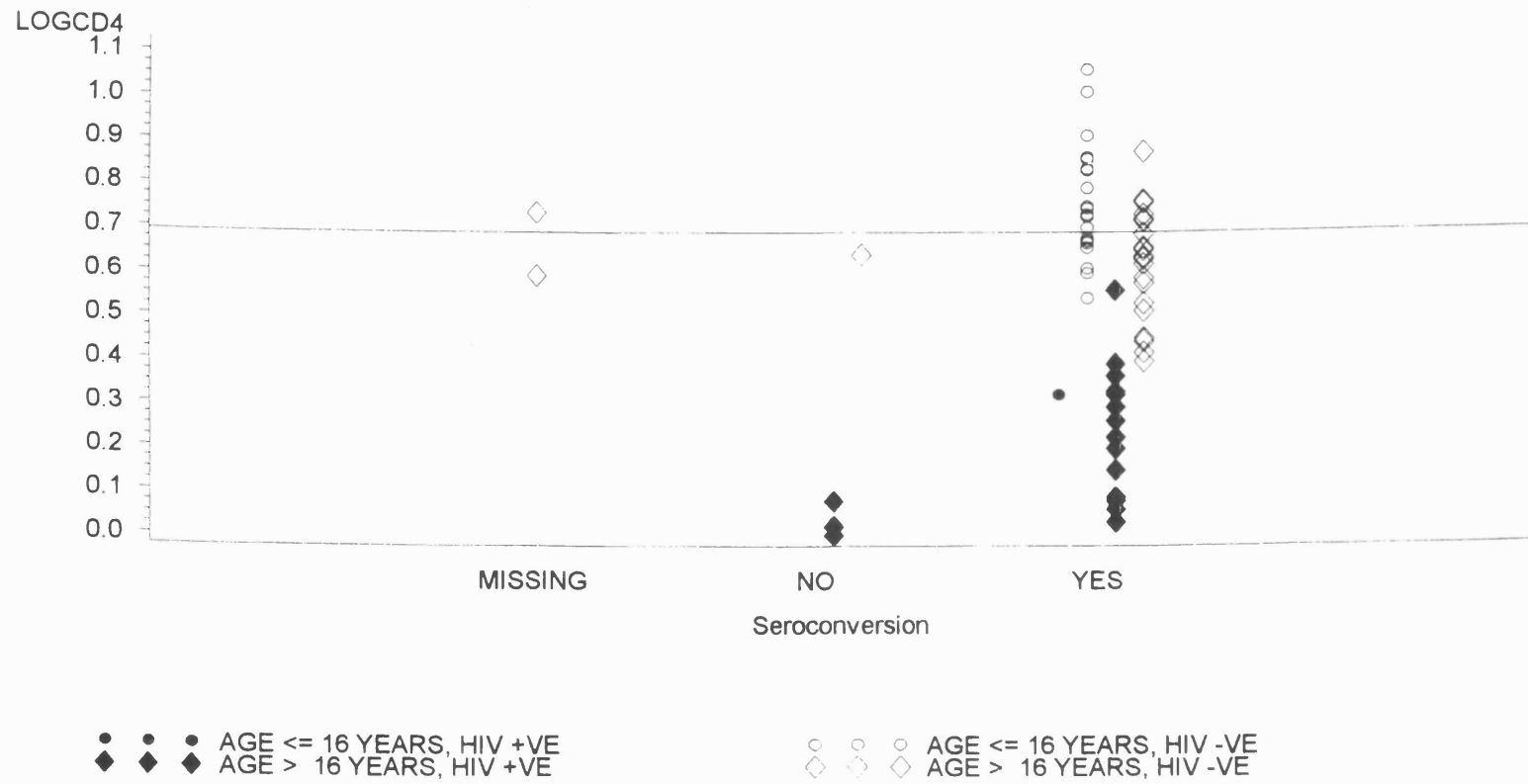
Figure 7:
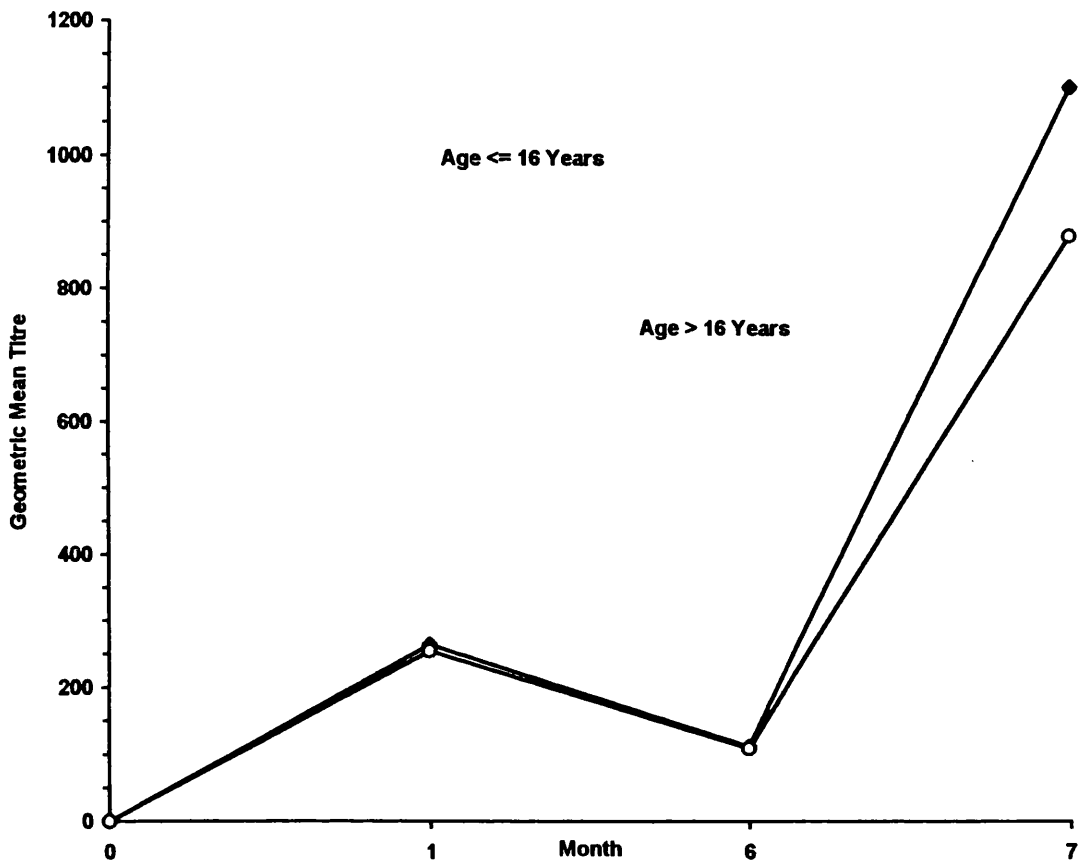
GEOMETRIC MEAN TITRES OF HEPATITIS A ANTIBODY AFTER IMMUNISATION IN ADULTS AND CHILDREN

Figure 8:

REFERENCE LINE CD4=2.0 IS SHOWN AT LOGCD4=0.69

This process inactivates enveloped viruses such as HIV, HCV and HBV, but hepatitis A virus is a small non-lipid unenveloped, heat stable virus which is unlikely to be inactivated by this method.

Several hypotheses were put forward to explain the occurrence of these cases although no direct causal association was established between infection occurring through use of the concentrate and the reported clusters of acute hepatitis A (Shouval and Gerlich 1992). Seroprevalence studies undertaken in haemophiliacs in the United Kingdom did not demonstrate an increased susceptibility in this group compared with the local population (Clarke et al. 1995). However, the UK Haemophilia Centre Directors recommended, as a precautionary measure, that all recipients of clotting factors derived from plasma pools who are susceptible to hepatitis A virus should be offered inactivated hepatitis A vaccine (Communicable Disease Report 1992), and similarly in the USA (Centers for Disease Control 1996).

Consequently, this study was undertaken to determine the immunogenicity and reactogenicity of two doses of 1440 ELU inactivated hepatitis A vaccine, for adults, and 720 ELU for children, administered by the subcutaneous route.

The vaccine was well tolerated and highly immunogenic in subjects with congenital coagulation disorders. The side effects recorded were generally mild and transient and not dissimilar to those reported with other generally available vaccines.

Overall, 90% of the subjects seroconverted at month 7, with seroconversion rates of 79% and 73% at months 1 and 6 respectively. The overall geometric mean titres reflected similar findings with the maximum titre of 950 IU/l at month 7. Both groups reached maximum seroconversion rates at month 7 with the titres in children always being higher at each time point in the study.

One difficulty throughout this study was that a large number of subjects at each visit were ineligible for inclusion in the per protocol analysis, because their visits did not fall within the necessary time intervals. However, the intention-to-treat analysis did not differ significantly from the per protocol analysis and so it is unlikely that this reduction in the data obtained had any adverse effect upon the subsequent analysis.

Failure to mount a satisfactory B lymphocyte immune response and consequently an inadequate antibody response to T-cell dependent vaccines in HIV-infected subjects is a well recognised phenomenon (Rhoads et al. 1991; Santagostino et al. 1994). This study confirms previous reports of lower seroconversion rates following hepatitis A vaccination occurring in association with low CD4 counts in HIV-infected haemophiliacs (Wilde et al. 1995; Hess et al. 1995).

In conclusion, subjects with congenital coagulation disorders may be at risk from hepatitis A transmission of the virus from pooled plasma products.

Such individuals are also at risk from other bloodborne viruses including hepatitis B and hepatitis C which in many cases result in chronic liver disease and its sequelae although recombinant Factor VIII concentrates, which will abolish the risk of bloodborne viruses, have now become available (Tuddenham and Laffan 1995).

The study demonstrates that administration of a rapid immunisation schedule using an inactivated hepatitis A vaccine (1440 ELU and 720 ELU) is safe and highly immunogenic when given at high dose by the subcutaneous route. It is recommended, therefore, that all susceptible patients with congenital coagulation defects be included in a rapid immunisation programme as prophylaxis against acute hepatitis A not only to preclude infection as such but also to reduce complications in patients with haemophilia and other coagulation defects who had been exposed in the past to a severe viral burden (hepatitis B, hepatitis C, HIV and others) and may have underlying liver disease.

A multicentre clinical trial of two hepatitis A vaccines

prepared from the HM 175 and the GBM strains of HAV

<u>INTRODUCTION</u>

A multicentre European Phase III clinical trial of two hepatitis A vaccines was carried out in order to compare the immunogenicity and safety of the new Pasteur Merieux (PM) inactivated hepatitis A vaccine prepared from the GBM strain of hepatitis A virus with the only licensed hepatitis A vaccine in Europe (Havrix) prepared from the HM 175 strain by SmithKline Beecham Biologicals (SB). The SB vaccine was used as the reference product. The clinical study was a large trial in healthy volunteers of both sexes recruited by five centres in four countries, England, Belgium, France and Germany.

The trial was conducted in accordance with the latest version (1989) of the Declaration of Helsinki and the European Good Clinical Practices. Approval was obtained from the French Viral Safety Committee (France being the country where the vaccine was prepared) and by the Local Ethical Practices Committee in each centre. Volunteers were enrolled with informed consent from September 1993 to December 1993 and the last follow-up visit was in June 1994.

<u>Participating Centres</u>

The five centres participating in this trial were:

- The Royal Free Hospital School of Medicine, London NW3 2PF, UK

- Laboratoire de Microbiologie, AZ-VUB, Brussels, Belgium;

- Centre Hospitalier A. Mignot, Le Chesnay, France;

- Centre Hospitalier Intercommunal, Poissy, France, and

- Klinik and Poliklinik für Innere Medizin, Universität Rostock, Rostock,Germany

Trial objectives

The primary aim was to compare the immunogenicity of the PM vaccine with that of the reference vaccine in healthy adult volunteers without detectable hepatitis A antibody, 2 weeks after the first inoculation of the vaccine (early response) and 8 weeks after primary immunisation (one dose of the PM vaccine and two doses of the reference SB vaccine). The secondary objectives were to establish the immunogenicity of the new vaccine and the reference product after the booster inoculation and to compare the local and systemic reactogenicity of the two vaccines in volunteers without detectable HAV antibodies at the time of inclusion.

Screening for HAV antibodies was not undertaken before inclusion in the study. If a subject was enrolled in the trial with HAV antibodies at the first visit, that person was only excluded from the analysis of the primary evaluation criterion. Analysis of the secondary evaluation criteria was carried out on all volunteers according to their serological status. The reactogenicity was evaluated as a function of anti-HAV antibodies status at the time of enrollment. Local and systemic reactions after each inoculation, expressed as percentage of subjects who experienced local or systemic reactions after vaccination was assessed by means of standardised check lists.

Design of the trial

The trial was designed as a multicentre, controlled, randomised, open trial with two study groups: one group (Group A) to receive the new PM vaccine and the other (Group B) to receive the reference SB vaccine.

This was a Phase III trial with direct individual benefit based on previous data which showed good immunogenicity with high seroconversion rate and safety. The trial plan is summarised in Table 7.

Selection of volunteers

Volunteers at the Royal Free Hospital and School of Medicine were sought from among the staff and undergraduate medical students and nursing students by distribution of information leaflets and by addressing groups of students.

At the first visit (day 0) volunteers were informed about the risks/benefits of the vaccine, the design of the trial and informed consent was obtained. After signing the informed consent, a full medical history was taken, each subject was examined clinically and inclusion and exclusion criteria were assessed. The inclusion and non-inclusion criteria were as follows:

Inclusion criteria

- Signed informed consent;

- Age ranging from 18 to 60 years old (inclusive);

- In health compatible with vaccination.

Non-inclusion criteria

- Subject presently enrolled in another trial;

- Active moderate or severe illness, including a fever $\geq$ 38°C on the day of inclusion

- Breast feeding mother

Table 7

Visit Number	1	2	3	4	5	6
Day	0	14	28	56	168	168 + 28 days
Time limit		+/- 4 days	+/- 4 days	+/- 7 days	+/- 7 days	+/- 7 days
Purpose of Visit	Inclusion Vaccination	Follow up	Group B Vaccination	Follow up	Vaccination	End of Follow up
Informed consent	●					
Medical Interview	●	●		●		●
Blood sample	●	●		●	●	●
Checking for inclusion and non-inclusion criteria	●					
Assignment of identification number	●					
Randomisation	●					
Vaccination	Groups A and B		Group B only		Groups A and B	

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- Known allergy to aluminium;

- Uncontrolled coagulopathy;

- Known or planned pregnancy;

- Known immunological deficiency, including HIV infection, or treatment with immunosuppressive drugs (including corticosteroids);

- Known previous history of hepatitis A (a history of jaundice was not a non-inclusion criteria);

- Other vaccinations scheduled on the day of visit 1, visit 3 or visit 5;

- Subject not able to adhere to planned visit interval;

- Administration of human immunoglobulin within the last four months;

- Subject with hepatomegaly or splenomegaly.

A blood sample was collected from each enrolled volunteer, and each was assigned an identification number corresponding to randomisation to Group A or B. Each subject was then vaccinated with one dose of the allocated vaccine and monitored for 15 minutes for local and systemic reactions. Each subject was given a self-monitoring form for recording any subsequent local and systemic reaction and an appointment was made for the next visit.

