INVESTIGATION OF A SEROLOGICAL MARKER DETECTED IN BLOOD FROM A DONOR TWICE IMPLICATED IN THE TRANSMISSION OF NON-A, NON-B VIRAL HEPATITIS

by

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DECLARATION

The work reported here was designed and conducted by myself, with the following exceptions:-

I. Inoculation of marmosets (Part 3.2) was kindly performed by Dr. Hazel Appleton (Central Public Health Laboratory, Colindale) who also bled the animals and assayed the serum for ALT.

2. Some electron microscopy (Part 3.2) was performed by Professor C.R. Madely, Newcastle University.

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SUMMARY

Enzyme immunoassays for the detection of M2Ag, anti-M2 IgM and anti-M2 IgG were developed, using an antigen purified from the blood of a donor twice implicated in the transmission of non-A, non-B viral hepatitis. Application of the assays revealed an apparent association between M2 markers and a form of non-A, non-B viral hepatitis. The prevalence of M2Ag, anti-M2 IgM and anti-M2 IgG is approximately 2 percent for each marker in blood donors from the South-East of Scotland. Retrospective investigation has shown that transfusion of either M2Ag or anti-M2 IgM positive blood can result in a hepatitis episode with an incubation period of 3-7 weeks.

M2 markers were found in approximately 33 percent of 'sporadic' acute phase non-A, non-B hepatitis sera, but absent in appropriate controls (ie. hepatitis caused by HAV or HBV and hepatitis of non-viral origin). Likewise, M2 markers were present in 48 percent of patients with chronic hepatitis of an unknown viral aetiology, but absent in patients with chronic non-viral hepatitis. Two patients who died of fulminant non-A, non-B viral hepatitis during the course of this study also possessed anti-M2 IgM in their serum.

Biochemical and biophysical characterisation of M2Ag have indicated a molecular weight between 3.5×10^6 daltons, and 4.5×10^6 daltons, a sedimentation coefficient of 118S

to I35S and a buoyant density in caesium chloride of I.25g/ml. There are four major polypeptides of molecular weights ranging from 25,000 to 9I,000 daltons. Electron microscopy has revealed 20nm-40nm spherical particles similar to (but antigenically distinct from) 22nm HBsAg bearing particles.

M2Ag is immunogenic in animals but cannot be transmitted to marmosets.

Further epidemiological investigation has indicated that a significantly higher proportion of M2 markers are present in groups at high risk of exposure to HBV, indicative of a similar route of transfusion of both agents.

The evidence presented in this thesis suggest that M2 markers may well be of value in identifying transmitters of one form of non-A, non-B viral hepatitis and adds weight to the case for a prospective study to define whether these markers are predictive of donor infectivity.

ABBREVIATIONS USED THROUGHOUT TEXT

HAV	Hepatitis A virus
HBV	Hepatitis B virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B 'e' antigen
HBsAg	Hepatitis B surface antigen
CMV	Cytomegalovirus
EBV	Epstein-Barr virus
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
RNase	Ribonuclease
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CEA	Carcino-embryonic antigen
E.R.	Endoplasmic reticulum
SER.	Smooth endoplasmic reticulum
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Fc	'Fragment crystallizable'
Fab'	'Fragment antigen binding'
RF	Rheumatoid factor
IAHA	Immune adherence haemagglutination assay
EIA	Enzyme immunoassay
RIA	Radioimmunoassay
Ag	Antigen
O.D.	Optical density
C.V.	Coefficient of variation

S.D.	Standard deviation
M2Ag:AP	Anti-M2 alkaline phosphatase conjugate
T:N	Test to negative ratio
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
PAGE	Polyacrylamide gel electrophoresis
EDTA	Ethylenediaminetetra-acetic acid disodium salt
BSA	Bovine serum albumin
PEG	Polyethylene glycol
CRYO	Cryoprecipitate
PTA	Phosphotungstic acid
NHS	Normal human serum
ISG	Immune serum globulin

PART ONE

INTRODUCTION

I.I VIRAL HEPATITIS

The earliest description of hepatitis (epidemic jaundice) is usually attributed to Hippocrates. More detailed observations were not recorded until the seventeenth and eighteenth centuries, when outbreaks of jaundice were noted, particularly in association with military campaigns (Monro I764, von Borman et al I943). The infectious nature of the disease was not widely accepted until the early part of the twentieth century. Although acute inflammation of the liver may be associated with other viral infections (e.g. herpes virus group, coxsackie virus and yellow fever virus), epidemiological investigations of naturally occurring outbreaks of hepatitis and transmission studies in the decade from I940 pointed to the existance of two distinct agents associated with 'infectious' (type A) and 'serum' (type B) hepatitis. (MacFarlane and Chesney I944, Neefe et al I946, MacCollum et al I95I).

Despite rapid developments in hepatitis research over the past two decades, the overall picture relating to viral hepatitis has still to be fully elucidated. There is now firm evidence that it is not possible to account for all illnesses currently diagnosed as viral hepatitis on the basis of infection with hepatitis A virus (HAV) or hepatitis B virus (HBV). Causative agent (or agents) of non-A, non-B hepatitis clearly exist and have yet to be defined.

In view of the abundance of recently published information

concerning HAV and HBV, the introduction of this thesis will consider only briefly relevant information concerning these agents before reviewing in detail the available information relating to non-A, non-B viral hepatitis.

I.2 HEPATITIS A VIRUS

Feinstone et al were among the first to detect HAV in faecal extracts of infected patients (Feinstone et al I973, Gravelle et al I975). Definitive characterisation of the virus was facilitated by the subsequent development of tissue culture systems (Frösner et al I979, Provost and Hilleman I979, Flehmig I980, Kojima et al I98I). HAV purified from stool, infected liver or tissue culture harvests appears as a spherical unenveloped particle 27-32nm. in diameter, with a surface structure probably consisting of 32 capsomeres. (Siegl I982). The virus genome is a linear single stranded RNA with a molecular weight of I.9 x $I0^6$ to 2.8 x $I0^6$ daltons, depending on whether it has been isolated from stool, liver or cell culture. (Siegl and Frösner I978, Coulepis et al I98I, Siegl et al I98I). Four polypeptides (3I-33,000, 26-27,000, 2I-23,000 and 6-I4,000 daltons) are observed following denaturation with sodium dodecyl sulphate and polyacrylamide gel electrophoresis (SDS-PAGE). (Coulepis et al I980, Tratschin et al I98I, Coulepis et al I982). Other characteristics of HAV are listed in Table I.2a.

HAV is transmitted primarily via the faecal-oral route. The incubation period is between I4 to 40 days, mortality is low, about 0.I percent in otherwise healthy individuals. (Lucke I944, Rakela et al I978). There is no evidence of development of a carrier state or progression to chronic liver disease (Lindberg et al I978, Rakela et al I978).

TABLE 1.2a

BIOPHYSICAL AND BIOCHEMICAL CHARACTERISTICS OF HEPATITIS A VIRUS

Size		27nm (range 27-33nm)
Capsid symmetry		cubic
Virion		unenveloped
Capsid assembly		cytoplasm
Density of CsCl		1.36-1.43g/ml
Sedimentation constants in sucrose gradients		160S (complete particles)
Nucleic acid		
Type		single stranded RNA
Molecular weight		1.9×10^6 - 2.8×10^6 daltons
Polypeptides (SDS-PAGE)		
VP1		31,000 - 33,000 daltons
VP2		26,000 - 27,000 daltons
VP3		21,000 - 23,000 daltons
VP4		6,000 - 14,000 daltons
Stability		
Temperature - 20° to -70°		stable for years of storage
4°		stable for weeks to months of storage
	50°C 60 minutes	stable
	60°C 60 minutes	mostly stable
	60°C 10 hours	mostly inactivated
	100°C 5 minutes	inactivated
Ether 20%	4°C 4 hours	stable
Acid pH 3.0		stable
Formalin 1:4,000	37°C 72 hours	inactivated
U.V. irradiation		inactivated
Chlorine 1mg/ litre 30 minutes		inactivated

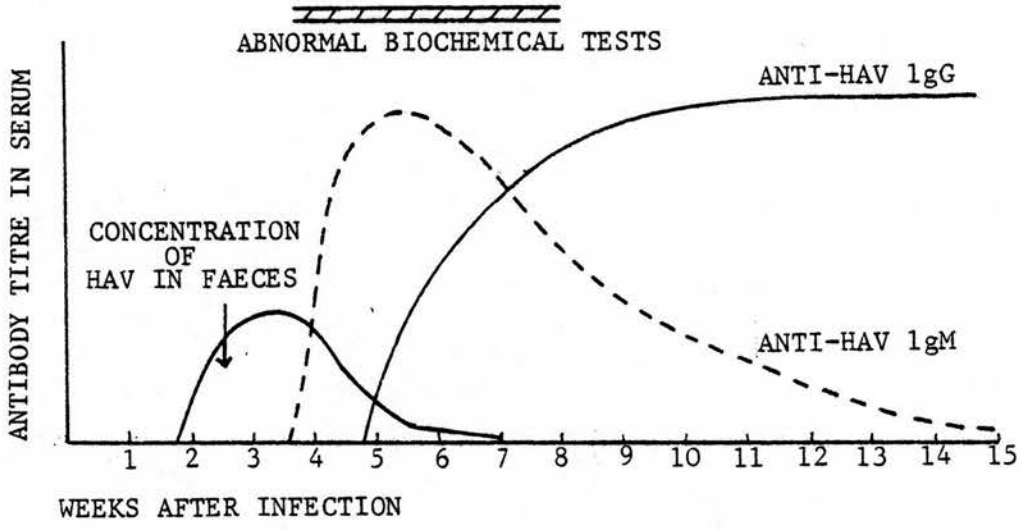
Adapted from: McCollum and Zuckerman (1981). Viral Hepatitis: Report on a WHO informal consultation. Journal of Medical Virology, 8, 1-29.

Figure I.2i indicates the sequence of serological events most frequently observed during acute HAV infection. Antigen can be isolated from faeces two weeks prior to the onset of symptoms, (McCollum and Zuckerman I98I) at which point 50 percent of patients may be excreting virus (Carl et al I982). As the acute phase progresses excretion of infectious virions declines sharply, probably resulting from the influence of secretory and other antibodies, with the result that most patients may be non-infectious within one week of the onset of symptoms. (Krugman et al I959). Transient viraemia occurs prior to the onset of symptoms which, together with the often mild nature of the disease (Krugman et al I962), probably accounts for the rare occurrence of HAV related post-transfusion hepatitis (Seeberg et al I98I, Barbara and Briggs I983). Anti-HAV IgM is always present at the onset of disease, peaking within a few weeks and declining over the ensuing weeks or months. In contrast anti-HAV IgG peaks during the late acute and convalescent phases and remains elevated for many years. Anti-HAV IgA shows a similar, though less pronounced serum profile to anti-HAV IgM (Overby et al I982). Secretory anti-HAV IgA has been found in the stools of hepatitis A patients (Yoshizawa et al I980a).