The Vaccines

The new Pasteur Merieux vaccine was prepared from a strain of hepatitis A virus named GBM, identified in faeces (Figure 9) from a patient with the initials M.B. in Gomaringen, Germany (Frosner et al. 1977) and studied extensively since then (Flehmig et al. 1981; Heinricy et al. 1983;

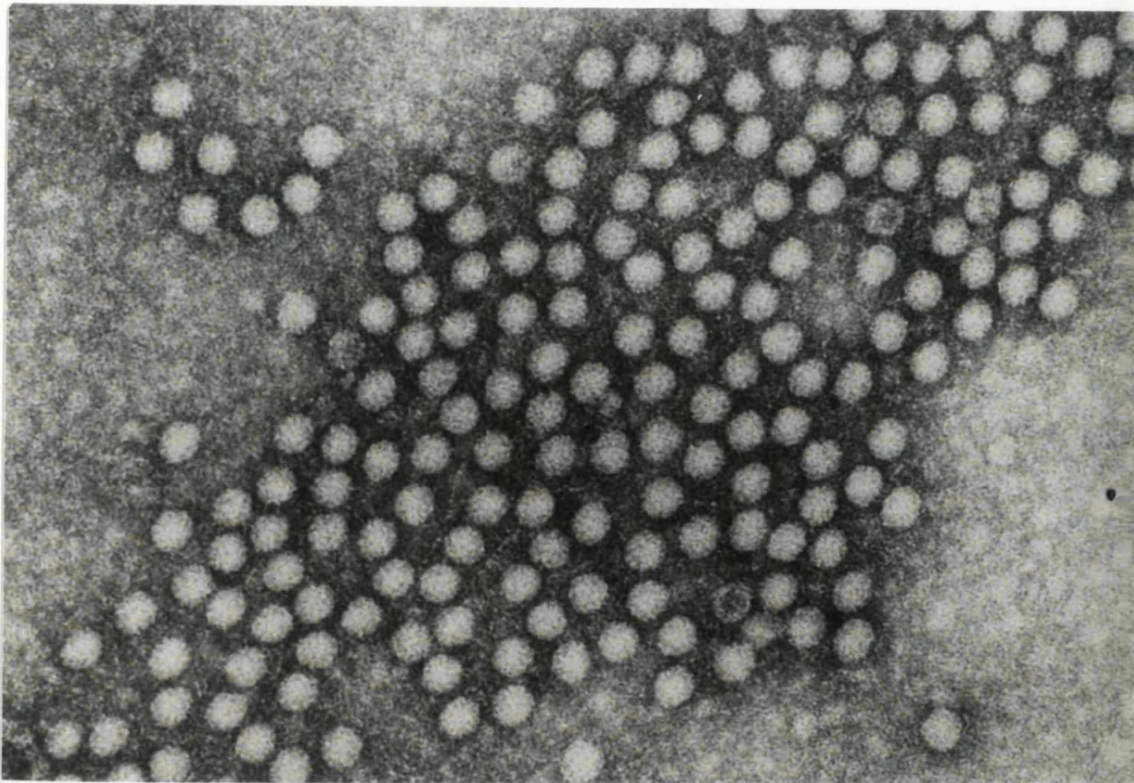**Figure 9:** Electron micrograph of the original faecal extract of GBM
showing hepatitis A virus particles measuring 24-29 nm
(average 27 nm) in diameter. Most of the particles are full.
(From a series by A. Thornton and A.J. Zuckerman)

Graff et al. 1994a; Graff et al. 1994b). The virus was shown to be attenuated (Flehmig et al. 1988) and immunogenic in animals and humans (Flehmig et al. 1987; Flehmig et al. 1988a). A candidate inactivated vaccine produced by Professor B. Flehmig was given to healthy volunteers in Germany, and was shown to be safe and immunogenic (Flehmig et al. 1987; Flehmig et al. 1989; Flehmig et al. 1990).

After viral propagation and amplification, a filtered harvest obtained by cellular lysis was purified by chromatography and concentration by tangential ultrafiltration. Purified HAV was inactivated by formaldehyde at a dilution of 1:4000 for 14 days at 37°C with constant agitation. The bulk vaccine antigen was filtered at 0.22 μm, adjusted for antigen content (160 ELISA units/dose) and adsorbed to 0.5 mg aluminium hydroxide and 0.5% 2-phenoxyethanol was added as preservative. The vaccine was formulated in 0.5 ml doses (batch S2831) and was administered as one injection of one dose given intramuscularly into the deltoid. A booster injection was given 6 months after the primary immunisation.

The vaccine has been the subject of a Phase I safety trial in healthy adult volunteers, and a Phase II dose/response trial was carried out in order to determine the optimum antigen dose for further clinical development (Garin et al. 1995). The optimal antigen dose was shown to be 160 HAV antigen units per dose. On the basis of these results, it was decided to conduct the multicentre European Phase III Study using 160 units of HAV antigen per dose in order to compare the immunogenicity and safety of this vaccine with

the reference hepatitis A vaccine (720 ELISA antigen per dose), produced by SmithKline Beecham which was the only licensed product at the time.

The preparation and characteristics of the reference HAV vaccine produced by SmithKline Beecham have been described in Section 1 (Andre et al. 1992; Peetermans 1992). Briefly, the vaccine contained purified inactivated HM 175 strain of HAV formulated to contain 720 ELISA antigen units/dose adsorbed to 0.5 mg aluminium hydroxide with 0.5% 2-phenoxyethanol added as a preservative in a total volume of water for injection of 1 ml per dose. The vaccine was given intramuscularly into the deltoid, with two injections of one vaccine dose administered at an interval of one month for primary immunisation and a booster dose was given 6 months after primary immunisation (Table 7). Commercial batches of the SB vaccine were purchased from a local distributor. Different batches were used in each country.

Immunisation schedule and follow-up

During visit 1 (day 0) all enrolled subjects were bled for determination of total antibodies to HAV, and then inoculated with one dose of the allocated vaccine. Each subject was monitored for 15 minutes for local and systemic reactions and issued with a self-monitoring form.

At visit 2 (day 14 ± 4) all subjects were interviewed, examined for local and systemic reactions after the first inoculation, and were bled.

During visit 3 (day 28 ± 4), which was arranged only for the volunteers inoculated with the SB vaccine (Group B), each subject was vaccinated with one dose of the SB vaccine, monitored for 15 minutes and issued with a self-monitoring form.

During visit 4 (day 56 ± 7) all the volunteers (Groups A and B) were bled. All Group B subjects were interviewed and examined for local and systemic reactions following the second inoculation.

During visit 5 (day 168 ± 7) all subjects who had no HAV antibodies at the time of inclusion in the trial were bled for HAV antibodies and were immunised with one dose (booster) of the allocated vaccine, monitored for 15 minutes and issued with a self-monitoring form to record any local and systemic reaction.

During visit 6 (28 days ± 7 days after visit 5), all the volunteers were bled, and interviewed and examined for local and systemic reactions after the booster inoculation.

Laboratory tests for hepatitis A antibodies

Serological tests for hepatitis A antibodies and all titrations were carried out blinded by a central laboratory, BARC Laboratories, Ghent, Belgium using a commercial radioimmunoassay kit (HAVAB, Abbott Laboratories, North Chicago, Illinois) as described by Stafford et al. (1980).

The assay was modified in order to increase sensitivity (Miller et al. 1993),
and the results were converted into International Units by comparison with a
reference curve generated from the WHO Reference HAV Globulin No. 1
(Gerety et al. 1983). The detection cut-off value was about 10 IU/l, and a
value of 5 IU/l was given to all subjects below the detection limit in order to
calculate the geometric mean titre (GMT). Two measurements were used to
assess the immunogenicity of the vaccines: the seroconversion rate,
defined as an increase in total HAV antibody titre from < 20 IU/l to
≥ 20 IU/l, and the GMT.

Evaluation criteria

The primary evaluation criterion was the HAV antibody titre expressed in IU/l,
measuring the percentage of seroconversion in each group and the GMTs.

The secondary evaluation criteria were the HAV antibody titre, and the local
and systemic reactions after each inoculation, expressed as the percentage
of subjects with reactions. Seropositivity was defined as HAV antibody titre
≥ 20 IU/l and immunogenicity was assessed on the basis of the percentage
of seropositive subjects in each group.

Safety was assessed by the following three criteria:

1.   Percentage of subjects who experienced immediate reactions during
      the first 15 minutes after injection. An immediate reaction was defined
      as one or more of the following:

      •   Spontaneous pain at the injection site lasting more than
           15 minutes after the injection;

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- Allergic reaction;

- Malaise;

- Headache;

- Nausea/vomiting

2. Percentage of subjects who experienced local reactions after injection. Local reaction was defined as one or more of the following:

- Spontaneous pain at the injection site within 14 days after injection;

- Redness ≥ 3 cm at the injection site within 14 days after injection;

- Haematoma ≥ 3 cm at the injection site within 14 days after injection;

- Presence of a nodule at the injection site, at the visit following injection

3. Percentage of subjects who experienced systemic reactions after vaccination. A systemic reaction was defined as one or more of the following, within 7 days after injection:

- Fever;

- Fatigue;

- Headache;

- Myalgia, arthralgia;

- Nausea, vomiting, abdominal pain, diarrhoea

Statistical analysis

The sample size required to detect a difference of at least 5% on the mean of $\log_{10}$ titre, with a power of 95% and a two-tailed significance level of 5%, based on the method of Cochran and Cox, was 164 subjects per group. Given an expected seroprevalence rate of HAV antibody of 25-60% in enrolled volunteers and lost to follow-up, it was estimated that 200 subjects would be available for analysis of the primary evaluation criterion. The characteristics of the subjects between groups stratified by centre were considered before analysis of the primary evaluation criterion in order to assess the success of randomisation and comparability of the two groups. Variance analysis was used to test the serological results, seroconversion rates and GMTs at week 2 and 8, with adjustment for age, sex, weight, height and smoking as concomitant variables. The analysis of the reactogenicity of the vaccine took into consideration the number of subjects with one or more clinical reactions, and the results were examined according to the presence or absence of HAV antibody at the time of inclusion in the trial. The data were analysed using SAS software (6.08 Windows).

The data base

Data were recorded on standard case report forms and laboratory results of tests for HAV antibody were recorded on a separate form. The data were validated by an independent monitor. The information was transferred to the Methodology and Statistics Department of Pasteur Merieux Sérums and Vaccins, Marnes-La-Coquette, France and entered into a PS2 computer using Climed software (3.6 version MS-DOS) by double data entry. The data were filed in a Vax 3400 computer (Oracle).

RESULTS

Characteristics of the volunteers

210 volunteers were enrolled at the Royal Free Hospital School of Medicine

and their characteristics are shown in Table 8:

	Sex	Mean Age in yrs (range)	Mean Height in cms	Mean Weight in kg
Group A PM Vaccine	54 (51.4%) M	24.3 (18.1-48.4)	177	73.2
	51 (48.6%) F		165	58.9
Group B SB Vaccine	45 (42.9%) M	23.3 (18.3-63.0)	179	72.9
	60 (57.1%) F		164	58.2

The characteristics of the subject enrolled in Belgium, France and Germany

are shown in Table 9.

There were no statistically significant differences between the volunteers in

the four countries in terms of the sex ratio, mean age, mean height and

mean weight and there were no differences between Group A (PM vaccine)

and Group B (SB reference vaccine).

The effect of tobacco smoking could not be determined since overall

620 (74%) of the 840 volunteers enrolled by all the centres did not smoke,

59 (7%) smoked 1-5 cigarettes daily, 65 (7.7%) smoked 6-10 cigarettes daily

and 95 (11.3%) smoked more than 10 cigarettes daily.

Table 9

	Country	Sex	Mean Age in years (range)	Mean Height in cms	Mean Weight in kg
Group A PM Vaccine	Belgium	36 (34.3%) M	28.1 (17.9-51.1)	176	69.5
		69 (65.7%) F		166	61.0
	France	26 (25.0%) M	36.5 (20.0-58.5)	173	72.8
		78 (75.0%) F		164	60.8
	Germany	27 (25.7%) M	29.0 (18.0-56.0)	179	80.2
		78 (74.3%) F		167	62.6
Group B SB Vaccine	Belgium	37 (35.2%) M	27.5 (18.0-35.0)	177	73.5
		68 (64.8%) F		166	60.0
	France	25 (23.8%) M	36.3 (21.0-60.1)	174	74.1
		80 (76.2%) F		163	61.0
	Germany	25 (23.8%) M	29.4 (18.2-54.8)	179	77.5
		80 (76.2%) F		167	62.3

Seroprevalence of hepatitis A antibody at the time of inclusion in the study

The seroprevalence of hepatitis A antibody among the 210 volunteers in

London at the time of inclusion was 34 (16.2%), with 18 (17.1%) in Group A

and 16 (15.2%) in Group B. As expected the seroprevalence rates were

different in each country, with the lowest rates observed in Germany and the

highest in France (Table 10). The seroprevalence by age is shown in

Figure 10 for the London centre and for the other countries.

The frequency distribution of anti-HAV titres of 839 volunteers at the time of

inclusion in the study is shown in Figure 11. 620 subjects had HAV

antibody titres < 20 IU/l. These were considered as seronegative and were

included in the trial. 176 subjects of the 210 volunteers who were enrolled

in London were seronegative.

840 subjects were enrolled in this multicentre study, 210 in each country.

One person in France did not receive the first vaccine dose so that the total

study population comprised 839 volunteers. 419 volunteers in Group A (PM

vaccine) and 420 volunteers in the reference vaccine Group B received the

first injection, 418 volunteers received the second injection of the reference

vaccine and 668 received booster injection (336 in Group A and 332 in

Group B). All volunteers with variation in the dates of visits were excluded

from the analysis of the primary evaluation criteria. Of the 620 anti-HAV

seronegative volunteers available for this analysis, 559 were eligible.

Table 10:

<u>Seroprevalence of hepatitis A antibody at inclusion</u>

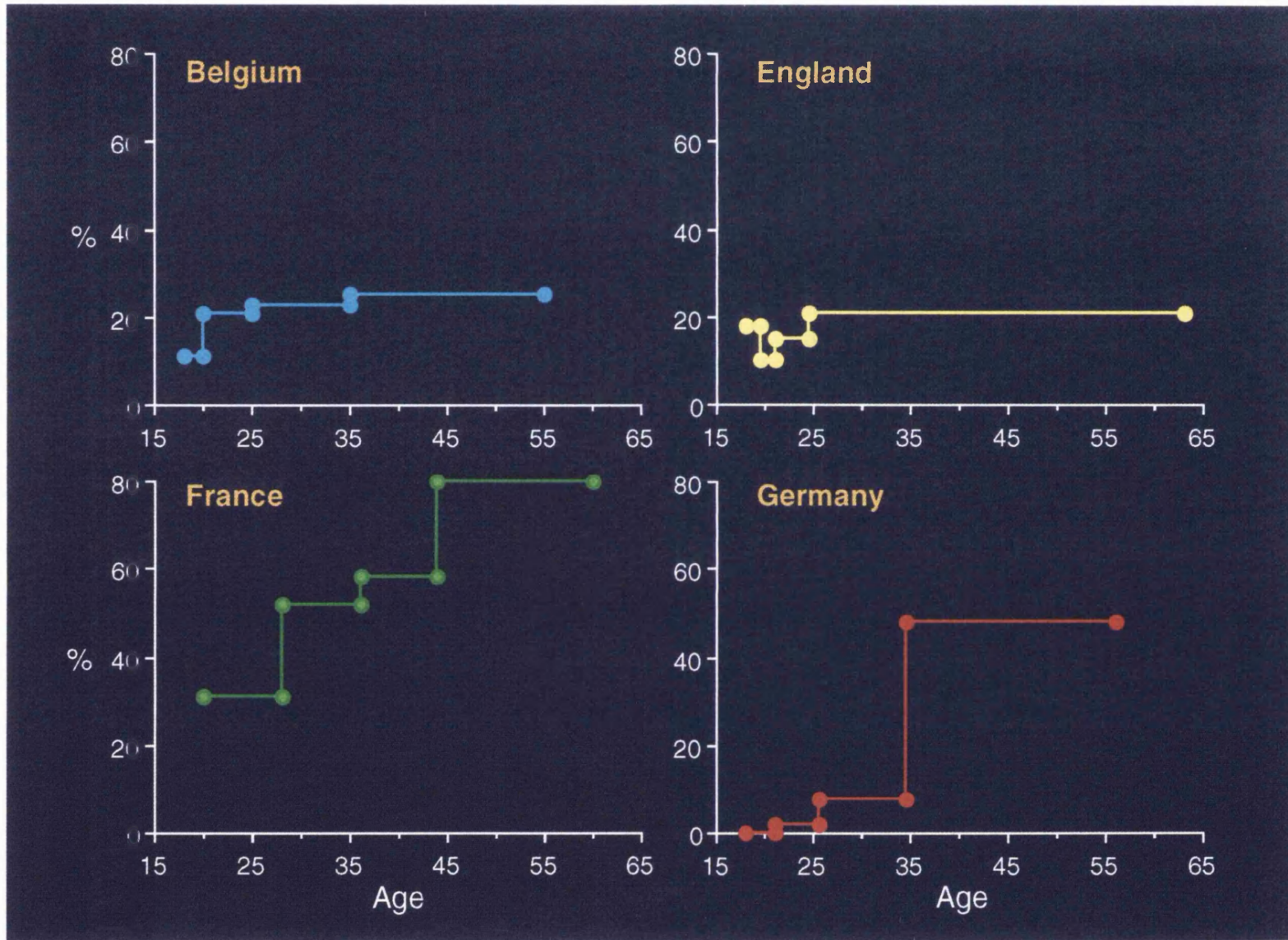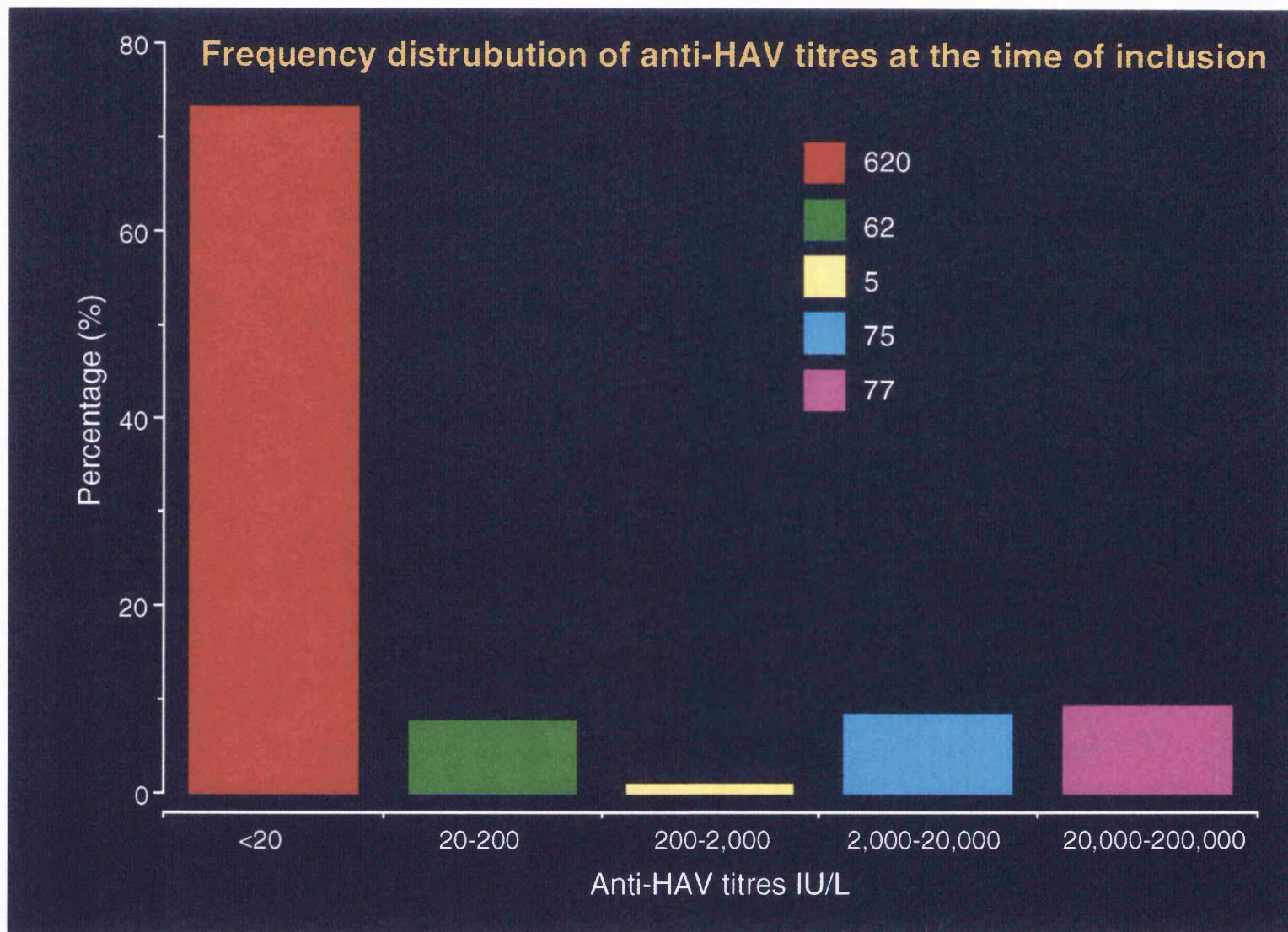| Country	Group A (PM Vaccine)	Group B (SB vaccine)	Total
England	18 (17.1%)	16 (15.2%)	34/210 (16.2%)
Belgium	21 (20.0%)	21 (20.0%)	42/210 (20.0%)
France	57 (54.8%)	56 (53.3%)	113/209* (54.1%)
Germany	15 (14.3%)	15 (14.3%)	30/210 (14.3%)

Figure 10: Seroprevalence of hepatitis A antibody in the volunteers in Belgium, England, France and Germany

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Figure 11:

Frequency distrubution of anti-HAV titres at the time of inclusion

A description of the characteristics of these 559 volunteers showed no differences from the 620 volunteers included initially and there were no differences between the two vaccinated groups in terms of sex ratio, mean age, mean height and mean weight, nor between the different centres. The data were therefore pooled for analysis. Among the 62 volunteers with anti-HAV titres between 20 and 200 IU/l, 59 received primary and booster vaccination, since their immune status was not definitely established. Immunogenicity data obtained from these volunteers were not used for statistical analysis of the primary evaluation criterion. The remaining 157 volunteers with HAV antibodies (titres above 200 IU/l) received primary immunisation only.

Six volunteers withdrew during the trial (3 in Group A and 3 in Group B). Three volunteers were lost to follow-up. A decision to stop the trial in six volunteers was taken by investigators, three because of an adverse event, one because of enrollment with a non-inclusion criteria, one because of pregnancy and one because of suspected pregnancy.

Adverse reactions

The summary of clinical reactogenicity in seronegative and seropositive volunteers (number and percentages of volunteers with one or more reaction) are shown in Tables 11 and 12. A vaccine dose-related increase in reaction rate was not observed in either anti-HAV seronegative or seropositive volunteers. The comparison between the percentage of volunteers in the two vaccine groups who experienced local or systemic reactions after the first dose and those experiencing such reactions after the booster dose showed no statistically significant differences.

Table 11: Clinical reactions in seronegative subjects

Type of reaction	After first vaccine dose		After 2nd vaccine dose	After booster	
	PM	SB	SB	PM	SB
Immediate	3/308 (1.0%)	5/312 (1.6%)	1/311 (0.3%)	1/303 (0.3%)	1/306 (0.3%)
Local	76/308 (24.7%)	61/311 (19.6%)	51/309 (16.5%)	57/303 (18.8%)	49/305 (16.1%)
Systemic	84/308 (27.3%)	78/311 (25.1%)	41/309 (13.3%)	49/303 (16.2%)	33/305 (10.8%)

Table 12: Clinical reactions in seropositive subjects

Type of Reaction	After first vaccine dose		After 2nd vaccine dose	After booster	
	PM	SB	SB	PM	SB
Immediate	2/111 (1.8%)	1/108 (0.9%)	1/107 (0.9%)	0/33 (0)	0/26 (0)
Local	22/110 (20.0%)	13/107 (12.1%)	12/107 (11.2%)	7/32 (21.9%)	3/26 (11.5%)
Systemic	20/110 (18.2%)	16/107 (15.0%)	10/107 (9.3%)	4/32 (12.5%)	3/26 (11.5%)

A dose-related increase in reaction rates was not observed in the group without HAV antibodies and the group with HAV antibodies at the time of inclusion in the trial. Comparison between the percentage of subjects in the two groups who experienced local or systemic reactions after the first dose and those with such reactions after the booster dose showed no statistically significant differences in the two populations.

Among the ten adverse events reported during the trial (three in volunteers vaccinated with the PM vaccine and seven in volunteers vaccinated with the SB vaccine), two were considered to be possibly vaccine-related, non-serious adverse reactions by the investigators: in one volunteer in France who received the PM vaccine, a rise in enzyme levels (aspartate aminotransferase: 60 U/l [N<40]; alanine aminotransferase: 95 U/l [N<50]; lactate dehydrogenase: 306 U/l [<60]; alkaline phosphatase: 199 U/l [N<110]; gamma-glutamyl-transpeptidase: 128 U/l [N<25]) was observed 3 weeks after the booster vaccination. This episode was accompanied by headache, myalgia and fatigue. Complete recovery was observed four weeks later. A second volunteer who received the reference SB vaccine had fever and fatigue 2 days after the booster vaccination. The remainder were considered to be non-vaccine related adverse events.

The three non-vaccine related adverse effects which occurred at the Royal Free centre were as follows:

1.   Acute infectious mononucleosis, confirmed by EB virus serology, 6 weeks after the second dose of the SB vaccine and one week after immunisation against hepatitis B (Engerix B). This subject was not given a booster immunisation.

2.   A volunteer in Group A who required cardiac surgery 6 months after the first dose of the PM vaccine. This subject was not given a booster dose.

3.   A subject in Group B who underwent nasal surgery 3 weeks after booster immunisation with the SB vaccine.

<u>Immunogenicity</u>

Because of variations in visit-dates, 608 volunteers were available at week 2

for measurement of seroconversion rates and GMTs, 565 subjects at

week 8, 609 subjects at month 6 (booster) and 609 at month 7. The

seroconversion rates and the GMTs (IU/l) with the range and the 95%

confidence interval after vaccination are shown in Table 13. At week 2,

93.4% of subjects seroconverted after the PM vaccine (GMT: 59 IU/l) versus

76.1% with the reference vaccine (GMT: 30.8 IU/l). Logistic regression

analysis revealed a significant vaccine effect on the percentages of

seroconversion at week 2 ($p < 0.0001$). The same conclusion was drawn

from the GMT analysis by multivariate regression. At weeks 8, 24 and 28

the seroconversion rates and GMTs were similar in both groups. As

expected, a significant sex ($p < 0.001$) and age ($p < 0.0001$) effect on

antibody response was observed.

Females were better responders than males and younger subjects responded

better than older ones. The age variable was analysed by using the median

age of the total population eligible for analysis (23.7 years) as the limit

between the two classes. The study population was not very heterogeneous

for age, since older subjects were poorly represented, but this did not

prevent the demonstration of the effect of this variable on the antibody

response. In 62 volunteers with titres between 20 and 200 IU/l before

vaccination, who were classified as possibly non-immune, it is interesting

to note that in four the antibody titres observed at week 2 increased by

more than 10 fold (21 IU/l to 361 IU/l, 21 to 305, 103 to 1436 and 169 to

5859 mIU/ml) suggesting that these volunteers were already immune and

that the vaccine had a marked booster effect on antibody titres.

Table 13: Seroconversion rates (SC) and geometric mean titres (GMTs) of HAV antibodies (IU/l) in initially seronegative volunteers

		Week 0	Week 2	Week 8	Month 6	Month 7
Group A PM Vaccine	Number	308	302	287	303	303
	SC rates	0%	93.4%	99.3%	99.7%	100.0%
	95% CI Titre range	5-19	90.4-96.3 5 - 1661	97.5-99.9 5 - 7070	98.2-99.9 16 - 7128	99 - 100 163 - 63627
	GMTs	7.1	59.0	138.4	279.6	4189.6
	95% CI	6.7 - 7.5	54.4 - 64.1	124.5 - 153.9	250.0 - 312.8	3792.3 - 4628.6
Group B SB Vaccine	Number	312	306	278	306	306
	SC rates	0%	76.1%	100%	99.0%	100%
	95% CI Titre range	5-19	71.2-81.1 5 - 402	99-100 24 - 7373	97.2-99.8 5 - 7854	99-100 57 - 57570
	GMTs	7.2	30.8	161.6	244.1	3163.4
	95% CI	6.8 - 7.7	28.1 - 33.7	143.8 - 181.5	216.9 - 274.8	2799.0 - 3575.2

95% CI = 95% confidence interval

In contrast, in the 58 remaining volunteers, whose anti-HAV antibody titres ranged between 20 and 82 IU/l at the time of inclusion, titres noted at week 2 showed a 0.3 to 6.5 fold rise, suggesting that these volunteers were non-immune. In the 157 subjects with titres over 200 IU/l, classified as immune, the GMTs increased after vaccination, demonstrating that in immune volunteers vaccination boosts antibody levels. The analysis of the serological results in these seropositive volunteers are shown in Table 14.

DISCUSSION AND CONCLUSIONS

This is the first comparative evaluation carried out by a multicentre European clinical trial of two inactivated hepatitis A vaccines, one produced by Pasteur Merieux using the GBM strain of hepatitis A virus, and the other vaccine employed as a reference preparation using the HM 175 strain manufactured by SmithKline Beecham.

Vaccine administration could not be blinded because the formulations of the two vaccines differed (0.5 ml for the PM vaccine versus 1 ml for the SB reference vaccine) as did the vaccination schedule (2 doses for the PM vaccine and 3 doses for the SB vaccine). Randomisation was correct, yielding two comparable populations eligible for statistical analysis of the primary evaluation criterion. The antibody titration method used blindly by an independent laboratory was used previously in other clinical trials of hepatitis A vaccine (Ellerbeck et al. 1992; Zaaijer et al. 1993).

Table 14: **Geometric mean titres of HAV antibodies (IU/l) in seropositive volunteers (titre > 200 IU/l at inclusion) after one (PM) or two (SB) vaccine doses**

		Week 0	Week 2	Week 8
Group A PM Vaccine	Number	78	76	77
	Titre range	693 - 124230	4326 - 106731	8337 - 160600
	GMTs	16656.6	25612.3	44406.4
	95% CI	13226.7 - 20976.0	21577.6 - 30401.3	37559.0 - 52502.0
Group B SB Vaccine	Number	79	76	70
	Titre range	1052 - 129987	2709 - 135420	7644 - 238360
	GMTs	17697.9	20752.8	46766.6
	95% CI	13895.0 - 22541.5	16725.0 - 25750.7	39218.1 - 55767.9

GMTs   =   geometric mean titre
95% CI =   95% confidence interval

The anti-HAV seropositivity cut-off was set at 20 IU/l, so as to be comparable with other PM vaccine trials conducted earlier. The safety of both vaccines was monitored after each inoculation and the reaction rates observed were similar to those reported previously (Wiedermann et al. 1990; Theilmann et al. 1992; Horng et al. 1993). The similar safety profile of the two vaccines confirmed the good tolerability of the PM vaccine, which was similar to other adsorbed adult vaccines such as inactivated polio vaccine and hepatitis B vaccine. The reaction rates with the PM vaccine decreased with successive vaccine doses (24.7% to 18.8% for local reactions and 27.3% to 16.2% for systemic reactions) indicating that the vaccine did not induce hypersensitisation. This confirms previous observations from the phase I study where volunteers received four vaccine doses. Another important point is the fact that the HAV serological status does not influence the safety profile of the vaccines. The kinetics of antibody response after the first vaccine dose were clearly different for the two vaccines, with a higher seroconversion rate observed at week 2 with the PM vaccine. This difference was no longer evident at week 8 and thereafter. The antibody titres observed after the booster injection were similar for both vaccines (4189 and 3163 IU/l) indicating that the duration of protection conferred by both vaccines should be equivalent. As demonstrated previously (Nalin et al. 1993), age and sex have been identified as concomitant variables influencing antibody response to inactivated hepatitis A vaccine, with a better response in females and in young subjects. The age variable was analysed by using the median age of 23.7 years as the limit between the two classes. The total study population was not heterogeneous for age since older subjects were under presented.