The virus is endemic in countries where standards of hygiene are low. (Table I.2b). In developed countries (with higher standards of hygiene) antibody prevalence increases with population age, until approximately 80 percent of the population over 60 years of age are immune to

FIGURE 1.2i

TYPICAL COURSE OF HEPATITIS A INFECTION



HAV. (Iwarson et al I978). However outbreaks can occur in areas of low herd immunity related to ingestion of contaminated food or water. (Batik et al I968, Zachoval et al I98I, Hadler et al I982). Male homosexual behaviour also appears to be associated with an increased risk of HAV infection. (Corey and Holmes I980).

There is evidence that HAV is responsible for a recent increase in notifications of viral hepatitis in the United Kingdom. (Parry, J.V. personal communication I983). The four-fold rise reported in Scotland for the period I980-I98I mainly associated with the under I3 age group, could have resulted from imported infections, declining living standards or reflect a natural I0 year cycling of the virus. (Follet I983).

Protection is afforded by normal immune globulin (Mosley et al I968, Maynard I982), due to significant levels of anti-HAV in donor serum. (Table I.2b). (Frösner et al I977, Smallwood et al I980). Anti-HAV levels in Scottish normal immune globulin have remained approximately constant over the last 20 years. (Hopkins I983).

The recent advances in tissue culture of HAV has made vaccine development feasible, while cloning of DNA copies of the HAV genome in bacterial plasmids will aid development of a synthetic vaccine (van der Helm et al I98I).

The virus was first serially transmitted to two species

TABLE 1.2b

PREVALENCE OF ANTIBODY TO HEPATITIS A ANTIGEN IN VARIOUS POPULATIONS

City/District	State/Country	Assay	Number tested	Percent Anti-HAV positive
Philadelphia	Pennsylvania	IAHA	197[a]	12
North London	U.K.	RIA	100	16
Lillehammer	Norway	RIA	175	17
Bern	Switzerland	IAHA	98	24
New York	New York	IAHA	629	41
Corpus Christie	Texas	RIA	538	44
Tokyo	Japan	IAHA	400	50
Victoria	Australia	RIA	1053	51
Tubingen	Germany	RIA	661	55
West Scotland	U.K.	RIA		57
Warsaw	Poland	IAHA	128	60
Melbourne	Australia	RIA	959	62
San Jose	Costa Rica	IAHA	300	72
Dakar	Senegal	IAHA	102	75
East Scotland	U.K.	RIA		78
Miscellaneous	Mediterranean	RIA	70	81
Leuven	Belgium	IAHA	133	87
Taipai	Taiwan	IAHA	138	88
Machakos	Kenya	IAHA	138	88
Tel Aviv	Israel	IAHA	112	94
Sarajevo	Yugoslavia	IAHA	100	97

IAHA - Immune adherence haemagglutination

RIA - Radio immunoassay

a - High socio-economic group

Dienstag, J.L., Szmuness, W., Stevens, C.E. and Purcell, R.H. (1978). Hepatitis A Virus Infection : New Insights from Seroepidemiologic Studies. Journal of Infectious Diseases, 137, 328-340.

of marmosets (Saguinus fusciollis and Saguinis nigricollis) by Dienhardt et al (I967). Differences in susceptibility appear to exist between marmoset species, S. mystax being the most susceptible. The liver appears to be the only site of replication of the virus in marmosets (Krawczynski et al I98I). Chimpanzees are also susceptible to HAV infection (Thornton et al I975) exhibiting a cyclic excretion of virus particles. (Bradley et al I977). Other susceptible primates include the stump-tailed monkey (Mao et al I98I) and the Panamanian owl monkey (Lemon et al I982).

There have been recent, though unconfirmed, reports indicative of more than one antigenic strain of HAV. (Stakhanova et al I979, van der Akker and Hekker I980). To analyse the genetic and antigenic relationship between individual isolates of HAV, 7 strains of HAV originating from Middle and North America, North Africa, Europe, Australia and China were propagated in PLC/PRF/5 (human hepatoma derived) and MRC-5 (human diploid embryonic lung) cells. The viruses could be distinguished on the basis of their host cell specificity as well as by their predilication for replication at either 32°C or 37°C. Genetic variation could be demonstrated directly by two dimensional mapping of RNase T_I- resistant oligonucleotides of the viral genomes. The respective results revealed minor but distinct differences between individual HAV isolates. (Weitz and Siegl I984). Gruer et al I982 reported a relapsing

illness in 5 hepatitis A patients, 7 to IO weeks following the onset of initial illness. It is not clear whether this represents reinfection of a second antigenic strain of HAV or an agent of non-A, non-B hepatitis.

I.3 HEPATITIS B VIRUS

Characterisation of HBV began in I965 when Blumberg et al (I965) demonstrated precipitin lines using serum from a haemophiliac as a source of antibody and serum from an Australian aborigine as the antigen source. The antigen was designated 'Australia antigen'. Prince (I968) showed Australia antigen to be present in patients with serum hepatitis. The antigen was subsequently demonstrated in serum during the acute phase of hepatitis B and in the serum of apparently healthy 'carriers'. (Giles et al I969). The nomenclature was finally modified to hepatitis B surface antigen (HBsAg) when it was discovered that this marker was expressed on the lipoprotein coat of the complete (infective) virus particle.

Three characteristic morphological structures, each bearing HBsAg determinants, are frequently observed in reactive serum (Bayer et al I968, Dane et al I970). Twenty to 25nm. diameter spherical particles usually predominate with occasional filamentous structures which may be several hundred nanometers in length, while 42-45nm. diameter 'Dane' particles are believed to represent the infectious hepatitis B virion.

SDS-PAGE analysis of purified 22nm. HBsAg particles indicate seven polypeptides ranging in molecular weight from 23,000 to 97,000 daltons. (Gerin and Sher I978, Peterson I98I). In addition to a common group determinant 'a', HBsAg is associated with two pairs of determinants d/y and w/r, such

that four major subtypes of HBsAg are possible : adw, adr, ayw and ayr, while pleotypic antigens such as ady, awr, adyr, and aywr have been described. The w antigen is further subdivided into w_1, w_2, w_3 or w_4. (Le Bouvier I97I, Bancroft et al I972, Soulier and Courouce-Pauty I973, Courouce-Pauty et al I978).

The Dane particle has a complex morphology consisting of an outer HBsAg coat and an internal 27nm. diameter core, HBcAg. (Almeida et al I97I). A subpopulation of Dane particles possess cores with endogenous DNA polymerase activity and circular double stranded DNA. (Kaplan et al I973, Robinson et al I982). Core particles possess a major polypeptide of molecular weight I7,000 to I9,000 daltons and one or more minor polypeptides of higher molecular weight. (Budkowska et al I977, Hruska and Robinson I977, Neurath et al I978, Takahashi et al I979 Shih et al I980). Core particles are found in the nuclei of HBV infected hepatocytes, their presence usually being signalled by detectable anti-HBc in serum. Other biochemical and biophysical properties of HBV are listed in Table I.3a.

HBeAg is a complex of soluble proteins of varying sizes (Fields et al I978), and represents the third immunological system associated with HBV infection. It was first reported by Magnius and Espmark (I972), and is derived from HBcAg. (Lam et al I977). HBeAg exists in two forms. One is freely circulating and can be physically separated

TABLE 1.3a

BIOPHYSICAL AND BIOCHEMICAL CHARACTERISTICS OF HEPATITIS B VIRUS

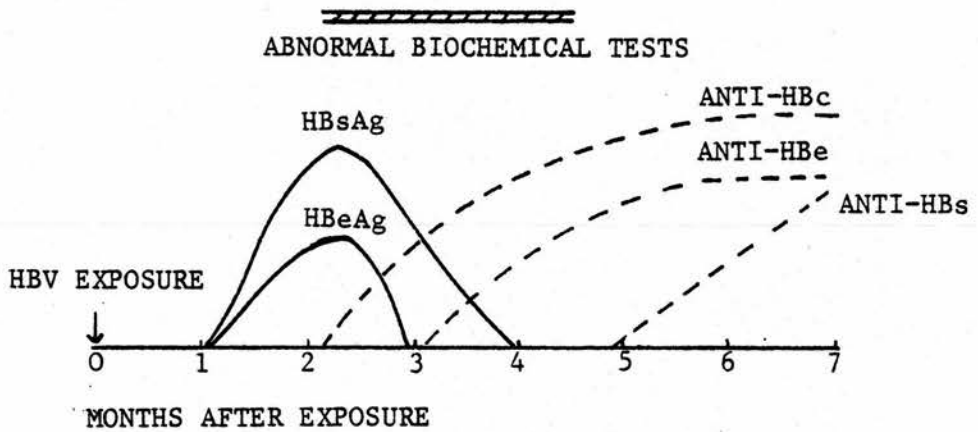
Size and morphology	20-25nm spherical particles
	20nm diameter rod like structures; length several hundred nm.
	42-45nm 'Dane' particles.
Density in CsCl.	1.19-1.21g/ml surface antigen 1.24g/ml Dane particle 1.27g/ml core antigen
Sedimentation constants in sucrose	110S surface antigen 280S Dane particle
Nucleic acid - type	double stranded circular DNA
molecular weight	1.8×10^6 daltons
Polypeptides - 22nm surface antigen	22-26,000
	25-32,000
	38,000
	49,000
	69,000
	97,000
45nm core particles	17-19,000
	25,000
	38,000
Stability as determined by infectivity	
100°C 30 mins.	inactivated
98°C 1 min.	partially inactivated
60°C 1 hour	resistant
60°C 10 hours (albumin)	inactivated
56°C 30 mins.	resistant
-10°C to -20°C 1½ years	resistant
-10°C to -20°C 4½ years	resistant
Ultra violet irradiation	Equivocal
Phenol-ether (Equal parts) 0.5%	resistant
10% ether 24 hours 4°C	resistant
Triple ether extraction of serum	resistant
Merthiolate 1:2,000	resistant
Nitrogen mustard (500mg/l)	resistant
Trioesol 0.2%	inactivated
Formalin	inactivated
B-propiolactone 4mg/ml	inactivated
Ethylene oxide (liquid or gas)	inactivated

from HBcAg, while the other is associated with Dane particles and is released by detergent treatment. Three antigenic types of HBeAg (HBeAg/I; HBeAg/2; HBeAg/3) are known. (Williams and Le Bouvier I976, Murphy et al I978). Persistance of HBeAg beyond the acute phase of illness is one prognostic index of the chronic carrier state; while seroconversion to anti-HBe usually indicates the onset of convalescence and resolution of infection (Villarejos et al I978, Overby et al I982). Circulating HBeAg suggests high infectivity while anti-HBeAg implies the reverse (Werner et al I977). Figure I.3i indicates the general pattern of serological markers observed during acute infection with HBV and the chronological order in which they appear.