This did not affect the demonstration of the effect of age on this variable. The role of another concomitant variable (height in male volunteers) is much more difficult to interpret because of other unknown concomitant variables. A negative influence of tobacco consumption on seroconversion rates and GMTs was not observed, as is the case with hepatitis B vaccination, but only a few volunteers were heavy smokers. In conclusion, this first comparative trial between two inactivated hepatitis A vaccines demonstrated equivalent safety and immunogenicity after primary immunisation (week 8) and after booster inoculation. However, a more rapid increase in antibody levels was observed with the PM vaccine, using a two injection schedule instead of three inoculations of the reference vaccine.

In the absence of a stable international standard of hepatitis A antigen, it is not possible to compare the antigen content of the two vaccines, 160 ELISA units in the PM vaccine and 720 ELISA units in the SB reference vaccine. Although SmithKline Beecham kindly provided purified HAV antigen for comparison, the instability of the preparation prevented its use. The Merck, Sharp & Dohme internal HAV antigen standard is highly purified (to a purity greater than 95% and expressed as ng protein). However, the antigen incorporated in the PM vaccine is semi-purified and therefore the two preparations could not be compared, nor could a comparison be made with the SmithKline Beecham HAV antigen. It should be noted, nevertheless, that earlier studies based on the induction of antibodies to HAV antigen in guinea pigs suggested that the antigenicity of the PM and the SB vaccines in this model were similar, and more antigenic than the Merck, Sharp & Dohme

antigen preparation. Further studies are clearly required when a stable international reference HAV antigen vaccine preparation becomes available.

In summary, a multicentre, controlled, randomised, open, comparative trial of 839 healthy adult volunteers was carried out in order to compare the immunogenicity and reactogenicity of two vaccines against hepatitis A during primary immunisation and after booster injection. The first vaccine was produced by Pasteur Merieux (PM), and the reference vaccine by SmithKline Beecham (SB). The vaccination schedule consisted of two doses (month 0, 6) with the PM vaccine and three doses (month 0, 1 and 6) with the reference vaccine. Two weeks after the first dose, the seroconversion rates among initially HAV seronegative subjects (n=608) were 93.4% and 76.1% with the PM and the SB vaccine respectively, the corresponding geometric mean titres (GMTs) were 59 IU/l versus 30.8 IU/l. Two months after the beginning of immunisation (one dose versus two doses) the GMTs were 138.4 and 161.6 IU/l respectively. At month 7, the seroconversion rates were 100% with both vaccines, and the GMTs were 4189 and 3163 IU/l respectively. After the first dose of vaccine, 24.6% and 19.6% of those inoculated with the PM and SB vaccines respectively reported local reactions. The rates for systemic reactions were 27.2% and 25.0% respectively. Lower rates of local and systemic reactions were observed after booster injections and there were no statistically significant differences between the two vaccines. Logistic regression analysis revealed a significant vaccine effect on seroconversion rates only at week 2 (p < 0.0001).

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The same conclusions were drawn from the analysis of GMTs by multivariate regression. When both week 2 and week 8 were analysed together, a statistically significant effect of interaction between time and vaccine was observed, indicating that the kinetics of antibody responses were different (Goilav et al. 1995).

Follow-up of the subjects one year after the booster dose given 6 months after the primary inoculation revealed a decrease in the titre of anti-HAV with both the PM vaccine and the reference vaccine, with GMTs measured blindly by the modified RIA technique of 1556.1 IU/l and 1021.2 IU/l respectively. The kinetics of the antibody decrease were similar for both vaccines, with a higher GMT with the PM vaccine under trial. Variance analysis of GMTs showed no significant effect of group/time interaction.

A significant group (p < 0.0001) and time (p < 0.0001) effect was observed confirming higher GMT with the PM vaccine group and a decrease in antibody titres with time. All subjects in both groups retained protective levels of anti-HAV.

IMMUNISATION AGAINST HEPATITIS A

General discussion and conclusions

The three studies which are described above indicate clearly that the

inactivated hepatitis A vaccines, the 720 ELU per dose and the 1440 ELU

per dose prepared from the HM 175 strain of HAV (SB vaccine) and the 160

antigen units per dose preparation of the GBM strain of virus (PM vaccine),

are safe with minor transient side-effects and highly immunogenic when

given by the intramuscular route into the deltoid muscle. The schedule of

administration of the vaccine varied according to the manufacturer with

3 doses of the SB vaccine and 2 doses with the PM vaccine. At month 7,

the seroconversion rates were 100% with both vaccines, and in the

comparative study the GMTs at that time were 3163 IU/l with the SB vaccine

and 4189 IU/l with the PM vaccine. One year after the booster (18 months

after primary immunisation) all subjects who received either of the two

vaccines were seropositive with GMTs of 1021 IU/l and 1556 IU/l

respectively. Variance analysis at 7 months and 18 months revealed that

the GMT with the PM vaccine was significantly higher, and that the kinetics

of antibody decline were equivalent for the two vaccines. Studies with the

SB vaccine have been conducted over a longer period of time and the

antibody titres recorded 2 and 3 years after immunisation with this vaccine

(Berger et al. 1993) confirmed an estimate of antibody persistence for

10 years. A more recent study projected a much longer period of

protection for at least 20 years (van Damme et al. 1994b). The comparative

study showed that antibody levels induced by the PM vaccine were higher

implying that a similar long term protection can also be expected with this

preparation. It should nevertheless be noted that even the substantial level

of antibody response to inactivated HAV vaccines is well below the antibody titre of 150,000-1,000,000 IU/l which follows infection with wild-type virus.

Although this difference in concentration of circulating antibody to HAV is unlikely to be of significance in terms of protection against infection in the first 10 years or so after immunisation, only long term follow-up will establish whether the lower level of antibody responses reflect a comparable level of immunological memory and a shorter period of immunity.

The study in patients with congenital coagulation defects using a rapid two dose immunisation schedule with the 1440 ELU per dose SB vaccine in adults and 720 ELU per dose in children demonstrated that the vaccine is safe and highly immunogenic when given subcutaneously. The overall seroconversion rate was 90% and the GMT was 950 IU/l at month 7. Seroconversion occurred in all the children compared with 85% of the adults one month following the booster dose. Statistical comparison of the rate of seroconversion and GMTs with the CD4 cell counts demonstrated that those with low CD4 counts did not appear to seroconvert. However, several adults who did not seroconvert also had low CD4 counts so that it was not possible to determine a causal relationship. It is recommended that all patients with congenital coagulation effects without antibodies to HAV should be immunised against hepatitis A not only to preclude infection as such, but also to reduce complications in these patients who had been exposed in the past to a heavy viral burden with hepatitis B, C, and the more recently identified hepatitis G/GBV-C, HIV and others, and who may have underlying liver disease of varying severity.

<u>Other considerations</u>

<u>Pre-vaccination testing for susceptibility and post-vaccination testing for serological response</u>

Screening for anti-HAV before vaccination may or may not be cost-effective depending on several factors, including the expected prevalence of anti-HAV in the population, the cost of the screening test and the cost of the vaccine. For example, anti-HAV prevalence is generally greater than 50% among persons born in developing countries and those born in industrialised countries before 1945. Prior testing in these persons may be cost effective. Anti-HAV prevalence among persons born in industrialised countries after 1945 is generally lower than 25%, and therefore vaccination without pre-screening is usually cost-effective in these persons.

In view of the high immunogenicity observed with inactivated hepatitis A vaccines, post-vaccination testing for serological response is not indicated generally.

<u>Development of immunisation strategies</u>

In highly endemic countries, it is difficult and impractical to identify risk groups for transmission of hepatitis A and any intervention strategy must take into account that children below the age of 5 years are the single most important group in this setting. However, taking into consideration the relative light disease burden from hepatitis A in this group and the current cost of the vaccine, the cost-benefit of mass immunisation of infants may be too low for most developing countries in the light of other pressing public health problems. However, the increased hepatitis disease burden with increasing age of infection will lead to reassessment of the position.

Meanwhile efforts to improve water supplies and sanitation should have a high priority in order to reduce the infection rate with hepatitis A.

In intermediate endemic areas infection with HAV occurs with high rates in older children and adults and can result in significant morbidity. Hepatitis A occurs endemically and in large community outbreaks. Priority groups for immunisation should be defined initially based on the local situation. Ideally, when vaccine costs permit the ultimate aim should be mass vaccination.

In low endemic areas, in the industrialised countries circulation of HAV is low in the general population. Hepatitis A continues to occur, however, in certain groups such as travellers to endemic areas, sexually active male homosexuals, intravenous drug abusers, semiclosed institutions, communities with low socio-economic conditions and in some countries, day-care centres for young children, and historically at least in institutions for the mentally-disabled. Hepatitis A vaccine affords protection of longer duration than immune globulin and offers many advantages in these groups and settings. Other groups include persons with chronic liver disease, sewage treatment plant workers and it has been suggested that economic analysis should be undertaken to determine whether pre-exposure vaccination of food handlers is indicated. Nosocomial outbreaks from infected patients to health care workers are rare, but health care workers and students seconded for work in endemic areas should be immunised.

Future considerations

Several questions remain regarding inactivated hepatitis A vaccines, including whether such vaccines may provide protection when given shortly after exposure (for which there is preliminary supporting evidence), the duration of protection and the extent of immunological memory, and the cost-effectiveness of various strategies for the use of these vaccines.

The epidemiological characteristics of hepatitis A suggest strongly that effective control of hepatitis A in all populations could only be achieved by universal immunisation. This could be realised by the development of vaccines combining inactivated hepatitis A antigen with other vaccine antigens in a single formulation e.g. with hepatitis B or diphtheria-pertusis-tetanus (DPT). Alternatively, live attenuated HAV vaccine, or potentially recombinant live-vector hepatitis A vaccines may offer advantages in terms of costs, production, administration and acceptability.

Section 4:

Overcoming non-responsiveness to hepatitis B immunisation

in health care workers using a novel recombinant vaccine

INTRODUCTION

Systematic vaccination of individuals at risk of exposure to hepatitis B virus has been the main method of controlling the morbidity and mortality associated with hepatitis B. The first hepatitis B vaccine was manufactured by the purification and inactivation of hepatitis B surface antigen (HBsAg) obtained from the plasma of chronic HBV carriers (Szmuness et al. 1981; Hadler et al. 1986; Jilg et al. 1988). This was soon followed by the production of HBsAg using recombinant DNA techniques and expression of the S antigen component in yeast cells.

All studies of the antibody response to currently licensed plasma-derived hepatitis B vaccines and hepatitis B vaccines prepared by recombinant DNA technology have shown that between 5% and 10% or more of healthy immunocompetent subjects do not mount an antibody response (anti-HBs) to the surface antigen component (HBsAg) present in these preparations (non-responders) or that they respond poorly (hypo-responders) (Dienstag et al. 1984; Craven et al. 1986; Westmoreland et al. 1990; Wood et al. 1993). The exact proportion depends partly on the definition of non-responsiveness or hypo-responsiveness, generally less than 10 IU/l or 100 IU/l respectively against an international antibody standard.

It is considered that non-responders remain susceptible to infection with hepatitis B virus. While several factors are known to affect adversely the antibody response to HBsAg including the site and route of injection, gender, advancing age, body mass (overweight), immunosuppression and

immunodeficiency, the mechanisms underlying non-responsiveness to the S component of hepatitis B surface antigen in humans remain largely unexplained although evidence is accumulating that there is an association between different HLA-DR alleles and specific low responsiveness in different ethnic populations. Considerable experimental evidence is available that the ability to produce antibody in response to specific protein antigens is controlled by dominant autosomal Class II genes of the major histocompatibility complex (MHC) in the murine model (reviewed in Alper et al. 1989; Milich 1991; Kruskall et al. 1992). Much effort has been devoted to overcoming Class II-linked non-responsiveness to current hepatitis B vaccine (for example Milich et al. 1985a; Milich et al. 1986; Arif et al. 1988).

There is evidence that the pre-S1 and pre-S2 domains have an important immunogenic role in augmenting anti-HBs responses, preventing the attachment of the virus to hepatocytes and eliciting antibodies which are effective in viral clearance, stimulating cellular immune responses, and circumventing genetic non-responsiveness to the S antigen (Milich et al. 1985a; Klinkert et al. 1986; Alberti et al. 1988; Gerlich et al. 1990). Thus a number of studies indicated that the inclusion of pre-S components in recombinant or future synthetic vaccines should be developed. For example, the pre-S2 region is more immunogenic at the T and B cell levels than the S regions in the mouse model (Milich et al. 1985a; Milich et al. 1985b), as is the case with pre-S1 in the mouse (Milich et al. 1986) and in man (Ferrari et al. 1992) and circumvent S region non-responsiveness at the level of antibody production.

Indeed, Milich et al. (1986) demonstrated in the murine model that the independence of MHC-linked gene regulation of immune responses to pre-S1, pre-S2 and S regions of hepatitis B surface antigen would assure fewer genetic non-responders to a vaccine containing all three antigenic regions. Studies conducted in humans with experimental recombinant hepatitis B vaccines containing all three S components of the viral envelope polypeptides demonstrated the enhanced immunogenicity of such preparations when compared with conventional yeast-derived vaccines (Yap et al. 1992; Shouval et al. 1994; Yap et al. 1995) although several earlier studies with vaccines containing the S, pre-S1 and pre-S2 components revealed significant differences from preparations containing only the S antigen (Marescot et al. 1989; Ferrari et al. 1992; Clements et al. 1994; Suzuki et al. 1994).

A single centre study of health care personnel who have previously failed to mount a surface antibody (anti-HBs) response to immunisation against hepatitis B with currently licensed hepatitis B vaccines containing the S antigen was carried out at the Royal Free Hospital School of Medicine using a novel recombinant DNA hepatitis B vaccine (Hep B-3) containing pre-S1, pre-S2 and S components of hepatitis B surface antigen. The trial was conducted between October 1994 and December 1995 in accordance with the 1989 Hong Kong amendment of the Declaration of Helsinki and the Good Clinical Practices Guidelines after approval of the Local Ethical Practices Committee of the Royal Free Hospital and the School of Medicine.

Volunteers who failed to respond previously to hepatitis B immunisation were recruited from various hospitals in London and other regions of the country between 27th October 1994 and 12th December 1994.

The Hep B-3 recombinant DNA hepatitis B vaccine

The vaccine was manufactured by Evans Medical, a subsidiary of Medeva Plc, UK and was developed originally in Germany by Exogene Pharma Forschung GmbH as a third generation hepatitis B vaccine containing pre-S1, pre-S2 and S antigenic components of the hepatitis B surface antigen protein of hepatitis B virus subtypes adw and ayw. It is believed that by incorporating all three surface antigen components, produced in mammalian cells as the host system, the new vaccine will emulate more closely the physico-chemical properties and the antigenicity and immunogenicity associated with the natural viral coat protein.

The vaccine is produced in a continuous mammalian cell line, the mouse c127 clonal cell line, after transfection of cells with recombinant HBsAg DNA using a bovine papillomavirus vector (Yoneyama et al. 1988; Samanta and Youn 1989). It was shown that the plasmids are integrated into the host cell chromosomal DNA, resulting in stable expression of HBsAg. Following the transfection of suitable expression plasmids into the c127 cells, clones secreting S and pre-S1 antigenic components were selected for establishing a Master Cell Bank.

The c127 cell line is a non-transformed clone derived from a spontaneous

mammary tumour of an R III mouse (Lowy et al. 1978). The cell line has

been judged suitable for the expression of foreign genes introduced into the

cells by transfection of the corresponding DNA. A variety of gene products

have been expressed in c127 cells, including human plasminogen activator,

human growth hormone, human chorionic gonadotrophin and human

interferon beta 1 and human interferon gamma. The Master Cell Bank has

been characterised and tested on the basis of current Requirement of the

EEC Guidelines, the Bureau of the Food and Drug Administration (FDA),

USA and the Japanese Ministry of Health and Welfare.

The transfected cell line clone secretes particles consisting of three

monomers of HBsAg (which make up the product of the vaccine):

1.   The S protein, 226 amino acids in length;

2.   The pre-S1-S monomer, 227 amino acids in length consisting of the

      S protein and the pre-S1 epitope; and

3.   The pre-S2-S monomer, 274 amino acids in length consisting of the

      S protein and the pre-S2 epitope.

The HBsAg protein was characterised by polyacrilamide gel electrophoresis,

Western blots, amino acid composition analysis, UV absorption, amino

terminal analysis, buoyant density, electron microscopy, enzyme linked

immunosorbent assays, and epitope mapping using monoclonal antibodies.

The composition of the final filled vaccine product formulated in 1 ml unit dose in a pre-filled syringe is as follows:

HBsAg purified assembled particles	0.01 mg
Aluminium hydroxide (0.5 mg aluminium)	1.44 mg
Sodium chloride in water for injection 0.9% w/v to	1.0 ml

Study objectives

The objectives of the study were to assess the immunogenicity, reactogenicity and the kinetics of the surface antibody response (anti-HBs) of four different doses 5, 10, 20 and 40 μg of the vaccine, in true non-responders to currently available hepatitis B vaccines and to evaluate the anti-HBs response following a second dose of vaccine given two months later.

Selection of volunteers

Written and telephone information about the vaccine and the study were provided to Occupational Health Units of Hospitals in the London area and other cities within reach of London seeking adult health care volunteers from among the staff and medical students who failed to mount an anti-HBs response after multiple immunisation with currently licensed hepatitis B vaccines containing the S antigen. Volunteers were interviewed individually at the Royal Free Hospital School of Medicine and were provided with full information about the vaccine and the design of the study, which was to be carried out entirely at the Royal Free centre. A signed informed consent was obtained from each subject.

Inclusion criteria

- Males or females aged 18-70 years old;

- Females who were demonstrably not pregnant or lactating;

- A history of non-seroconversion (< 10 IU/l anti-HBs antibody) following at least 4 and usually multiple injections of an S containing hepatitis B vaccine

Exclusion criteria

- Any clinically significant findings on physical examination or clinically significant concurrent illness that in the opinion of the investigator precluded inclusion into the study;

- Subjects who were immunocompromised due to illness;

- Subjects who were receiving any medication that may affect their immune function (e.g. corticosteroids or chemotherapy);

- Subjects who had a known history of anaphylactic shock after vaccination;

- Subjects who had a history of hepatitis B infection or had serological markers of past or current infection with hepatitis B virus;

- Subjects with clinically significant abnormal laboratory assessments.

Subjects were allowed to receive concomitant medication, excluding immunosuppressant treatment, during the study. If such treatment was given, the subject was to remain in the analysis up to the point at which excluded therapy(ies) began.

Study design and randomisation

Subjects were allocated in a double blind, randomised fashion into four groups to receive either 5, 10, 20 or 40 μg of the vaccine at time 0 and 2 months according to the procedure outlined in Table 15.

The following routine haematological and biochemical tests were carried out at the Royal Free Hospital at screening:

- Approximately 10 mls of blood were taken for the following haematological and biochemical analyses:

Haematology:	Haemoglobin, white blood cell count, platelets, differential count
Biochemistry:	Urea and electrolytes, liver function tests, creatinine
Urine:	Pregnancy screen (HCG, Clearview™)

Laboratory abnormalities considered clinically significant were recorded on the Case Report Form. In addition to this, the pregnancy screen was repeated prior to each vaccination in females of child bearing age. This screen was omitted on Study Day 0 if this was within 1 day of the screening visit.

Laboratory tests for antibody determinations

A 10 ml blood sample was taken for anti-HBs and anti-core (anti-HBc) determinations and the serum was aliquoted into four vials. Samples were stored at -20°C at the Royal Free Hospital until required for testing.

Table 15:

Procedure	Screening	Study Day 0 (Month 0)	Study Day 2 (Month 2)	Follow-Up Month 6
Written informed consent	●			
Demographic Data	●			
Medical History	●			
HBV vaccination history (including response)	●			
General physical examination	●			
Venepuncture for haematology/biochemistry	●			
Pregnancy screen (females of child-bearing potential only)	●	● (if > 1 day since screening)	●	
Venepuncture for anti-HBs, anti-HBc determination	●	●	●	●
Vaccination with Hep B-3		●	●	
Vital Signs pre- and 15 min post-vaccination		●	●	
Assessment of local/ systemic reactions		●	●	
Spontaneous complaints of adverse effects		●	●	●
Provide diary card		●	●	
Review diary card			●	●

Two aliquots of each sample were also sent in batches to the Evans Medical Laboratories.

The anti-HBs determinations were carried out in the Department of Virology of the Royal Free Hospital using a sandwich ELISA developed by Biokit, SA (Barcelona, Spain), and repeated independently by the WHO Collaborating Centre for Reference and Research on Viral Diseases, Royal Free Hospital School of Medicine using enzyme immunoassay reagents purchased from Abbott Laboratories (Ausab EIA, Abbott Laboratories, North Chicago, Illinois).

All sera from enrolled volunteers were tested for total antibody to hepatitis B core antigen (anti-HBc) by a competitive enzyme immunoassay (Bioelisa anti-HBc, Biokit, Spain).

Replacement policy
A subject who received both vaccinations and completed the study visits was to be deemed evaluable. Subjects who were withdrawn for any reason were not to be replaced since the sample size calculation allowed for a degree of loss from the study.

The study was monitored independently by Medeva Scientific and Regulatory Affairs and by Clinical Research Officers of Euro Bio-Pharm, Chippenham, Wiltshire.

Statistical analysis

Sample size justification

The study contained no placebo group, since it was considered that a placebo response was sufficiently improbable that it could be ignored. However, since it is not impossible, the size calculation assumed a 5% placebo response, and calculated group sizes which would be necessary to have 90% power to detect a 45% response in the active groups ($p < 0.05$; 2 tailed). This calculation gave group sizes of 23, which rounded up to 28 would allow for up to five drop-outs per treatment group. Thus, it was planned to enrol 28 subjects into each of the 4 groups.

Statistical methods

Subjects were considered evaluable for analysis if they received both inoculations of the vaccine and completed the study visits.

Serological responses among the 4 dose groups were compared using the unpaired Student's t test with logarithmically transformed data. The dose was allowed to be both categorical and continuous to test for the presence of a trend to the response. The proportion of individuals seroconverting and protected was assessed by logistic regression. 95% confidence intervals were calculated for the geometric mean titres by dose group and month. Descriptive statistics were also used to compare the reactogenicity and safety between all the groups.

RESULTS

Subject accounting

100 volunteers, 50 males and 50 females were enrolled in this study between 27th October 1994 and 12th December 1994. The last volunteer completed the final visit on 25th May 1995. Each of the four groups comprised 25 subjects. However, 14 volunteers violated the original protocol in that they were found on testing to have baseline anti-HBs titres > 10 IU/l. A summary is provided in Table 16:

| | Vaccine dose | | | | Total |
	5 μg	10 μg	20 μg	40 μg	
Number of subjects enrolled	25	25	25	25	100
Protocol violators (baseline anti-HBs > 10 IU/l)	3	2	5	4	14
No. of subjects - intent to treat efficacy analysis	25	25	25	25	100
No. of subjects - per protocol efficacy analysis	22	23	20	21	86
No. evaluable for reactogenicity	25	25	25	25	100

Antibody to hepatitis B core antibody (anti-core, anti-HBc) was detected by enzyme immunoassay in the serum of four subjects on completion of serological screening. These sera were retested and 1 of the 4 sera was found negative to anti-HBc by a second independent laboratory, whereas the other 3 sera yielded equivocal results. The sera were tested for HBV-DNA by the nested-polymerase chain reaction (PCR) and were found to be negative. There was, therefore, no evidence of latent or silent hepatitis B infection in any of the per protocol subjects.

Demographic data

The data are shown in Table 17. There were no differences between the four groups for any of the demographic features tested at baseline. The majority of previous hepatitis B vaccinations had been carried out using Engerix B (SmithKline Beecham).

Vaccine administration

All 100 subjects received two doses of vaccine as planned, according to the randomisation group to which they were assigned. Checks on subject compliance were carried out as part of the normal monitoring procedures, and no issues arose.

In summary, 100 subjects were entered into the study, 14 of whom were not eligible for inclusion into the per protocol analysis. Therefore, the per protocol population consisted of 86 subjects with anti-HBs titres < 10 IU/l.

The median age at entry into the study was 38 years, and the mean number of immunisations each subject had received of commercially available hepatitis B vaccines containing the S component was 6.0.

All the subjects completed the study and the safety data was obtained from the intention-to-treat population which included all 100 subjects who received two doses of the Hep B-3 vaccine. There were no significant differences in either age or sex within the four antigen dose groups.

Table 17:

Demographic features

Antigen μg	5	10	20	40
Age (SD)	36 (12.0)	39.0 (11.5)	37 (11.8)	40 (11.9)
Male/Female	8/17	15/10	15/10	12/13
Weight Kg (SD)	72 (15.5)	74.6 (13.0)	77 (14.8)	76 (14.7)
Number of previous inoculations (Median)	5	5	5	6

(SD) = standard deviation

Reactogenicity and safety of the vaccine

Local or systemic reactions to the first inoculation of the vaccine were experienced by 15% of the subjects. These reactions comprised local pain at the site of injection (11 subjects) or local burning (4 subjects), chills, dizziness and headache (1 subject each). Fewer reactions were observed after the second inoculation: local pain was reported by 8 subjects, local burning by 5 and dizziness and nausea by 1 subject each.

No statistically significant changes were noted in pulse, respiration rate or blood pressure between dose groups after the first and second vaccination.

Subjects were also asked to record their body temperature for 72 h post-vaccination in their diary cards. Analysis of the data collected revealed no differences between dose groups, although were were apparent trends at some time points, particularly after the first vaccination. However, the mean values from which comparisons were made were all within normal range and the differences between treatment groups amounted to no more than 0.4°C.

Finally, it is interesting to note that adverse reactions considered to be related to the Hep B-3 vaccine by the investigator occurred more frequently with the lower doses. All general symptoms such as chills, dizziness, headache, nausea and diarrhoea occurred within 24 hours of immunisation and were transient. No serious adverse events occurred during the study.

Immunogenicity

Two months following a single dose of vaccine, 64% (55/86) of subjects seroconverted with an anti-HBs titre of > 10 IU/l. The seroconversion rates for each of the four antigen dose groups was 45.5% (10/22), 69.6% (16/23), 60% (12/20) and 81% (17/21) respectively (Table 18). The overall rate of seroconversion four months following the booster dose was 66% (57/86), with rates ranging from 55-76% across the different groups, the highest occurring in the 40 μg antigen dose group.

There was no significant difference in overall seroconversion rate between the four groups. However, only the 5 μg dose was significantly different from the 40 μg dose at 2 months (p = 0.02). By 6 months, there were no significant differences between the different doses (Figure 12).

The overall rate of arbitrary seroprotection, defined as the presence of anti-HBs titres of > 100 IU/l, was 22% (19/86) after the first immunisation and 21% (18/86) after the booster (Figure 13). A pairwise comparison showed a significant difference between the 40 μg and the 5 and 10 μg antigen doses at month 2, and similarly between the 5 μg and 20 and 40 μg antigen doses at month 6. There was a significant trend with dose at 6 months (p = 0.03) with seroprotection levels ranging from 5% in the lowest group to 33%. At 2 months, there was a significant difference between the 5 and 40 μg groups (p = 0.04) and between 10 and 40 μg groups (p = 0.02). However there was no significant difference between the 20 and 40 μg groups (Table 19).

There was significantly higher geometric mean titre levels within the 20 and 40 μg antigen dose groups, 36 IU/l and 63.1 IU/l respectively at month 2 compared with 6.4 IU/l and 22.5 IU/l for the 5 and 10 μg antigen dose groups (Figure 14). At 2 months, there was a significant difference in geometric mean titres by dose (p = 0.004). By 6 months this had become less apparent although it remained significant (p = 0.03), due mainly to a drop in geometric mean titres in the 40 μg group. At 6 months, only the 5 μg group had a significantly lower geometric mean titre than the 40 μg group (Figure 15). No other differences were significant (Table 20).

The proportion of subjects seroprotected was greater in the 40 μg antigen dose group, 33.3% (7/21) than with the 20 μg dose 30% (6/20). These differences were not different statistically.

Administration of a booster dose of the vaccine at 2 months did not produce a corresponding rise in geometric mean titres within any of the antigen dose groups. The cumulative seroconversion rates increased by 14% for the lowest two dose groups, with a maximum increase of 5% observed at month 6 in the 20 and 40 μg dose groups. There were no significant differences in the anti-HBs antibody titres obtained by the two different assay systems used (Figures 16 and 17).