Acute HBV infection may vary from an asymptomatic infection to fatal fulminant disease (Zuckerman I979). Up to I0 percent of acute patients become carriers of HBsAg (Hoofnagle et al I978a), they may be 'healthy' with normal liver function and histology, or may have abnormal liver function with intermittant or continuously abnormal liver histology (Feinman et al I982). The hosts immune response is involved in the elimination of HBV from infected hepatocytes and may be responsible for liver injury (Tiku et al I978). The existence of a healthy HBsAg carrier state with normal liver function and histology suggests that HBV related disease is variable ranging from mild chronic persistent hepatitis that eventually resolves to a severe chronic active illness that can rapidly lead to cirrhosis. (Fox

FIGURE 1.3i

SEROLOGICAL COURSE OF UNCOMPLICATED ACUTE HEPATITIS B WITH RECOVERY



et al I969, Redeker I975, Boyer I976, Hoofnagle et al I978a) and possibly hepatocellular carcinoma. (Prince et al I975, Tabor et al I977).

HBV is a worldwide infection with an estimated reservoir of 200 million human carriers (Gust I982). The carrier rate varies with geographic location: in the United States and United Kingdom it is about 0.I percent of the population compared to I5 percent of the population in some areas of Africa and the Far East. Transmission is predominantly by overt parenteral (injection, transfusion) or inapparent parenteral (e.g. sexual) means. Vertical transmission also plays an important role in the maintainance of the virus in the population in areas of high endemicity. There is no direct evidence of aerosol transmission. Groups at very high risk of HBV infection include sexual or household contacts of acutely ill patients or certain carriers. (Redeker et al I975 Szmuness et al I975, Ohbayashi et al I977), sexually active male homosexuals (Skinhöj et al I979, Schroeder et al I982), infants born to HBV carriers, (Schweitzer et al I983, Beasley et al I977, Gerety and Schweitzer I977), oncology units (Wands et al I974), institutions for the mentally retarded (Cancio-Bello et al I982), patients and staff in renal dialysis units (Marmion et al I982), intravenous drug abusers (Blanck et al I979, Miller et al I979) and regular recipients of blood products e.g. haemophiliacs (Holsteen et al I977, Burrell et al I978).

The introduction of sensitive immunoassays for screening blood donations has considerably reduced the likelihood of transmitting hepatitis B. Recent studies indicate that as few as I0 percent of cases of transfusion associated hepatitis are now due to hepatitis B infection. (Alter et al I975, Knodell et al I975), resulting in an eight-fold reduction in fatalities from post transfusion hepatitis. (Goldfield et al I975). However, to reduce the incidence of HBV transmission via blood further assays with a higher sensitivity are required possibly in conjunction with tests for anti-HBcAg. (Dike I98I, Hopkins et al I982).

Chimpanzees are very susceptible to HBV infection, although the acute disease is generally milder than in man (Shikata et al I980). Like man, they can become chronic HBV carriers. These animals provide excellent models for HBV inactivation studies, safety and effiacy testing of vaccines and cross challenge infectivity studies. (Purcell and Gerin I975, Buynak et al I976).

Summers et al (I978) were the first to report a virus, closely resembling HBV, associated with hepatitis and hepatoma in the North American Woodchuck, Marmota monax. More recently other similar viruses have been discovered in the Beechy Ground Squirrel (Marion et al I980) and the Pekin Duck (Mason et al I980). Serological studies of woodchuck hepatitis virus and HBV indicate partial cross reactivity of varying degrees. (Werner et al I979, Feitelson et al I98I, Galibert et al I98I, Millman et al

I982 and Stannard et al I983).

Although normal immune serum globulin usually contains small amounts of anti-HBs, hyperimmune globulin, prepared from specially selected blood donors, possesses considerably higher specific activity (Gerety et al I980a, Hopkins et al I98Ic). Protection afforded by the former, particularly that prepared before the introduction of HBsAg blood donor screening, may have resulted from passive-active immunisation (Hoofnagle et al I979). In certain settings hyperimmune globulin can prevent up to 75 percent of hepatitis B cases. (Maynard I978, Seeff and Hoofnagle I979). Hyperimmune globulin is most effective when given immediately after needlestick accidents or mucous membrane contamination.

The basis for active immunisation against HBV was established by Krugman et al (I970) who found that a I in I0 dilution of infective serum had its infectivity reduced, but retained antigenicity, when boiled for one minute, and prevented or modified infection in about 70 percent of vaccinees subsequently challenged with untreated serum.

Failure to propagate HBV in tissue culture has hampered development of a conventional vaccine and stimulated research to find other preparations capable of producing active immunity. Physical and chemical purification of 22nm. HBsAg spherical particles from the plasma of asymptomatic human carriers has provided a subunit vaccine. (Hilleman et al

I978). Placebo-controlled, randomised, double blind trials in susceptible male homosexuals (Francis et al I982), dialysis unit staff (Crosnier et al I98I) and others (Krugman et al I98I), Maupas et al I98I) have established the potential of these preparations for protecting against infection. The vaccine appears to be equally effective in preventing the development of an HBsAg carrier state in children (Maupas et al I98I).

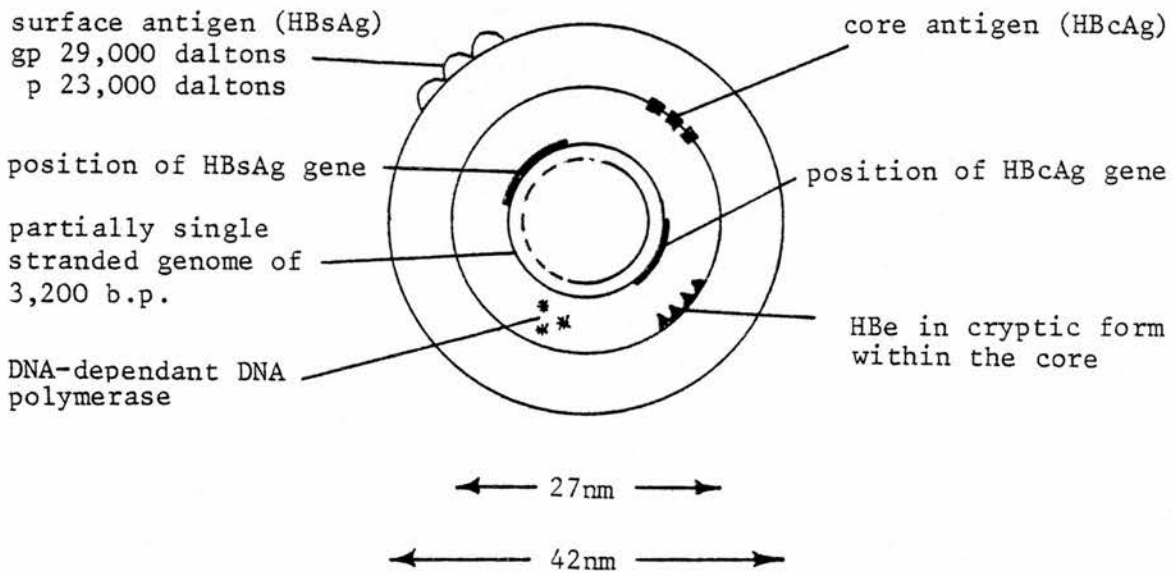
There have been numerous reports of the application of monoclonal antibodies for detection of HBsAg. Of particular interest is the application of monoclonal anti-HBs to simultaneous incubation (one step) systems. (Wands et al I980, Wands et al I98I, Goodall et al I982, Jackson et al I983). However the goal of exquisite specificity combined with significantly improved sensitivity has yet to be fully realised. (Wands et al I982).

The HBV genome has been cloned in prokaryotic and eukaryotic cells. (Burrell et al I979, Valenzuela et al I982). Both approaches have been successful but until now the yield of HBV gene products from bacteria has been too low to be useful with the exception of a bacterial clone which produces sufficient HBcAg for use in diagnostic tests (Stahl et al I982, Field I983). Much more promising is the use of yeast cultures; products are now under trial in man. Cloning experiments have made detailed genetic analysis of HBV DNA possible (Figure I.3ii). HBV DNA is a circular molecule approximately 3,200 nucleotides long (Szmuness I978, Mason

FIGURE 1.3ii

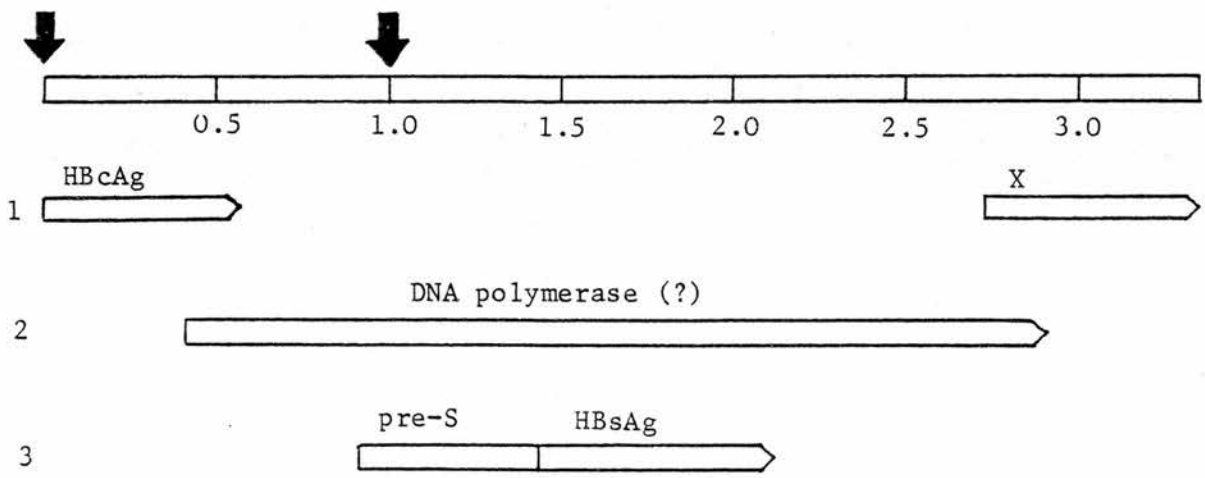
(a) DIAGRAMMATIC REPRESENTATION OF THE 42nm HEPATITIS B VIRION

HEPATITIS B VIRUS



(b) MAP OF HBV DNA IN LINEAR FORM BEGINNING AT THE FIRST NUCLEOTIDE OF THE GENE FOR HBcAg.