Figure 12: **Percentage of subjects who have seroconverted at months 2 and 6**
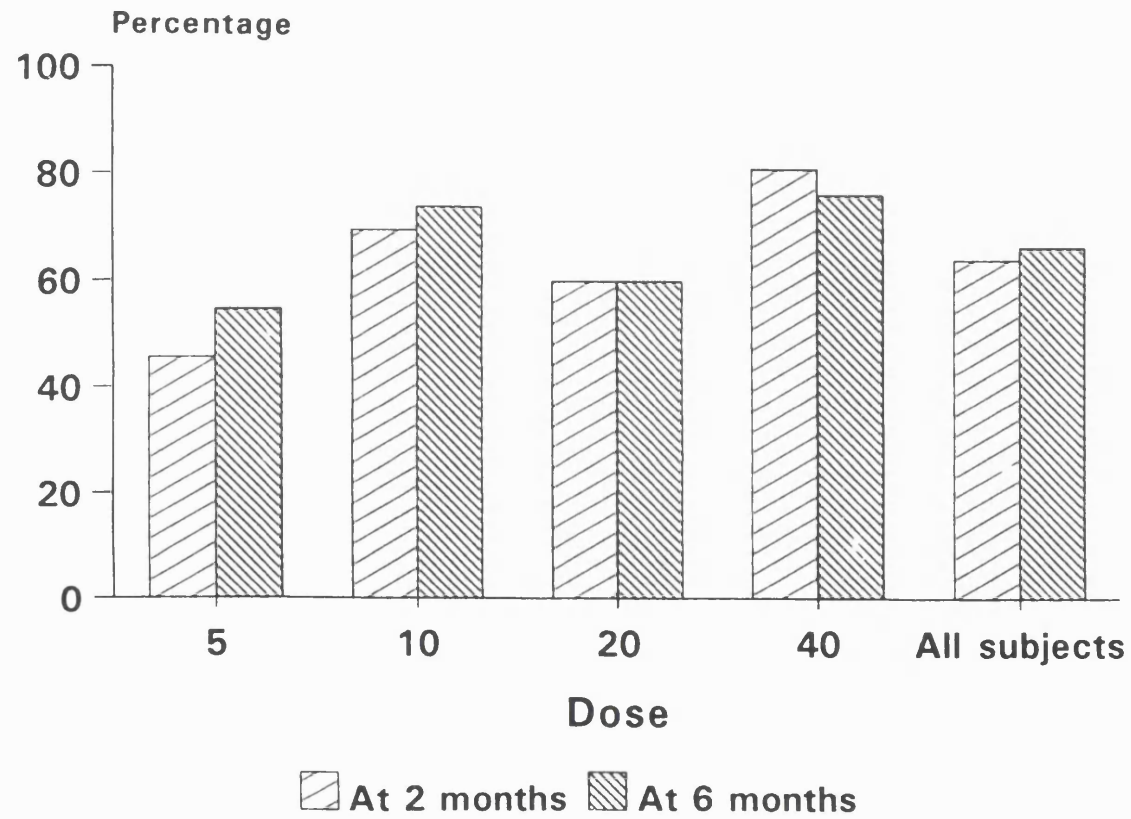
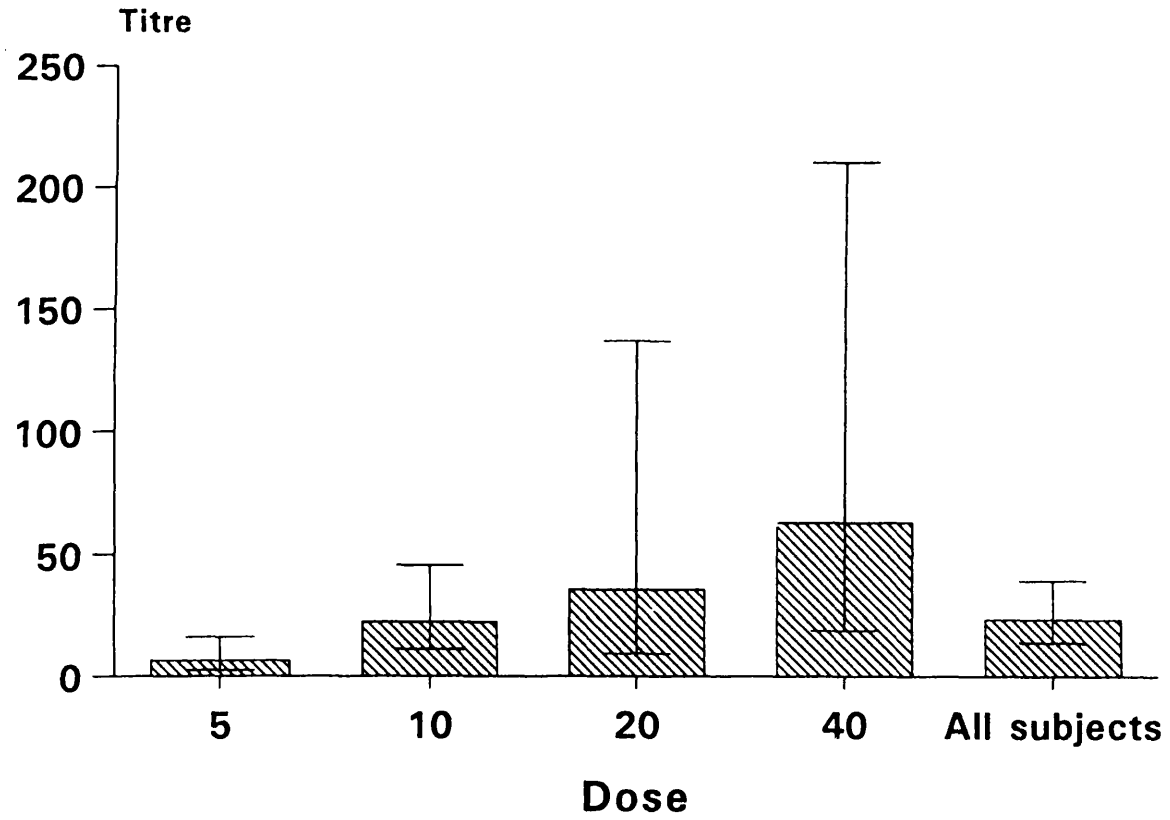Sample : 86 per-protocol subjects

Figure 13:   **Percentage of subjects who are protected at months 2 and 6**

Dose

At 2 months   At 6 months
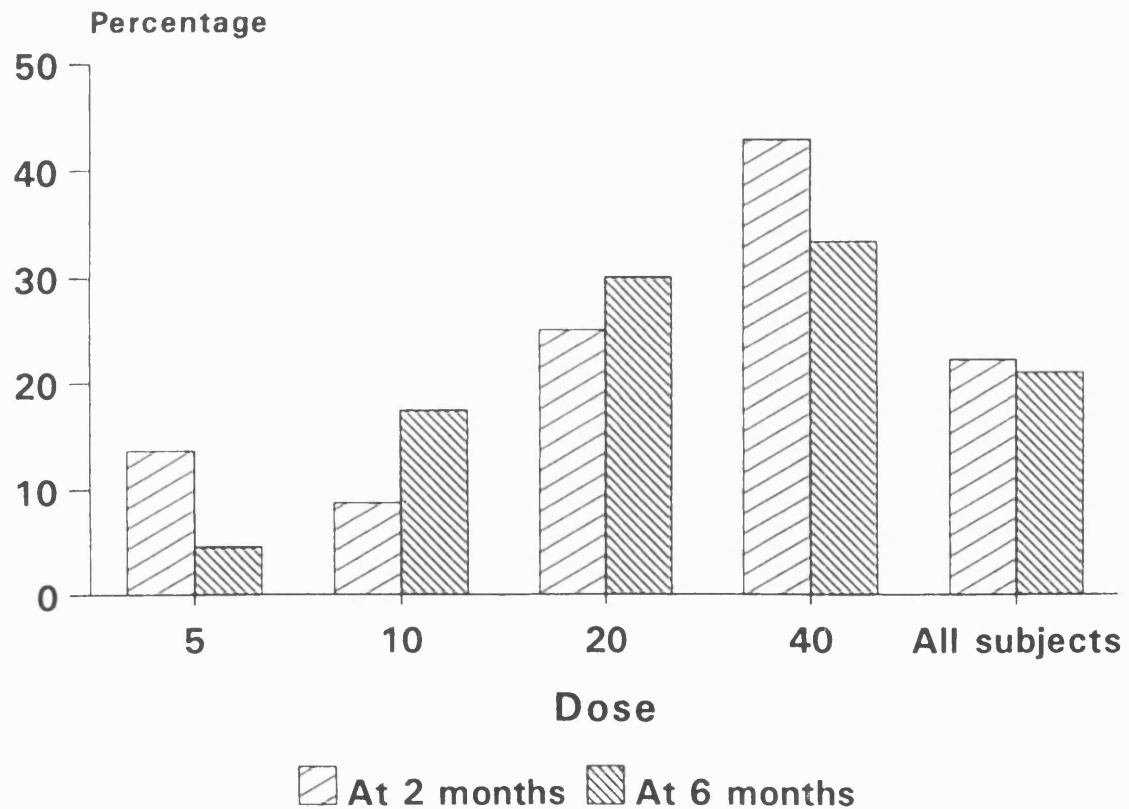
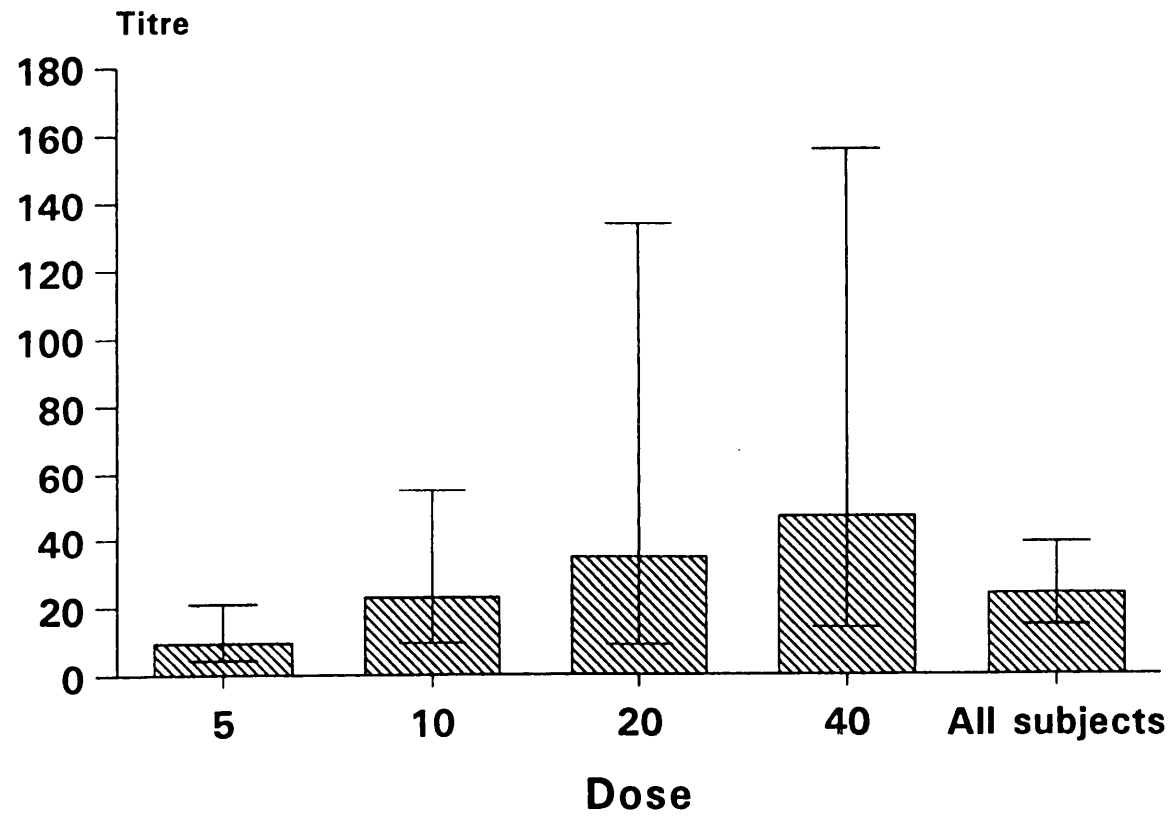Sample : 86 per-protocol subjects

Figure 14:

**GMTs (and confidence intervals)
at month 2**

Sample : 86 per-protocol subjects

Figure 15:

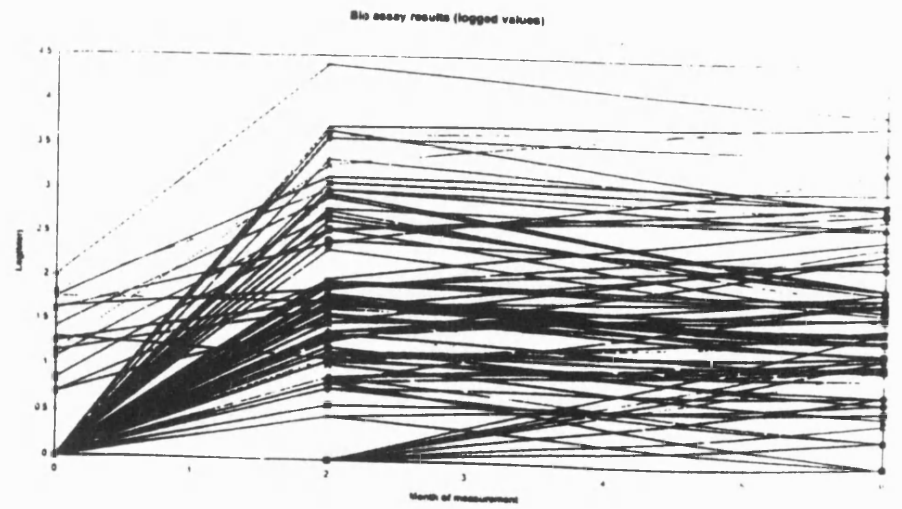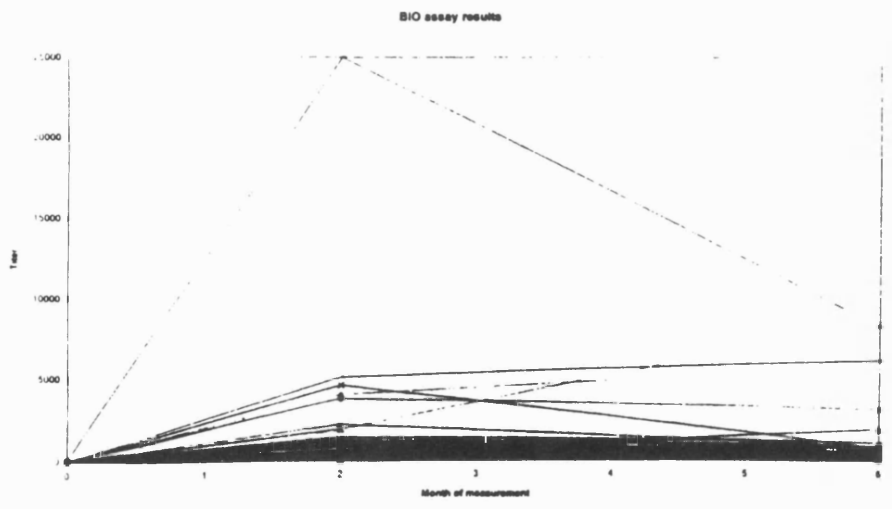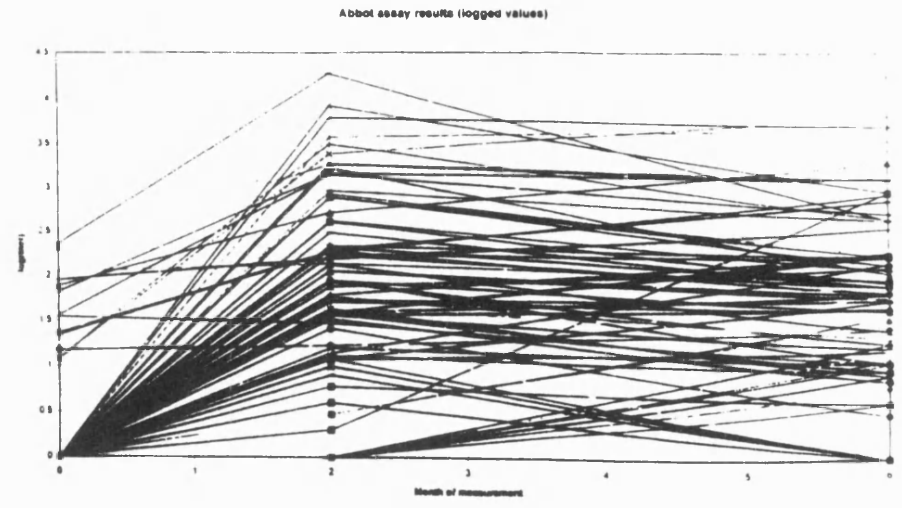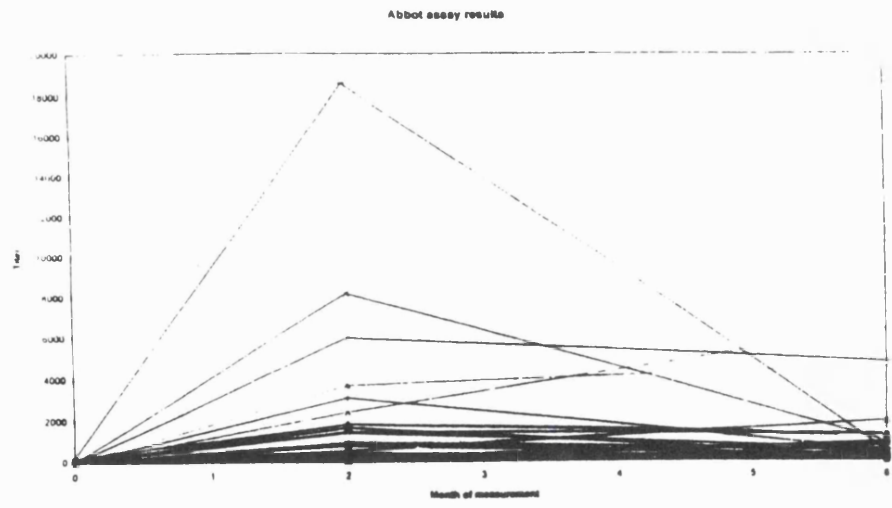# GMTs (and confidence intervals) at month 6