HBV GENOME



et al I980) with a single stranded region of variable length. Summers et al (I975) have proposed a model of two unequal strands, one being a long (L) strand of fixed length. Recent experiments have confirmed this model. (Mackay and Murray I983). The amount of DNA associated with HBcAg (I.8 x $I0^6$ daltons) is insufficient to code for the core antigen and the DNA polymerase enzyme in addition to the HBsAg determinants found in polypeptides isolated from HBsAg particles. It seems that either polypeptides of various sizes carrying HBsAg determinants are degradation products of a common primary polypeptide or the apparently high molecular bands found in SDS-PAGE are formed by the aggregation of smaller polypeptides. Evidence in favour of the latter hypothesis has been reported by Koistinen (I980).

THE DELTA AGENT. Delta antigen (δAg) appears to be exclusively associated with HBV infection. Originally described in Italy (Rizzetto et al I977), it has since been found in HBsAg carriers throughout the world (Rizzetto et al I980). The putative genome is a small ribonucleic acid and bears the protein coat of HBsAg (Rizzetto et al I980). Where infection occurs, sensitive assay techniques have demonstrated delta antigen in liver cell nuclei and anti-delta in serum (Rizzetto et al I977, Smedile et al I982a). Superinfection with delta agent may adversely effect the severity of HBV infection (Smedile et al I982b). Vertical transmission of delta agent has been reported

(Zanetti et al I982). Anti-delta IgM appears to be a useful serum marker for distinguishing active disease from past infection. (Smedile et al I982a).

HBsAg carriers repeatedly exposed to reinfection (such as drug addicts or haemophiliacs) have an increased risk of superinfection with delta agent. When this happens they are likely to experience a further acute hepatitis episode and frequently develop delta related progressive liver disease. (Smedile et al I98I, Rizzetto I982, Rizzetto et al I982). The implications of inadvertantly introducing delta agent in a population with a high natural prevalence of HBsAg carriers should be a primary consideration during safety testing of HBV vaccine.

I.4 NON-A, NON-B VIRAL HEPATITIS

DISCOVERY.

Indications that more than two types of viral hepatitis existed could have been drawn from early epidemiological observations of multiple attacks of hepatitis in drug addicts (Havens I956, Levine and Payne I960, Rosenstein I967), post transfusion hepatitis (Mosley I966), dialysis patients (Eastwood et al I968) and medical personnel (Gellis I95I). Without specific diagnostic tests for HAV or HBV, such interpretations would have been mere speculation. With the discovery that HAV does not cause prolonged viraemia and that shedding of virus in stool does not persist beyond the acute phase of the disease (Krugman et al I959, Zuckerman and Howard I979), it became clear that HAV had no significant role in parenterally transmitted hepatitis, a finding supported by the absence of a bimodal distribution of hepatitis cases, based on incubation periods of HAV and HBV (Grady et al I964, Mosley I965). The possibility that repeated attacks of hepatitis could result from different antigenic types of HBV, that immunity after hepatitis was not absolute, that a massive second exposure could overcome partial resistance, or that second or third attacks were exacerbations of a silent, persistent infection (Purcell et al I97I, Gocke I972, Hollinger et al I973, Iwarson et al I973, Koretz et al I973, Sutnick et al I973, Karrountzis et al I975), were at one time suggested to explain the phenomenon of what is now referred to as non-A, non-B hepatitis.

A reduction in the number of post-transfusion hepatitis cases was brought about by screening blood and blood products for HBsAg by sensitive 'third generation' assays (Gocke I972) and, particularly in the United States, by a significant reduction in the use of 'paid' donors (Alter et al I972). Residual hepatitis cases were at first thought to reflect an insensitivity of assays for HBsAg. Cases exhibiting an incubation period of under 40 days showed none of the epidemiological characteristics of HBV which led Ratazan et al (I97I) to postulate that such cases might 'represent a third entity, quite apart from infectious and serum hepatitis with distinct epidemiological characteristics of its own'.

Subsequently, by subdividing these post-transfusion hepatitis cases according to their HBsAg status, it was found that patients with HBsAg negative hepatitis had incubation periods longer than those generally observed for HAV, but significantly less than those found for HBV (Goldfield et al I972, Grady et al I972, Mosley I972). The failure to document overt hepatitis among household contacts of HBsAg negative cases, the relative ineffectiveness of immunoglobulin to abort the disease or significantly lengthen the incubation period (Prince et al I974) and the inability to incriminate HAV serologically as the causative agent of non-B post transfusion hepatitis (Feinstone et al I973) led to the term non-A, non-B hepatitis to describe such episodes.

EPIDEMIOLOGY.

Parenteral transmission.

In the absence of well defined serological markers, non-A non-B post transfusion hepatitis is diagnosed purely by exclusion of other hepatitis viruses (HAV, HBV, CMV, EBV), toxic liver damage and by the occurrence of at least 2 elevated alanine amino transferase (ALT) values occurring 2-24 weeks after the first blood transfusion. (Editorial I978). Parenteral transmission of non-A, non-B hepatitis agents was first recognised in a series of studies of post-transfusion hepatitis conducted in the mid I970's in the United States. (Prince et al I974, Alter et al I975, Knodell et al I975, Dienstag et al I977b, Seeff et al I977). Non-A, non-B hepatitis is now responsible for about 90 percent of hepatitis cases that follow transfusion of blood or blood products in the United States. (Alter et al I975). The shift from a post transfusion illness caused by HBV to one resulting from transmission of the agent(s) of non-A, non-B hepatitis coincided with a Federal requirement that all donor blood transfused after September I975 should be screened for HBsAg by test of 'third' generation sensitivity (Goldfield et al I975). Although effective in reducing the risk of exposure to HBV these measures have not resulted in a significant overall reduction in incidence of post-transfusion hepatitis. (Alter et al I978a, Aach and Kahn I980), inferring that non-A, non-B agents may always have been the major source of post transfusion hepatitis.

Non-A, non-B post transfusion hepatitis has now been reported to occur throughout the world exhibiting varying mean incubation periods. (Table I.4a). It has been suggested that 2 distinct agents can be differentiated by virtue of their incubation periods ('short' 3-5 weeks; 'long' 8-I2 weeks). (Shirachi et al I978). It soon became clear that transmission of non-A, non-B hepatitis was not limited to the transfusion of whole blood but also occurred following administration of certain blood products and components. Single and multiple bouts of short incubation acute hepatitis in haemophiliacs treated with Factor VIII concentrates have been described (Craske et al I978, Hruby and Schauf I978, Bradley et al I979). Although some have been attributed to an allergic reaction to antigenic protein derived from Factor VIII concentrates (Myers et al I980), there is evidence that clotting factor concentrates can harbour infectious non-A, non-B agents. Both Factor VIII and Factor IX concentrates implicated in cases of non-A, non-B hepatitis have been shown to contain transmissible agents, by experimental inoculation of chimpanzees (Bradley et al I979, Tsiquaye and Zuckerman I979, Wyke et al I979).

The disease has also been reported following infusions of fibrinogen (Yoshizawa et al I980b), when 2 patients developed non-A, non-B hepatitis 2I and 56 days after infusion of a commercial fibrinogen preparation manufactured in Japan. Haugen (I979) reported that packed red blood cells treated with a high glycerol concentration and

TABLE 1.4a

REPORTS OF PARENTERAL TRANSMISSION OF NON-A, NON-B HEPATITIS

STUDY	COUNTRY	NUMBER OF CASES	ATTACK RATE (percent[a])	PERCENT WITH JAUNDICE	INCUBATION PERIOD (weeks)
Prince et al 1974	U.S.A.	299	71%	39	2 - 7
Meyers 1977	U.S.A.	13	69%	N.A.	N.A.
Guyer et al 1979	U.S.A.	9	77%	60	4 - 7
Seeff et al 1978	U.S.A.	N.A.	N.A.	18	2 - 26
Alter et al 1975	U.S.A.	108	7.4%	31	5 - 20
Alter et al 1978a	U.S.A.	N.A.	N.A.	25	2 - 13
Tateda et al 1979	Japan	1082	10.7%	25	2 - 33
Katchaki et al 1981	Netherlands	380	3.4%	N.A.	5 - 11
Dienstag et al 1981	U.S.A.	N.A.	40%	N.A.	2 - 8
Cossart et al 1982	Australia	842	1.7%	14	4 - 12
Lagerstadt et al 1982	Finland	65	4.6%	N.A.	8 - 10
Hernandez et al 1983	Spain	230	72%	28	2 - 25
Ohori et al 1983	Japan	115	43%	23.1	2 - 3
Tur-Kaspa et al 1983	Israel	50	8%	25	4 - 13
Sugg et al 1983	W. Germany	97	12.4%	16.6	N.A.

*N.A. Information not available

a Determined by elevated serum ALT

stored at -80°C were capable of transmitting infection when infused after rethawing. An outbreak of hepatitis, originally thought to be HAV was described amongst donors and recipients of both plasma and platelets by Meyers et al (I974) with a mean incubation period of 27 days and a high attack rate in both donors (90 percent) and recipients (69 percent).

Transfusion related non-A, non-B hepatitis has been reported in transplant (Meyers et al I977), haemodialysis (Galbrieth et al I975, Gmelin et al I983) and oncology patients (Meyers et al I977), in medical personnel following accidental needlestick exposure (Ahtone et al I980), in parenteral drug abusers (Mosley et al I977) and in neonates who presumably acquired the disease from their infected mothers. (Tong et al I980). Outbreaks of non-A, non-B hepatitis have been reported in Germany among plasma donors participating in a plasmapheresis programme (Martini I979) and in Tennessee among frequently plasmapheresed participants in a red blood cell stimulation programme (Guyer et al I979).

Sporadic or community hepatitis.