Sample : 86 per-protocol subjects

Figure 16:   Anti-HBs titres obtained by two assays (Ausab and Bioelisa)
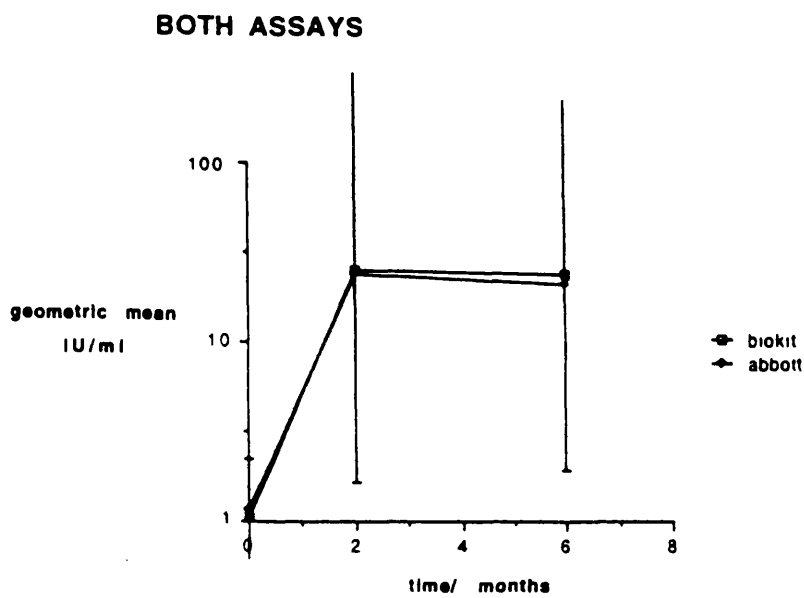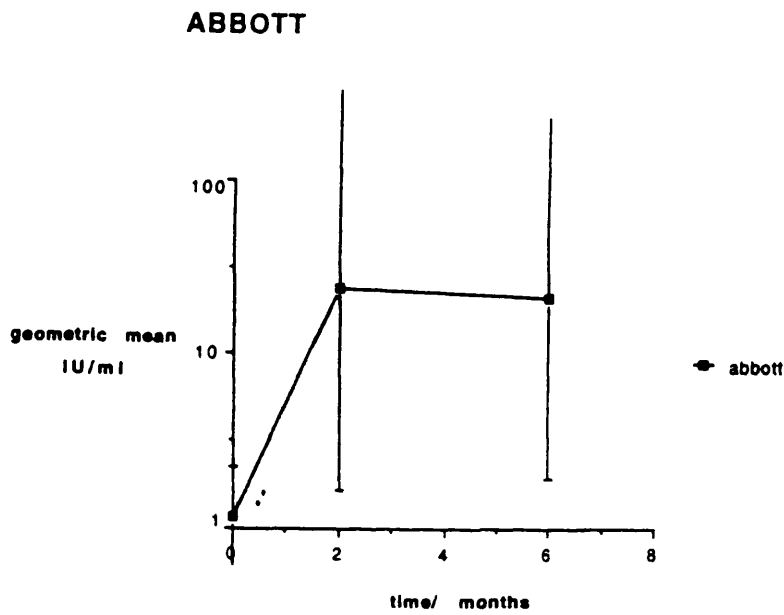
- 151 -
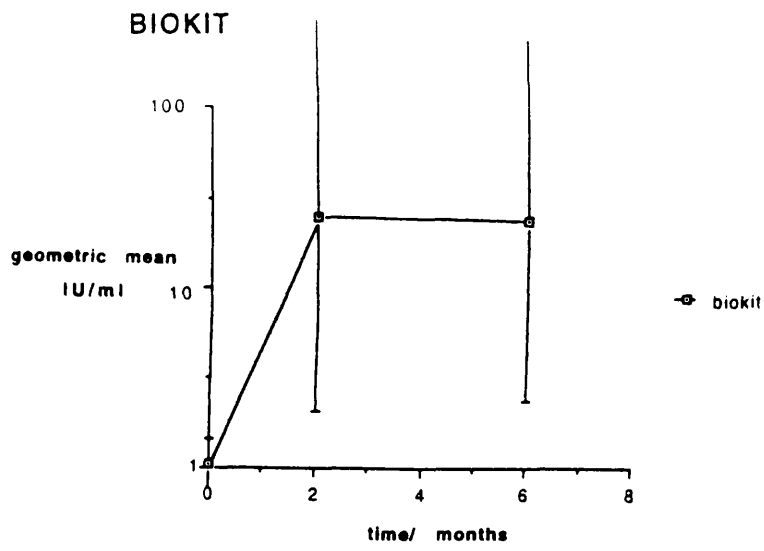
BIOKIT

ABBOTT

BOTH ASSAYS

Figure 17: Geometric mean titres of anti-HBs in IU/l obtained by
two assays (Ausab and Bioelisa)

Table 18:

Number (%) of subjects who have seroconverted at months 2 and 6
(per protocol subjects)

Dose	Number of Subjects	Month	
		2	6
5	22	10 (45.5%)	12 (54.5%)
10	23	16 (69.6%)	17 (73.9%)
20	20	12 (60.0%)	12 (60.0%)
40	21	17 (81.0%)	16 (76.2%)
Total	**86**	**55 (64.0%)**	**57 (66.3%)**
Test for trend		p = 0.05	p = 0.29
Test for group differences		p = 0.09	p = 0.36

Table 19:

Number (%) of subjects who have seroprotected at months 2 and 6
(per protocol subjects)

Dose	Number of Subjects	Month	
		2	6
5	22	3 (13.6%)	1 (4.5%)
10	23	2 (8.7%)	4 (17.4%)
20	20	2 (25.0%)	6 (30.0%)
40	21	9 (42.9%)	7 (33.3%)
Total	**86**	**19 (22.1%)**	**18 (20.9%)**
Test for trend		p = 0.007	p = 0.03
Test for group differences		p = 0.04	p = 0.05

Table 20:

GMTs (and 95% confidence intervals) at 2 and 6 months after baseline (IU/l)
(per protocol subjects)

Dose	Number of Subjects	Month			
		2		6	
		GMT	95% CI	GMT	95% CI
5	22	6.4	2.5 - 16.2	9.4	4.3 - 20.9
10	23	22.5	11.1 - 45.7	22.8	9.5 - 54.8
20	20	36.0	9.4 - 137.8	34.6	8.9 - 133.8
40	21	63.1	18.9 - 210.3	46.8	14.0 - 156.1
Total	**86**	**23.4**	**13.9 - 39.4**	**23.9**	**14.6 - 39.0**
Test for trend	p=0.004			p=0.03	
Test for group differences	p=0.01			p=0.11	

DISCUSSION

Inactivated hepatitis B vaccines have been available since 1981 and include plasma-derived as well as recombinant yeast vaccines. These vaccines have been shown to be both immunogenic and safe, although the main problem has been the lack of responsiveness in 5-10% or more of healthy individuals, and more recently the emergence of variants of HBV that are not neutralised by vaccine-induced anti-HBs.

Varying rates of non-responsiveness, between 5-10%, have been reported (see for example Dienstag et al. 1984; Craven et al. 1986; Westmoreland et al. 1990; Rogan and Duguid 1991; Cleveland et al. 1994). Several factors are known to play a role in failure to mount an antibody response to HBsAg, including the site of injection, the deltoid area being prefered to the buttocks as there is lack of antigen-presenting cells within the fat resulting in a delay in presentation of antigen to T and B cells (Zuckerman et al. 1992) increasing age, gender, smoking, overweight, immunosuppression and immunogenetic makeup.

However, following the identification of an immuno-dominant domain in the pre-S2 region of HBsAg (Neurath et al. 1984) and the observation that immunologically non-responsive mice developed antibodies corresponding to the pre-S epitope, vaccines have been prepared containing all three antigenic components; pre-S1, pre-S2 and S.

The recommendations for immunisation against hepatitis B are well known, and health care workers are at occupational risk of exposure to HBV. A proportion are non-responders and remain susceptible to infection. Recent guidelines from the UK Departments of Health concerning the immunisation of health care workers has led to improvements in immunisation programmes and consequently uptake of hepatitis B vaccine (British Medical Association 1995). This study was undertaken to evaluate the immunogenicity and reactogenicity of the new Hep B-3 vaccine in health care workers who had previously failed to demonstrate a humoral immune response following multiple doses of currently available hepatitis B vaccines.

There are reports in the literature that repeated injections or repeat courses of the vaccine in hypo-responders and in non-responders will result in the production of anti-HBs in a proportion of subjects varying from 10% to about 40% (see for example Fagan et al. 1987a; Fagan et al. 1987b; Westmoreland et al. 1990) and others. The study discussed now, however, concerns the immunogenicity of a new vaccine in true non-responders only who failed previously to produce antibody to hepatitis B vaccines despite repeated immunisation.

Hepatitis B surface antibody assays

Two different commercially available and widely used enzyme immuno-sorbent assays (Bioelisa and Ausab EIA) were selected for anti-HBs measurements because it has been suggested that most of the commercial reagents do not give accurate quantitative results particularly in a range

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greater than 100 IU/l, probably because many were designed at an earlier period when the range of interest was around 10 IU/l. For example, a comparison of two quantitative antibody measurements of anti-HBs (McCartney et al. 1993) showed a significant difference in the level of anti-HBs using two different technologies: enhanced chemiluminescence (Amerlite ECL) and micro-particle enzyme immunoassay (IMx Abbott Laboratories).

In general, the microparticle assay recorded both low and high levels of antibody when other tests were negative. However, with both enzyme immunoassays (Bioelisa and Ausab EIA) used in this trial, a known concentration of antibody calibrated in international units, was used throughout. It is recognised that different assays, although calibrated against an international anti-HBs standard have different regression curves when used to test serum dilutions. Changes in geometric mean titres were therefore employed (Figures 16 and 17), and the results of both assays were very similar. Furthermore, it is reassuring that the results of an independent comparative quantitation of anti-HBs using IMx Ausab, RIA Ausab and EIA Ausab (one of the two assays used in the present vaccine study) gave an overall qualitative agreement between IMx and EIA of 99.1%, and there was a quantitative agreement in anti-HBs concentration in the range from 1 to 30,000 IU/l with a linear correlation coefficient of 0.96 (Ostrow et al. 1991).

Immunogenicity

The Hep B-3 vaccine preparation produced a response in a group of persistent non-responders with an overall seroconversion rate of 64% after a single injection. There was no significant difference in the proportion of those subjects who seroconverted, i.e. anti-HBs 10 IU/l, in the antigen dose groups 10, 20 and 40 μg. There was insignificant differences between the proportion of subjects with antibody titres above 100 IU/l, 30% and 33%, founds between the 20 μg and 40 μg antigen dose groups.

Another mammalian cell derived vaccine (Chinese hamster ovary cells, CHO cells) containing S, pre-S1 and pre-S2 antigens was shown recently to induce seroconversion rates similar to the two currently licensed S antigen yeast-derived vaccines (Engerix B and B Hepatavac II) but with higher anti-HBs geometric mean titres (Shouval et al. 1994; Yap et al. 1995) in healthy children, healthy adults and patients including patients treated by haemodialysis. There are no reports of the effect of the CHO-cell vaccine in non-responders. The enhanced immunogenicity of the CHO-cell vaccine, as is the case with the Hep B-3 vaccine in the clinical study in non-responders which is described now, may be due to the generation of a T cell mediated helper function, and/or because the glycosylation of the HBsAg particles secreted by the transfected mammalian cells are more immunogenic than that of the non-glycosylated yeast cell-derived particles in the currently available vaccines.

Although the aim of this study was to determine the immunogenicity of this vaccine in non-responders, an intention-to-treat population was derived which included 14 subjects with an anti-HBs titre on screening of >10 IU/l but < 100 IU/l.

Of these, 10 of 14 subjects developed anti-HBs titres of > 100 IU/l after a single injection of the vaccine. A minority of subjects, 16% (5/31), whose antibody titre remained below 10 IU/l after one dose of the vaccine, did seroconvert following the booster dose. It was not possible to determine the cause of this delayed immune response. An unexpected observation was that the administration of a booster dose did not enhance significantly the immune response in any of the dose groups.

No empirical data are available for the anti-HBs titre required for protection against particular routes of infection or the size of the infectious inoculum. The minimum protective level following immunisation has been set in earlier protective efficacy studies at 10 IU/l or more of anti-HBs (Szmuness et al. 1981; Francis et al. 1982). In both studies most cases of HBV infection occurred in subjects who mounted little or no anti-HBs response. Specifically, a protective level of anti-HBs was defined as 10 IU/l against an international standard (Stevens et al. 1984; Centers for Disease Control, 1987). Various studies have also demonstrated that the risk of HBV infection increases as anti-HBs levels decline to 10 IU/l (Stevens et al. 1984; Coursaget et al. 1986; Hadler et al. 1986; Taylor and Stevens 1988).

For example, Hadler et al. (1986) reported in a follow-up study of vaccinated homosexual men an overall incidence of HBV infection of 2.9 per 100 person years with nearly 75% occurring in subjects with anti-HBs titres < 10 IU/l at the time of infection and only a few with anti-HBs titres > 50 IU/l. A lower and asymptomatic infection rate of 0.8 per 100 person years was observed after immunisation of health care workers in nephrology units who had antibody titres of < 50 IU/l (Courouce et al. 1988).

The kinetics of anti-HBs response

The titre of vaccine induced anti-HBs declines, often rapidly, during the months and years following immunisation. The highest anti-HBs titres are generally observed one month after booster vaccination followed by rapid decline during the next 12 months and thereafter more slowly (see for example Hilleman 1984; Jilg et al 1984; Ambrosch et al. 1987; Gibas et al. 1988; Nommensen et al. 1989; Wismans et al. 1989 and others). Mathematical models were designed and an equation was derived consisting of several exponential terms with different half-life periods.

It is considered by some researchers that the decline of anti-HBs concentration in an immunised subject can be predicted accurately by such antibody kinetics and preliminary recommendations before the next booster have been made (Jilg et al. 1984; Fagan et al. 1987a; Fagan et al. 1987b; Ambrosch et al. 1987; Nommensen et al. 1989 and others). If the minimum protective level is accepted at 10 IU/l, which is being debated, consideration should be given to the diversity of the individual immune response and the decrease in levels of anti-HBs as well as possible errors in quantitative anti-HBs determinations, then it would be reasonable to define a level of

> 10 IU/l and < 100 IU/l as an indication for booster immunisation. It has been demonstrated that a booster inoculation results in a rapid increase in anti-HBs titres within 4 days (Jilg et al. 1988). However, even this time delay might permit infection of hepatocytes (Nommensen et al. 1989).