Few patients with viral hepatitis recall a specific percutaneous exposure to blood or contaminated instruments e.g. syringes, tattooing or ear piercing. Such cases of sporadic hepatitis also include non-A, non-B hepatitis, first reported by Villarejos et al (I975) in Costa Rica where I2 of I03 patients presented with non-A, non-B hepatitis with only I case demonstrating a rise in titre of

anti-CMV. Several studies of patients with hepatitis have been conducted in urban centres in the United States, Australia, New Zealand, Western Europe, Scandinavia and Japan. (Table I.4b). In most reported studies non-A, non-B hepatitis accounts for approximately I5 to 20 percent of sporadic cases in adults, (Locarnini et al I976, Dienstag et al I977a, Müller et al I978) ranging from 9 percent in Athens (Papavangelou et al I979) to 82 percent in Tokyo (Suzuki I980). Such wide variation may reflect the stringency of diagnosis or a preponderance of one epidemiological setting over another. For example, the very low incidence in Athens may be related to the high prevalence of HBV in Greece. Similar factors may account for the low frequency of non-A, non-B hepatitis in Costa Rica. Low frequencies were also recorded in Boston and Copenhagen. However, in both studies the high proportion of male homosexuals may have favoured HBV infection. (Szmuness et al I975, Dietzman et al I977, Corey and Holmes I980, Schroeder et al I982).

Little detailed information is available concerning the transmission of non-A, non-B hepatitis to sexual contacts, Although evidence suggests that sexual transmission does occur. (Farrow et al I98I).

Epidemic non-A, non-B hepatitis.

Until recently it was assumed that HAV was the cause of large epidemics of viral hepatitis. With the introduction of sensitive serological tests for markers of current HAV

TABLE 1.4b

RELATIVE CONTRIBUTION OF HEPATITIS VIRUSES TO SPORADIC HEPATITIS IN URBAN ADULTS

STUDY	URBAN AREA	NUMBER OF CASES	PERCENT WITH NON-A, NON-B	HAV	HBV	OTHERS
Farrow et al 1981	W. London	368	13	58	27	
Bamber et al 1983	N. London	172	13	51	34	2 (EBV)
Busher et al 1981	Birmingham	50	36	18	46	
Hopkins 1982*	E. Scotland	N.A.	40-50	7	N.A.	N.A.
Carreda et al 1979	Milan	243	18	30	52	
Intonazzo et al 1983	Palermo	407	8.6	72.9	17.9	0.5 (NS)
Muller et al 1978	Hanover	N.A.	19	17	64	
Papavengelou	Athens	222	9	22	69	
Mathieson et al 1980	Athens	216	14	10	76	
Widmer et al 1980	Zurich	N.A.	13	25	50	12 (NS)
Norkrans et al 1978	Goteburg	148	16	27	57	
Mathieson et al 1979	Copenhagen	115	6	57	34	3 (NS)
von Erlich et al 1979	Heidleberg	N.A.	15	15	66	4 (NS)
Norkrans et al 1979	Goteburg	N.A.	13	22	62	3 (NS)
Froland et al 1982	Oslo	297	12.5	17.2	70.3	
Moritsugu et al 1978	Tokyo	98	82	18	N.T.	
Suzuki 1980	Tokyo	N.A.	45.5	12	42.5	
Locarnini et al 1976	Melbourne	N.A.	19	43	38	
Goldwater et al 1982	Auckland	94	26	23	27	
Shalit et al 1981	Jerusalem	182	16.5	67	14.8	
Shalit et al 1982	Jerusalem	N.A.	15.1	70.9	12.1	1.7 (EBV)
Dienstag et al 1977a	Los Angeles	45	44.4	44.4	4.4	6.8 (NS)
Hoofnagle 1978	Washington DC	N.A.	20	16	62	
Pawl et al 1981	Boston, Mass.	N.A.	11	28	52	
Alter et al 1980	Baltimore	253	44	10	46	
Farnika et al 1983	W. Bohemia	106	19	24	57	
Khuroo et al 1983	Kashmir	293	53	15	32	
Villarejos et al 1975	Costa Rica	103	10.6	N.A.	N.A.	0.9 (NS)

HAV = Hepatitis A Virus; EBV = Hepatitis B Virus; EBV = Epstein Barr Virus;
NA = Information not available; NS = Agents not specified; * = Personal communication.

infection, it is now established that at least some epidemics of viral hepatitis are caused by an agent (or agents) other than HAV.

Three common source epidemics have been described, each outbreak attributed to sewage contamination of drinking water. The outbreak in Delhi during I955-I956 (Viswanathen I957, Parri et al I973) showed subtle epidemiological differences compared to HAV outbreaks. These included a longer incubation period (mean 40 days), an age distribution skewed towards older individuals (mean 27 years), a low (for India) case fatality rate, except amongst pregnant women and cholestastis as a prominent histological histological change. Similar features occurred in an outbreak in Kolhapur in I982 (Sreenivason et al I982) and Kashmir in I978 (Khuroo I980). Follow-up of the Kashmir epidemic showed there was no tendency towards chronic hepatitis (Khuroo I980).

In contrast the epidemic in Amhedabad during I975-I976 was due to contamination of water supplies to many widely spread street pipes and residential taps (Sreenivason et al I978) and presented a different epidemiological picture (Wong et al I980). The epidemic appeared to be associated with a water borne virus but was characterised by 3 waves of infection in contrast to the single sharp peak of the Delhi epidemic. The mortality rate was also higher.

CLINICAL FEATURES.

Non-A, non-B viral hepatitis is clinically indistinguishable from either HAV or HBV, although some basic features, mainly regarding degree of severity and length of illness are beginning to emerge. In typical transfusion related non-A, non-B hepatitis, the serum alanine aminotransferase (ALT) levels tend to be only moderately elevated (8 to 25 times normal, 350 - I,000 I.U./ml) and have a characteristic fluctuating course (bi- or multiphasic) (Shimizu et al I979, Tateda et al I979, Yoshizawa et al I980b). This may be less common in sporadic non-A non-B hepatitis (Dienstag et al I977a, Norkrans I978, Lucas et al I980).

Non-A, non-B viral hepatitis is usually a milder disease than type B hepatitis, the anicteric to icteric ratio of non-A, non-B hepatitis being approximately 4-6 : I. (Alter et al I975, Seeff et al I978). In contrast the anicteric to icteric ratios for HAV and HBV are approximately I-2 : I (Hoofnagle I978, Routenberg et al I979). The fatality rate of non-A, non-B hepatitis cannot be well defined but would appear to be approximately I percent; less than the average for HBV (I-4 percent) but higher than that for HAV (0.I percent). (Skinhöj et al I977, Hoofnagle I978, Norkrans I978, Rakela et al I978). Fulminant non-A, non-B hepatitis has been reported (Mathieson et al I980b, Wyke and Williams I980, MacLeod I983). Complications such as liver failure, cirrhosis or hepatoma are relatively infrequent (The Copenhagen Hepatitis Acta Programme I982) but carry a high mortality.

However, there are an increasing number of reports which suggest that chronic sequalae (as defined by elevated ALT levels persisting for more than 6 months) are particularly frequent following transfusion related non-A, non-B hepatitis (Table I.4c). (Knodell et al I977, Aach et al I978, Seeff et al I978, Berman et al I979, Rakela and Redeker I979). In these 5 studies a total of 267 cases of acute non-A, non-B transfusion hepatitis were followed for a period of at least 6 months. Elevated ALT levels were demonstrated in 96 patients (36 percent) throughout the period of the study. Liver biopsy was performed on 25 patients with ALT elevations. Nineteen (76 percent) showed histological features of chronic active hepatitis and 3 also had cirrhosis. In these studies at least 20 percent of those who developed transfusion associated non-A, non-B hepatitis showed progression to chronic active hepatitis or cirrhosis.

Chronic non-A, non-B hepatitis may also develop in the absence of a transfusion history, although several authors have reported a lower incidence of chronicity following 'sporadic' acute disease (Aach et al I978, Norkrans I978, Rakela and Redeker I979). In the study by Rakela and Redeker (I979) 20 percent of sporadic non-A, non-B hepatitis patients developed persistent ALT elevations. However in those undergoing biopsy, chronic persistent hepatitis rather than chronic active hepatitis was consistently found. Norkrans (I978) reported the development of chronic liver disease in 4 of 59 patients with acute

TABLE 1.4c

CHRONIC LIVER DYSFUNCTION FOLLOWING TRANSFUSION ASSOCIATED ACUTE NON-A, NON-B HEPATITIS.

STUDY	NUMBER OF PATIENTS WITH ACUTE HEPATITIS	PATIENTS PROGRESSING TO CHRONIC HEPATITIS	PERCENT
Galbrieth et al 1975	29	8	27.6
Koretz et al 1976	47	29	61.7
Knodell et al 1977	44	10	23.0
Seeff et al 1978	119	31	26.0
Aach et al 1978	65	36	55.0
Rakela & Redeker 1979	13	7	54.0
Berman et al 1979	26	12	46.0
Koretz et al 1980	66	26	39.4
Bamber et al 1981a	12	9	75.0

non-A, non-B hepatitis of which 3 developed chronic persistent hepatitis, the only patient to develop chronic active hepatitis had received a blood transfusion. Müller et al (I979) estimated I2.5 percent of patients without a transfusion background developed chronic hepatitis in Germany, in contrast to 44.4 percent chronicity following acute non-A, non-B hepatitis among patients involved in a German plasmapheresis centre epidemic reported by Martini et al (I978). Galbrieth et al (I975 and I979) reported an outbreak of non-A, non-B hepatitis in a haemodialysis unit, 28 percent (8 of 29 patients) showed elevated ALT values for greater than six months. Liver biopsy revealed chronic hepatitis in 3 patients (of which 2 also had cirrhosis) and persistent hepatitis in 2 cases. One patient died in hepatic failure with cirrhosis six years after the acute infection.

It appears that transfusion associated non-A, non-B hepatitis has a propensity for the subsequent development of chornic active hepatitis and cirrhosis. The reason for this is not clear, multiple factors may be involved. For example, the large amount of viral protein administered during transfusions, or the possibility of more than one non-A, non-B hepatitis agent. Chronic hepatitis has not been observed to date following 'epidemic' non-A, non-B hepatitis (Khuroo I980).

ATTEMPTS TO IDENTIFY THE AGENT(S) OF NON-A, NON-B VIRAL HEPATITIS.

A variety of particles have been described associated with non-A, non-B hepatitis, in human serum, liver, urine and faeces and in the serum or liver of experimentally infected chimpanzees (Table I.4d). The great variety of structures observed could reflect the possibility that non-A, non-B hepatitis may be caused by more than one virus, each exhibiting different morphological forms or a variety of the hosts response to hepatocyte injury.

Perhaps the most relevant studies have developed from investigation of clotting factor concentrates and other plasma fractions (Bradley et al I979, Yoshizawa et al I980b). Bradley et al (I979) transmitted non-A, non-B viral hepatitis to four chimpanzees by infusion of three lots of Factor VIII concentrates, implicated in the transmission of non-A, non-B hepatitis to two human recipients (incubation period 37 days and 22 days respectively). Immune electron microscopy of fractions from a density gradient of the Factor VIII concentrates revealed several aggregates of particles ranging in diameter between 25-30nm. which appeared to be coated with antibody. All these structures appeared electron luscent ('empty') and ragged in appearance. ' Similar particles were observed in the acute phase serum of one patient and in the liver homogenate of one of the infected chimpanzees. Both

TABLE 1.4d

SUMMARY OF PUBLICATIONS DESCRIBING THE STRUCTURES OF POSSIBLE NON-A, NON-B VIRUSES.

STUDY	PARTICLE SIZE (nm)	LOCATION
Cossart et al 1975	23	human serum
Prince et al 1978	60-80	chimp liver
Bradley et al 1979	25-30	chimp liver
Shimizu et al 1979	20-27	chimp liver
Coursaget et al 1979	60*	human serum and urine
Bradley et al 1980	25-30	chimp liver
Gmellin et al 1980	27	human liver
Grimaud et al 1980	22-27	human liver
Yoshizawa et al 1980b	27	fibrinogen
Mori et al 1980	32*	human serum
Hantz et al 1980	15-25, 35-40	human serum and liver
Williams et al 1980	80-140	human serum
Dermietzel et al 1980	32.5-40.5, 50-72	human plasma
Gibo et al 1980	20-27	human serum
Burk et al 1981	20-22, 37	chimp liver
Yoshizawa et al 1981	22-28	human serum
Cabral et al 1981	4-14, 23-27, 35	human liver
MacFarlane et al 1981	20, 27-33, 50*	human serum
Balayan et al 1982	27-30	human stool
Screenivason et al 1983	26-29	human stool

* Enveloped

electron dense and electron luscent particles were seen in fractions with a density of I.3Ig/ml. after centrifugation of a liver homogenate in a caesium chloride density gradient. Some particles were also found coated with IgM antibody after reaction with PBS alone, suggesting that they had reacted with antibody in acute phase serum during homogenisation of the liver. It is interesting to note that HBcAg bearing particles from infected chimpanzee liver also became coated by circulating antibody during extraction. The finding could represent a similar phenomenon since these non-A, non-B related particles have not been observed in normal human serum or liver.

Yoshizawa et al (I980b) described 27±2nm. diameter particles purified from a batch of fibrinogen implicated in the transmission of non-A, non-B hepatitis to two human patients exhibiting an incubation period of 3 and 8 weeks, one recipient had developed biopsy proven chronic persistent hepatitis 2 years after the acute disease. Identical particles were also seen in the serum of a chimpanzee who developed hepatitis after the intravenous inoculation of 200mg. of this batch of fibrinogen. Eight of I00 asymptomatic blood donors with elevated serum ALT levels also possessed detectable numbers of similar 27nm. particles in their sera. Inoculation of one of the sera containing particles resulted in hepatitis in two chimpanzees. The particles in fibrinogen, as well as the human and chimpanzee sera, appeared uniformly electron dense

and had a buoyant density of I.28g/ml in caesium chloride.

Specific ultrastructural alterations in liver cells of experimentally infected chimpanzees were first described by Jackson et al (I979) and later by Shimizu et al (I979), Bradley et al (I980), Pfeifer et al (I980), Tsiquaye et al (I980), Yoshizawa et al (I980b) and Tsiquaye et al (I98I). These alterations were primarily classified as nuclear changes and cytoplasmic tubular formation thought to represent the characteristic fingerprints of different agents, designated 'F' and 'H' strains of non-A, non-B hepatitis respectively. (Shimizu et al I979). However, these changes have subsequently been found to occur sequentially (Pfeifer et al I980, Tsiquaye et al I98I) and are thought to represent non-specific alterations in the liver in response to infection. The tubular structures in the hepatocyte cytoplasm are, apparently, the result of a proliferative process involving the smooth endoplasmic reticulum. The tubules, which ranged in diameter from I50nm to 300nm. comprised a double unit membrane enclosing an as yet uncharacterised osmiophilic substance. Accompanying these structures are a number of other equally characteristic ultrastructural changes that are primarily confined to the hepatocyte cytoplasm (Pfeifer et al I980). Curved or highly convoluted endoplasmic reticulum (ER) is frequently found in infected hepatocytes and may precede the formation of the larger tubular structures. These convoluted membranes are also

a result of a proliferative process involving the smooth endoplasmic reticulum (SER) and may be found throughout the cytoplasm or, more frequently, within restricted spaces. The ultrastructural changes described have their counterparts in both plant and animal cells infected with viruses possessing RNA genomes.

Less frequently reported are cytoplasmic fibrillar-granular masses (or dense reticular inclusion bodies) first described by Pfeifer et al (I980). It is unclear whether these structures are actually aggregations of particulate, ribosome-sized particles or are accumulations of electron-dense highly convoluted strands similar to the dense reticular inclusions described for mouse hepatitis virus, an RNA containing corona-virus (David - Ferreira and Manaker, I965). The fibrillar-granular masses, which appear after the more granular structures are first observed in hepatocyte cytoplasm, are morphologically similar to the viroplasmic foci or virus factories found in influenza virus infected chick embryo fibroblasts.

Other ultrastructural changes observed in the cytoplasm of hepatocytes in non-A, non-B hepatitis include bundles of granular microtubules in which 25nm. diameter cross sectioned microtubules are visible. Bradley and Maynard (I983) have observed paracrystalline arrays identical in morphology to those observed in cells infected with an unenveloped RNA virus (reovirus). These arrays may

be comprised of the protein tubulin, a major constituent of the microtubules found in bundles in some non-A, non-B infected hepatocytes.

McCaul et al (I98I and I982) and Bradley et al (I980) have observed crystalline arrays of densely stained 25-30nm particles in endothelial cells during non-A, non-B infection. Examination of individual particles by the Markham rotation technique showed that the particles consisted of an electron dense core approximately I2nm. in diameter, surrounded by an envelope measuring 5.5nm. in thickness. The outer structure possessed I6-I8 divisions. However, the individual crystals are probably non-viral and the presence of crystalline arrays may reflect the pathological response of the host cell to infection (McCaul et al I982); early reports suggest that this may be the result of interferon production (Grimley et al I983).

THE SEARCH FOR SEROLOGICAL MARKERS OF NON-A, NON-B HEPATITIS AGENTS.

There have been numerous reports of serological markers for non-A, non-B viral hepatitis detected by immunological methods ranging from immunodiffusion through counterelectrophoresis to enzyme immunoassay. To date, none of these assays have had their specificity for non-A, non-B hepatitis proven. Research is hampered by lack of availability of specific antibody and the probability that the infectious agent(s) of non-A, non-B

hepatitis are present in a low titre in plasma rendering them undetectable even by sensitive techniques such as enzyme immunoassay or radioimmunoassay. A summary of the reported markers follows: for clarity they are presented according to the method of detection.

(a) Serological markers detected by precipitation in gel techniques.

Shirachi et al (I978) reported a I00,000 - 300,000 dalton antigen with a buoyant density of I.30g/ml. (HCAg) in the serum of all I3 cases of long incubation (mean 7 weeks) and in 4 of I0 cases of short incubation (mean 5 weeks) post transfusion non-A, non-B hepatitis. The antigen was also detected in the sera of I0 of 25 cases of primary hepatocellular carcinoma and 2 of 9 cases of hepatitis B. The marker remained detectable for 8-24 weeks in long incubation episodes whereas in patients with the short incubation disease it appeared 3-4 weeks after transfusion disappearing within a week. This was thought to represent a distinctive feature between long and short non-A, non-B post-transfusion hepatitis. Antibody to HCAg was not readily detectable, being present in only 4 of I3 patients with long incubation post-transfusion non-A, non-B hepatitis. HCAg was found in the 7S (IgG) globulin fraction. The occurrence of HCAg in pre-transfusion serum of previously untransfused patients led the authors to suggest that a symptom free carrier state may exist.

Other serological systems have been reported by Vitvitski

et al (I979), Overby et al (I980), Renger et al (I980), Hopkins (I98Ia) and Suh et al (I98I), using human sera. Exchange of coded sera between these authors revealed that most were studying the same (or similar) systems (Hopkins et al I98Ia). Further study of the immunodiffusion system showed that it was markedly dependent upon pH, agarose concentration, molarity and nature of the buffering system (Suh et al I98I). It was also suggested that such reactions could be detecting immune complexes which may or may not include specific viral components (Suh et al I98I).

A marker showing identity with that of Vitvitski (and, by inference, the markers reported above) was reported recently by Spertini and Frei (I982). Differences between this and the earlier marker systems are the presence of either antigen or antibody in all patients falling into the non-A, non-B category, and the finding that precipitin lines could be obtained in the presence of at least I0mM/EDTA below pH 8.0.

Hoofnagle (I98I) characterised another antigen-antibody system in patients with chronic non-A, non-B hepatitis. An exchange of reagents with Dr. Trepo yielded lines of identity. However, the latter's antigen corresponded to Hoofnagle's 'antibody' and vice versa. Reagents from Dr. Tabor (see below) also reacted and yielded lines of identity. Antigen was not found in I67 normal human sera, but was detected in 30-33 percent

of patients with acute or chronic non-A, non-B hepatitis. However, the antigen was also detected in a similar percentage of sera from patients with other forms of acute and chronic liver disease. Antibody was detected in the sera from healthy rabbits, guinea pigs, rats and also in liver homogenates from both humans and rabbits.

An immunodiffusion system associated with a particulate antigen was reported by Mori et al (I98I). The antigen banded at I.3g/ml. CsCl and was found in I5 of I6 patients with hepatitis characterised by biphasic elevation of serum enzymes; antibody was detected in I4 of these patients. Seven of I0 patients with monophasic elevations of liver enzyme had antigen which appeared at the time of significant SGPT elevations 2-I3 weeks after transfusion and disappeared within I-2 weeks. Antibody appeared in 7 of I0 patients. Virus-like particles were detected in the sera of all 4 patients with biphasic liver enzyme elevations and antigenaemia, suggesting a relationship between the observed particles and the detected antigen.

Immunodiffusion systems have also been reported by Chircu et al (I980), Williams et al (I980) and Villarejos (I982). Their relationship to the other markers mentioned is unknown.

(b) Serological Markers detected by counter-electrophoresis. Tabor et al (I979a, I980a) have investigated a serological

marker system by counterelectrophoresis in serum from both chimpanzees and humans. Their antigen, a 90,000 - 100,000 dalton glycoprotein was found in 6 of 7 animals during the acute phase of non-A, non-B infection, in the serum of 3 humans with chronic non-A, non-B disease whose blood was known to have transmitted infection, and in the serum of one individual known to be capable of transmitting disease over a six year period, even when aminotransferase levels were normal.