Several options are therefore under consideration for maintaining protective immunity against hepatitis B infection:

- Relying upon immunological memory to protect against clinical infection and its complications (Centers for Disease Control 1991, and reviewed in Tilzey 1995), a view which is supported by in vitro studies showing immunological memory for HBsAg in B cell derived from vaccinated subjects who have lost their anti-HBs but not in B cells from non-responders (van Hattum et al. 1991), and indeed one cannot recall what has never been memorised (McIntyre 1995).

- Providing booster vaccination to all vaccinated subjects at regular intervals without determination of anti-HBs. This option is not supported by a number of investigators because non-responders must be detected (McIntyre 1995, Tedder et al. 1993) and because while an anti-HBs titre of about 10 IU/l may in theory be protective, this level is not protective from a laboratory point of view since many serum samples may give non-specific reactions at this antibody level (Westmoreland et al. 1990; Tedder et al. 1993).

- Testing anti-HBs levels one month after the first booster and administering the next booster before the minimum protective level is reached, which is the preferred option. A protective level of 100 IU/l seems to be appropriate.

In the context of the results of the study of the Hep B-3 vaccine, an interesting and important finding is that a single dose of 20 μg of the triple S vaccine was shown to be as effective as either two doses of 20 μg or 40 μg in terms of seroconversion, seroprotection or geometric mean titres. The results of this trial have been based on the measurements of anti-HBs titres, and further analysis of the pre-S1 and pre-S2 antibody response will be undertaken when reproducible assays become available and have been validated.

Reactivation in non-responders

There are numerous reports in the literature that the administration of a fourth, fifth or six or more doses of vaccine in apparent non-responders or hypo-responders resulted in the production of anti-HBs in as many as 50% (see for example Craven et al. 1986; Fagan et al. 1987b; Pasko et al. 1990; Westmoreland et al. 1990 and others) although most reports concern a small number of subjects. A study of some 26 hypo-responders and non-responders in the Netherlands (Wismans et al. 1988) revealed that some developed anti-HBs after up to a further 6 inoculations while others failed to respond, leading to a comment that there is an (unexplained) qualitative difference between hypo-responders and 'real' non-responders. Several other explanations had been offered, apart from the administration of the vaccine into an inappropriate site such as the gluteal muscles or intradermally or improper storage of the vaccine. A 'slow response' has been considered, modulation of the immune response by genetic factors particularly in relation to HLA type, non-specific cellular immune defects, concurrent infections, preferential stimulation of T-suppressor cells and so on.

The present study, however, as outlined above was carried out in true

non-responders confirmed indirectly by immunogenetics.

HLA haplotypes in non-responders to hepatitis B vaccine and in response to the Hep B-3 vaccine

The HLA Class I and Class II alleles were determined in a collaborative

study in the 86 vaccine non-responders participating in the study by a

lymphomicrocytotoxicity technique for Class I HLA typing using

commercially available plates (Biotest, Germany), and by PCR for Class II

HLA typing. A control group of 115 subjects who were hepatitis B vaccine

responders, and an additional group of 125 Caucasian new volunteer

donors at the Anthony Nolan Bone Marrow Trust were included as further

control subjects.

A significant association was found between the HLA phenotype

B44;DRB1*0701; DQB1*0201 and the vaccine non-response (p = 0.02).

Those with the phenotype B44;DRB1*0701;DQB1*0201 are nearly four times

more likely to be antibody non-responders when compared to hepatitis B

vaccine responders (p = 0.01). Previous studies have identified the

association of the extended haplotypes B44; DR7; FC31 and B8; DR3; SC01

based on six out of 9 individuals who were non-responders to the

S vaccine. The correlation of B44; DRB1*0701; DQB1*0201 with hepatitis B

vaccine non-response was confirmed in the present study representing

more accurately the frequency of HLA in the caucasian population.

Despite this we could not identify a significant association with the

HLA phenotype B8; DR3 with non-response to HBsAg vaccination as described previously. The frequency of HLA allele DQB1*0201 was high in our non-responder population (p = 0.002), this allele is in strong linkage disequilibrium with HLA alleles DRB1*0701 and DRB1*0301 which are found in 85% of vaccine non-responders. The HLA alleles DRB1*1501 and DRB1*0101/02/03 were found at a lower frequency in the non-responders when compared to the HLA and vaccine control groups respectively, but were not significant when adjusted for the number of tests carried out. The DR1 antigen has been described previously by less stringent analysis (Walker et al. 1981). Interestingly, the HLA allele DQB1*0602 was represented as a significantly low frequency in the non-responder population (p = 0.02). This allele is in linkage with DRB1*1501 suggesting that there was an association between the molecular subtypes of DR1 and the response to hepatitis B vaccination.

In summary, a high frequency of HLA Class II allele DRB1*0701 and the phenotype B44;DRB1*0701;DQB1*0201 was found in non-responders compared to controls. There were low frequencies of DRB1*1501, DQB1*0602 and phenotype DRB1*1501;DQB1*0602 among non-responders. All the initial non-resonders expressing the phenotype B7;DRB1*1501;DQB1*0602 responded to the new vaccine with antibody titres > 100 IU/l. The majority of those who failed to mount an antibody response expressed the phenotypes B8;DRB1*0301;DQB1*0201 or B44;DRB1*0701;DQB1*0201.

Non-response to the additional pre-S1 and pre-S2 components in the vaccine may be due to dysfunctional interaction of specific HLA molecules with these antigens, lack of T-cell activation or deletion of specific T-cells during thymic education. Further study and typing for additional polymorphism within the HLA mediated immune response may provide an insight into the mechanisms of failure to respond to immunisation against hepatitis B (McDermott, 1996). However, it should be noted that immunogenetic analysis confirmed that an initially distinct group of non-responders was indeed included in the study of the new pre-S1, pre-S2, S vaccine.

Non-responders and susceptibility to infection with HBV

While it is accepted that about 5-15% of fully immunocompetent healthy individuals do not mount a humoral antibody response to currently available hepatitis B vaccines, and others are poor-responders, there is little in the more recent literature based on long-term follow-up to address the issue of whether such persons are susceptible to infection with hepatitis B virus. The kinetics of antibody response and the issue of post-vaccination testing had been discussed above, and it is undoubtedly of importance, for example for health care workers, their patients and their employers (under current UK Department of Health Guidelines) to be aware of their protection or lack of protection after immunisation. Equally reliance on adequate protective cell mediated immune response (which in the vast majority had not been measured) or primed (or otherwise) immunological memory to mount an anamnestic response may not be entirely satisfactory.

Further, it is easy to be too dismissive of the importance of symptomless HBV infection, for example based on anti-HBc seroconversion in vaccine responders and non-responders. The suggestion in an Editorial (Hall 1993) that "whether antibody responses after vaccination should be verified and subsequent decay documented will depend on local resources" is not acceptable in the interest of the public health, apart from other considerations.

An early placebo-controlled study was carried out with a plasma-derived vaccine in an HBV 'high-risk' setting in 353 staff, patients on maintenance haemodialysis and their relatives in France in 1975 (Maupas et al. 1979). Follow-up of 73 patients and 191 staff showed that vaccined subjects who did not respond to the vaccine by developing anti-HBs were infected at the same rate as the unvaccinated controls i.e. nearly 50% as indicated either by anti-HBc production alone (5%), transient antigenaemia (15%) or prolonged antigenaemia (25%). Many of the subjects who developed infection within 2 months of immunisation were patients, who tend to mount a delayed or slow anti-HBs response, and were likely to be incubating the infection (figure reproduced from the report). Thirteen staff members (60%) were non-responders and 9 became infected with HBV within 4-12 months after the first inoculation.

It should be noted that interpretation of parts of the report is difficult. Other studies referred to above (Stevens et al. 1984; Coursaget et al. 1986; Hadler et al. 1986; Courouce et al. 1988; Taylor and Stevens 1988 and others) have shown that the risk of HBV infection increases as anti-HBs

levels decline to 10 IU/l in responders. There are few reports concerning

non-responders. Nevertheless, the initial efficacy trials of the plasma-

derived hepatitis B vaccine (produced by Merck, Sharp & Dohme in the

USA) provide evidence of the continuing susceptibility of persons who

receive a complete course of vaccine but develop less than 10 IU/l of anti-

HBs. For example, the study conducted by Szmuness et al. (1981) revealed

that 7 of 21 (33%) of vaccinated non-responder male homosexuals became

infected during an 18 month period of surveillance. That compared with

92 of 426 (22%) placebo recipients infected during the same period. The

evaluation in another study of long-term protection by hepatitis B vaccine for

7-9 years revealed 36 HBV infections among 139 male homosexuals who

had no detectable anti-HBs after three doses of vaccine (Hadler et al. 1991).

In an earlier trial, the same investigators noted that HBV infection occurred

in 55 vaccinated subjects with a poor antibody response, and two became

carriers of HBV both of whom were non-responders (Hadler et al. 1986). In

another study there were four 'vaccine failures' among 15 babies born to

'high risk' mothers; one infant non-responder became infected after the age

of 10 months and one poor responder became infected at the age of

6.5 months and remained e antigen positive for 5 months of the

follow-up (Flower and Tanner 1988).

There are apparently no reports of a cohort of healthy non-responders to

vaccination who have been surveyed systematically for a sufficient number

of person-years to estimate closely susceptibility to infection. It is proposed

to follow-up by serological surveillance the 86 participants in the Hep B-3

vaccine over a period of several years.

Non-responders and silent infection

A brief report (Lou et al. 1992) noted that 6.4% of 214 subjects in China who were immunised with the Merck, Sharp & Dohme hepatitis B vaccine and 12.5% of 96 subjects who received a locally produced vaccine did not respond. Hepatitis B virus DNA was detected by PCR in over 60% of the non-responders in each group suggesting that non-responsiveness to hepatitis B vaccine may be due to immunotolerance or immunosuppression induced by latent HBV infection. Other reports suggested that HBV e antigen can cause immunotolerance and chronic HBV infection (Brunetto et al. 1991), and that HBV itself may cause immunotolerance by infecting directly T and B lymphocytes resulting in viral persistence (Oldstone 1989) or through different mechanisms triggered by viral infection leading to imbalance in immunoregulation (Paller and Mallory 1991).

No evidence of latent HBV infection was found in non-responders in the present study as shown by the repeated absence of serological markers of infection in the subjects (anti-HBc and HBsAg), absence of HBV DNA by nested-PCR in many of the non-responder subjects at the Royal Free Hospital and School of Medicine (Dr T.J. Harrison, personal communication) and in four volunteers with anti-HBc.

Attempts to overcome non-responsiveness by the use of immunomodulators

Attempts have been made to enhance the anti-HBs response following immunisation, particularly in patients treated by maintenance haemodialysis, but often with conflicting results or in limited studies, which have not been confirmed:

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- alpha-interferon (Grob et al. 1984, Goldwater 1994);

- interleukin-2 (Meuer et al. 1989, Jungers et al. 1994);

- thymopentin (Zaruba et al. 1983, Melappioni et al. 1992)

and other substances such as experimental oral adjuvants in mice and oestrogen. These are referred to for the sake of completion and will not be considered further in this dissertation.

CONCLUSIONS

The overall rate of seroconversion in terms of anti-HBs with a titre of > 10 IU/l was 66% of the 86 true non-responders to the S antigen vaccine, with rates ranging from 55-76% across different antigen dose groups.

A single 20 μg dose of the Hep B-3 vaccine was as effective as two doses of 20 μg or 40 μg in terms of seroconversion, seroprotection or geometric mean titres.

The work described in this thesis includes extensive evaluation of the safety, reactogenicity and immunogenicity of two preparations of an inactivated hepatitis A vaccine derived from the HM 175 strain of hepatitis A virus, which led in part to the approval for the use of this vaccine for the prevention of hepatitis A in the UK and elsewhere.

A Phase III Clinical Study, which was part of a Multicentre European trial (but only one centre in the UK) compared the safety, reactogenicity and immunogenicity of a two dose schedule containing antigen prepared from the GBM strain of HAV in comparison with a three dose course of the vaccine derived from the HM 175 strain. An 18 month follow-up period to-date is described. The kinetics of antibody response established that the newer vaccine induced higher GMTs of antibody. It is expected that this study will lead to licensing of this vaccine and introduction into medical practice in the UK in 1997. Strategies for hepatitis A immunisation are described.

The third study concerned the protection against infection with HAV adults and children with congenital coagulation defects using an accelerated programme of immunisation with a higher dose of antigen administered subcutaneously.

The safety, reactogenicity and immunogenicity of this preparation are demonstrated, and the importance of protecting these patients from infection with hepatitis A (another hepatotropic virus), because they may have underlying liver damage caused by previous infections with hepatitis B, C, D, G/GBV-C and other bloodborne viruses is important in practice.

Health care workers and others at risk who do not respond to immunisation against hepatitis B with currently available vaccines remain susceptible to the infection. A large number of non-responders were immunised with a new recombinant experimental vaccine containing pre-S1, pre-S2 and the S antigens of the surface protein of HBV. Previous studies involved principally a limited number of genetically defined mouse strains. The immunogenetics of the non-responder participants are being investigated as part of another collaborative study between the candidate and the Anthony Nolan Bone Marrow Trust. The finding that 66% of the 86 non-responders (range 55-76% across different antigen doses) developed anti-HBs with a titre > 10 IU/l is important. The kinetics of antibody response indicated that a single dose of the new vaccine was as effective as two doses. These results constitute a significant advance in protection against hepatitis B.

Acknowledgements

The study of HLA haplotypes is part of a continuing collaborative study with Dr A. Madrigal and Mr A.B. McDermott of the Anthony Nolan Bone Marrow Trust.

None of these investigations could have been carried out without the volunteers - health care personnel and medical students from the Royal Free Hospital and School of Medicine, and in the case of non-responders, volunteers from other hospitals.

I should like to thank most sincerely Miss Marion Roper who undertook the typing of this thesis with much skill and patience.

Finally, I am deeply grateful to my parents for their unfailing interest, encouragement and support.

A note on the role of the candidate in the studies described in this thesis

The Acknowledgements refer to the contributions to these studies by my colleagues. The more precise role of the candidate is summarised below:

Studies on Hepatitis A Vaccines: The candidate initiated the studies and with others designed and agreed the protocols. The candidate prepared and obtained ethical approval, recruited personally all the volunteers, carried out solely all the clinical examinations, inoculations, venepunctures and follow-up, and recorded all the data. The inoculation of the patients

with coagulation defects and venepunctures were carried out by a senior nurse of the Haemophilia Centre and Haemostatis Unit.

Serological assays were carried out in the candidate's laboratory for the first trial and random tests in the case of the comparative study. Routine biochemical tests were carried out in the Hospital's laboratory and CD4 cell counts in the Department of Haematology.

Statistical analysis was carried out with the help of a medical statistician at the Royal Free Hospital School of Medicine, in France for the multicentre trial, and in the Netherlands.

Hepatitis B non-responders: The candidate performed the same tasks as described above. Recruitment of subjects extended to many other hospitals and clinical examination, vaccination and venepuncture and follow-up were carried out by the candidate personally at different Hospitals and Occupational Health Units.

One of the immunoassays was undertaken in the candidate's own laboratory. Tests for HBV DNA by nested-PCR were undertaken by Dr T.J. Harrison of the Department of Medicine of the Royal Free Hospital and School of Medicine.

The candidate retains responsibility for the follow-up and assessment of all the participants in the studies described above.

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