Specific antibody was detected in convalescent sera from chimpanzees, a nurse infected by accidental needle-stick exposure and a haemodialysis patient. Preliminary data suggested that this was an identical system to that of Shirachi et al (I978). However, the interval between the disappearance of the antigen and detection of the antibody in one chimpanzee was I3 weeks, with the antibody persisting for more than I2 months, while in the Japanese patients the interval was in some cases as short as 2 weeks, and antibody appeared to be only transiently detectable.

(c) Markers detected by immunofluorescence.
Indirect immunofluorescence has been used by Kabiri et al (I979) to detect an antigen in the nuclei of infected hepatocytes using convalescent sera from a chimpanzee and a human. The possibility that this nuclear antigen was produced in response to activation of a latent chimpanzee virus seems to be ruled out by the detection of specific antibodies in the serum of a

nurse convalescent from non-A, non-B infection probably acquired by accidental parenteral exposure. The relationship between this antigen-antibody system and the antigen described in chimpanzee by Tabor et al (I979a, I980a) is not yet known.

Alberti et al (I980) also reported a nuclear antigen detected by direct immunofluorescence in hepatocytes of patients with acute and chronic non-A, non-B hepatitis and later in children with acute leukaemia and chronic liver disease (Locasciulli et al I983), antibody being present in the serum of 5 out of 6 patients.

(d) Markers showing similarities with HBV.
It has been suggested that at least one non-A, non-B hepatitis agent is related to HBV by virtue of similar morphology and cross reacting antigens (Hantz et al I980, Trépo et al I980, Trépo et al I98I). The antigen previously reported by Vitvitski et al (I979) was designated non-A, non-B e antigen as it appeared to cross react with the HBe3Ag of HBV and was detected in the serum of 5I out of 62 long incubation post-transfusion hepatitis and I6 out of 26 donors implicated in the transmission of non-A, non-B hepatitis. It was also detected in 11 out of 56 non-A, non-B hepatitis (acute phase) patients who had no known parenteral exposure. Amongst 'high risk' groups it was detected in 22 out of 48 patients with chronic active hepatitis, I4 out of I5 patients with chronic persistent hepatitis, II out of 48

haemophiliacs and 6 out of 42 haemodialysis patients. A core antigen, designated non-A, non-B core antigen, cross reacted with HBcAg by immunodiffusion but not by radioimmunoassay and was found in association with anti-non-A, non-B e antigen. Non-A, non-B core antigen appeared to be released in excess in the blood where it became readily detectable and was associated with 22 to 25nm. particles. Antibody to this non-A, non-B core antigen was detected in association with non-A, non-B e antigen in I4 out of 26 patients with chronic active hepatitis. Immunofluorescence and counterelectrophoresis tests suggested that these were two distinct antigens, both located primarily in the nuclei of infected hepatocytes. Detergent treatment of non-A, non-B core particles appeared to release non-A, non-B e antigen. Four chimpanzees, immune to HBV were challenged with Iml. of the non-A, non-B inoculum intravenously (Trépo et al I983). Elevation of ALT developed after 4 weeks, at 6 weeks non-A, non-B core antigen was demonstrated by immunofluorescence in the liver nuclei and antibody to non-A, non-B c in the serum, both markers persisting for over 4 months. Double unit tubule or structures were observed in the cytoplasm of hepatocytes. Inoculation of acute plasma and serum into 2 other chimpanzees was followed by the same sequence of events.

It has been implied that some HBV carriers may terminate detectable HBsAg production yet continue to carry HBV-DNA (Prince I982). However, Monjardino et al (I982)

using ^{32}P labelled cloned HBV-DNA as a probe, were unable to find homologous nucleic acid sequences in serum or liver of humans, chimpanzees or marmosets infected with a human non-A, non-B agent. It is possible that false positive results (using E.coli derived HBV-DNA) could arise from contaminating E.coli present in the test serum.

(e) Serological markers detected by radio or enzyme-immunoassays.

The application of radioimmunoassay (RIA) for the detection of possible non-A, non-B antigens was first described by Neurath et al (I980) using IgG from the serum of a multiply transfused convalescent patient. The antigen was subsequently found in 94 percent of individuals who developed non-A, non-B hepatitis but was also present in I3 percent of HBsAg negative sera of blood donors with normal ALT and 34 percent of HBsAg negative sera from blood donors with elevated ALT. The antigen had an approximate molecular weight of 45,000 daltons, buoyant density of I.23g/ml. and an isoelectric point of 7. Further studies (Neurath et al I98I) showed it to be an intracellular alloantigen, inherited by an autosomal dominant trait in Caucasians. It was designated cytoplasmic dominant antigen (CDA).

Further examples of the care needed in developing assays are provided by the work of Arnold et al (I980), Thomas et al (I983) and Duermeyer et al (I983). A RIA developed

by Arnold et al (I980) detected an antigen in I3 out 26 acute non-A, non-B hepatitis patients, I4 out of I6 chronic non-A, non-B hepatitis patients and II out of 20 sporadic acute phase non-A, non-B hepatitis patients. It was absent in I0 convalescent non-A, non-B hepatitis patients. However, antibody to this antigen was detected in 3 out of I0 convalescent patients after an acute attack. The antigen was absent in control groups of acute HBV and chronic active hepatitis patients or blood donors. On testing a coded panel of infectious non-A, non-B sera, probable infectious sera and negative controls (Alter et al I982) the assay detected 5 out of 8 proven infectious sera and 2 out of 3 probable infectious sera. There were no false positive results and the overall rate of correct response was 78 percent.

Thomas et al (I983) developed a RIA which detected an antigen apparently associated with at least one type of parenterally transmitted non-A, non-B hepatitis common in haemophiliacs (incidence of 40 percent after Factor VIII infusion). The antigen was present in 3 percent of normal hospital staff and blood donors, in contrast to 29 percent of sporadic cases of non-A, non-B hepatitis where it was associated in all but I case with drug abuse. An increased prevalence of the antigen-antibody system was also found in patients with chronic renal failure, homosexuals and prostitutes. An enzyme-immunoassay (EIA) described by Duermeyer et al (I983) detected an antigen (DS Ag) apparently related (establ-

ished via an exchange of coded sera) to that reported by Arnold et al (I980) and Thomas et al (I983). The antigen exhibited a buoyant density of I.32g/ml in caesium chloride, was transmissible to at least one susceptible chimpanzee and was present in 65 percent of post transfusion hepatitis cases. Antibody to DSAg was detected in a significant number of haemophiliacs (48 percent) compared with normal blood donors, findings indicative of a predominantly parenteral mode of transmission, reminiscent of HBV. Further studies indicate that DS Ag in human serum is an IgM antibody directed against the Fc fragment of IgG in a certain number of individuals. In addition, results of a prospective study showed that DS Ag was not an indicator of infectivity. (Hellings et al I983).

(f) Immune complexes in non-A, non-B hepatitis. Dienstag et al (I979) first proposed that viral antigen could be present but masked by antibody in immune complexes. Their presence might explain the failure of conventional techniques to detect virus specific antigens. Using the Raji cell immunoassay, immune complexes were detected in 59 percent of patients with non-A, non-B hepatitis and in one of two inoculated chimpanzees. These complexes were present immediately before, coincident with or during the return to normal of raised ALT activity. Seven of I0 chronic non-A, non-B hepatitis patients also exhibited detectable complexes at levels which paralleled changes in serum ALT activity.

Use of the Raji cell immunoassay and Clq agar gel diffusion led Ohori et al (I982) to detect immune complexes in acute, convalescent and post recovery sera of non-A, non-B hepatitis patients. SDS-PAGE analysis revealed an 80,000 dalton protein indicating that this might be an antigenic determinant of a non-A non-B hepatitis agent.

ANIMAL MODELS.

The transmission of non-A, non-B hepatitis to the chimpanzee (Pan troglodytes) has proved invaluable for investigation of this disease and potential characterisation of its causative agent(s). In I978, two groups independently reported the first successful and reproducable transmission of non-A, non-B hepatitis to chimpanzees by the inoculation of serum obtained from acutely or chronically infected humans (Alter et al I978b, Tabor et al I978). Further studies (Hollinger et al I978, Prince et al I978, Bradley et al I979, Wyke et al I979) confirmed transmissibility via human sera and showed that the agent(s) could be transmitted to chimpanzees by Factor VIII concentrates, Factor IX complex, cryoprecipitate and liver tissue from an infected chimpanzee. The study of Hollinger et al (I978) indicated that such agent(s) could also be transmitted by intramuscular or intrahepatic inoculation, as well as intravenously. Cross challenge studies have shown there is no relationship between non-A, non-B hepatitis agent(s) and HAV, HBV and the G.B. agent, a cause of hepatitis in marmosets

and perhaps in some humans (Alter et al I978b, Tabor et al I978, Tabor et al I980b).

In the chimpanzee the disease resembles that in humans with an incubation period of 2-I2 weeks, monophasic or multiphasic ALT elevations and comparable histological changes in the liver. However, a particularly disturbing feature of the chimpanzee model is the development of a carrier state in which the disease may be transmitted even after prolonged resolution of enzymatic and ultrastructural abnormalities (Bradley et al I98I, Bradley et al I983a). This may parallel the situation in man as several investigators have succeeded in transmitting non-A, non-B hepatitis to chimpanzees with sera from implicated and presumably chronically infected donors (Alter et al I978b, Hollinger et al I978, Tabor et al I978). Tabor et al (I980a) have documented the persistence of an infective agent in the blood of one patient for at least six years, even during a period when serum ALT had returned to normal. The similar incubation period, pattern of ALT elevations, serological findings of an antigen - antibody system presumed to be associated with a non-A, non-B agent and histopathological changes in liver biopsy material suggested that the same agent was present in this patient's blood throughout the six year period.

The existence of at least two non-A, non-B hepatitis agents is indicated by chimpanzee cross-challenge exper-

iments (Tsiquaye and Zuckerman I979, Hollinger et al I980, Yoshizawa et al I98I). Studies by Alter (personal communication I983) indicate that the 'F' and 'H' strain inocula which result in typical post transfusion hepatitis in humans contain at least one common agent. A second, probably short incubation agent, may be present in many clotting factor concentrates.

Brotman et al (I983a) administered faecal material intravenously and orally into four susceptible chimpanzees. The inoculum was obtained from a chimpanzee with acute phase blood borne non-A, non-B hepatitis. None of the animals challenged in this way developed abnormal serum enzyme levels or exhibited ultrastructural alterations in hepatocellular cytoplasm characteristic of this disease inferring that faeces did not transmit this human strain of (normally) blood borne non-A, non-B hepatitis.

In contrast, Balayan et al (I983) reported the transmission of epidemic (faecal-oral) type non-A, non-B hepatitis to a human volunteer by oral transmission and two cynomologus monkeys by intravenous inoculation of stool extract. This may be the result of differences in susceptibility between chimpanzees and cynomologus monkeys, or distinct agents similar in properties to HAV (faecal/oral non-A, non-B) and HBV (parenteral non-A, non-B). HAV can be transmitted both by the faecal-oral

and parenteral routes (Hadler et al I982, Barbara et al I983) whereas HBV is transmitted primarily by parenteral means and not readily by the faecal-oral route.

Bradley et al (I983b) induced two separate and distinct episodes of non-A, non-B hepatitis in two chimpanzees, one containing a chloroform - resistant agent and the other containing a chloroform - sensitive agent. Both agents were recovered from liver tissue and plasma obtained from a single chimpanzee during the acute and chronic phases respectively following inoculation of a factor VIII concentrate. The chloroform resistant agent did not cause unique changes in hepatocytes: in contrast, the chloroform sensitive agent did induce the formation of cytoplasmic tubules convoluted endoplasmic reticulum and dense reticular inclusion bodies. The latter changes are similar in character to those induced in infected cells by some mammalian enveloped RNA viruses. These conclusions were based in part on the assumption that the chimpanzees used in these studies did not have latent viral infections that might have been reactivated during the experiments.

Tabor et al (I979b) investigated the transmission of a non-A, non-B inoculum documented to be infectious in chimpanzees to certain marmoset species, Saguinis mystax and Saguinis labiatus. Twelve weeks after inoculation, non-A, non-B hepatitis could not be observed in any of the

marmosets. It is possible that a larger challenge dose of infectious agent is needed, although on a body weight basis the infectious dose given to the marmosets was greater than that administered to the chimpanzees. However, Feinstone et al (I98I) reported the transmission and serial passage of the 'F' and 'H' strain non-A, non-B agents in S. mystax and S. labiatus but only after a prolonged incubation period of 22 to 24 weeks. They concluded that marmosets are not as susceptible as chimpanzees to the agents of non-A, non-B hepatitis, judged by the lower proportion of animals in which infection could be documented (2 of 6 animals receiving 'F' strain and 3 of 7 animals receiving 'H' strain) and by the very long incubation period.

Karayiannis et al (I983) transmitted non-A, non-B hepatitis to two colony born marmosets (S. labiatus), using the 'H' inoculum and a factor VIII concentrate. Both animals showed histological and ultrastructural evidence of viral hepatitis with raised ALT levels from the second week after inoculation through to the end of follow-up, 3 months later.

PREVENTION OF NON-A, NON-B HEPATITIS.

Approximately I0 percent of all blood recipients develop biochemical evidence of non-A, non-B hepatitis (Aach and Kahn I980) and half continue to exhibit protracted liver dysfunction (Berman et al I979). If the majority of episodes are due to infectious agents then the overall

frequency of non-A, non-B 'carriers' among blood donors likely to be much higher than for HBV. The transition from 'paid' to volunteer blood donors had a considerable impact on the transmission of HBV, and evidence suggests that the remaining 'paid' blood donors may be contributing significantly to an increased risk of non-A, non-B transmission (Stevens I978). A 'history of jaundice', which still excludes the acceptance of potential blood donors in many countries, does not appear to carry an increased risk of HBV transmission (Follett et al I980, Hopkins I98Ib) its relevance to non-A, non-B transmission is not yet clear. Reports from Conrad et al (I977) and Cossart et al (I982) describe an increased association of non-A, non-B hepatitis following transfusion of anti-HBc and/or anti-HBs positive blood, which may reflect the likely exposure to non-A, non-B agents of persons already carrying evidence of exposure to HBV.

A report by the Transfusion Transmitted Viruses Study Group (Stevens I978) revealed that non-A, non-B hepatitis occurred in 3.4 percent of recipients of units of blood with normal ALT levels compared to an incidence of 38.9 percent in recipients receiving at least one unit of blood with an elevated ALT value. A continuation of this study (Aach et al I98I) confirmed that non-specific screening of blood donations for raised serum ALT levels would reduce, but not eliminate, the incidence of post-transfusion non-B hepatitis. The non-specificity of ALT elevations and the lack of knowledge concerning the

prevalence of elevated ALT levels among otherwise healthy adults, as well as the likelihood of elevated and normal ALT levels in sequential samples from a single individual, dictate that answers to those questions must be assessed before this test can be used as a means to identify blood donors more likely to transmit non-A, non-B hepatitis.

The use of tests for carcino-embryonic antigen (CEA) to identify prospective blood donors at risk of transmitting hepatitis has been suggested (Molnar and Gitnick I977). Data from this study of donors matched with normal donors indicated that blood with elevated CEA levels transmitted hepatitis to 46 percent of recipients, compared with I6 percent receiving blood with normal CEA levels. These findings have not been confirmed (George et al I980) and the value of the CEA level as a screening test for preventing transmission of non-A, non-B hepatitis would appear unfounded.

A possible future approach is to attempt to prevent the disease by means of active or passive immunisation. However, the development of a specific vaccine (active immunoprophylaxis) requires purification of a causative agent, as yet to be identified. The possibility for passive protection exists since recent studies suggest that a protective antibody is produced following infection. Hoofnagle et al (I977) reported that five individuals who had developed non-A, non-B hepatitis following inoculation

with known infectious serum were resistant to reinfection upon challenge with the same serum. Essentially similar findings have evolved from a series of studies using chimpanzees (Tabor et al I978, I979c). Yoshizawa et al (I980b) also reported that chimpanzees who had been infected with non-A, non-B hepatitis via human fibrinogen appeared resistant to reinfection when the same preparation was re-injected.

Tabor and Gerety (I980) reported loss of infectivity for non-A, non-B hepatitis following treatment of 0.Iml. aliquots of serum with I:I,000 formalin at 37°C for 96 hours. Inoculation into chimpanzees did not result in recognisable non-A, non-B infection during seven months' observation. However, all the chimpanzees remained susceptible to non-A, non-B hepatitis when subsequently challenged with the untreated serum 3I weeks after inoculation with the formalin treated serum. Dilution of formalin to I in 2,000 had a similar effect, while pasteurisation (60°C for I0 hours) reduced infectivity but allowed the retention of sufficient antigenicity to stimulate some apparent immunity.

Chloroform extraction of 'H' strain non-A, non-B plasma resulted in complete loss of infectivity when inoculated into susceptible chimpanzees (Feinstone et al I983).

However, the ability of the extraction to produce a protective immune response was lost since sham extraction

of the 'H' strain plasma was shown to maintain its infectivity in chimpanzees that had been initially inoculated with the chloroform extracted plasma. The agents(s) in the 'H' strain inoculum appear to be totally inactivated by chloroform extraction, implying not only that the agent(s) contain essential lipids, but that treatment of plasma pools with chloroform may eliminate a proportion of non-A, non-B post-transfusion events. Preliminary data also suggests that beta-propiolactone combined with ultra violet light treatment may also be effective. (Stephen et al I983).

In the late I970's, several double blind, randomised studies of immunoprophylaxis in non-B post-transfusion hepatitis were conducted (Kahns I976, Knodell I976 et al I976, Seeff et al I977). The incidence of anicteric non-A, non-B hepatitis in these studies was not significantly different between blood recipients who had been given immune serum globulin (ISG) and those that had received a placebo, while in two other studies (Knodell et al I976, Seeff et al I977) the incidence of icteric non-A, non-B hepatitis was significantly lower in recipients of ISG than in those receiving a placebo. Furthermore, progression of acute to chronic non-A, non-B hepatitis occurred significantly more often in placebo treated (I0 percent) than in the globulin treated (0.5 percent) patients (Knodell et al I977).

In a trial undertaken in Japan (Kikuchi et al I980)

250mg. gamma globulin prepared for intravenous use was added to each unit of blood, one hour before transfusion. The incidence of hepatitis was significantly higher ($p \geq 0.01$) in the control group (13.7 percent) than in the group receiving gamma globulin (5 percent). The use of a human immunoglobulin preparation did not prevent infection in two experimentally inoculated chimpanzees. A prolonged incubation period of 15 weeks was observed in one animal when ALT elevation was used as a criteria of infection (Spichtin et al 1983). Typical tubular structures were observed in the hepatocytes. This may infer that if antibody to a non-A, non-B agent was present in the immunoglobulin, it was non-neutralising, or it is possible that infectious immune complexes were formed as a result of administration of antibody.

RELATEDNESS OF THE AGENTS OF HUMAN VIRAL HEPATITIS.

HAV, HBV and the agents of non-A, non-B hepatitis all have the liver as their primary target organ. Epidemiologically there are similarities between HAV and epidemic non-A, non-B hepatitis (Khuroo 1980) possibly indicating a common or related ancestry. Although not antigenically related further studies relating to nucleic acid hybridisation, polypeptide and amino acid sequencing may clarify the situation. There also appear to be epidemiological similarities between HBV and the non-A, non-B agents responsible for the majority of post transfusion and possibly sporadic hepatitis cases (Prince et al

I974, Alter et al I975, Dienstag I977a, Farrow et al I98I).

Serological cross reactivity between one putative non-A, non-B agent and HBV has been reported (Trepo et al I980) though not confirmed. Infectivity studies in chimpanzees suggests the existence of interference between the human hepatitis viruses. Chronic non-A, non-B infection appears to interfere with subsequent HAV and HBV challenge (Bradley et al I983c, Brotman et al I983b, Tsiquaye et al I983). In the absence of specific serological tests, it has been suggested that this viral interference may be a means of identifying or quantifying the presence of non-A, non-B viruses. The mechanism of interference remains unclear - de novo synthesis of an interferon like substance or alteration of the mechanisms responsible for virus absorption have been proposed. The reduction in DNA polymerase activity during HBV infection could suggest that non-A, non-B suppresses the expression of the viral genes responsible for the production of the complete HBV virion.

The order of viral challenge may influence the severity of illness in certain patient groups since infection with non-A, non-B and HBV was thought to be a possible cause of the high mortality rate in the hepatitis outbreak in the Edinburgh renal transplant/dialysis unit in I969-I970 (Marmion et al I982).

I.5 AIMS OF RESEARCH

M2Ag was first reported by Hopkins (I98Ia) having been discovered some eighteen months earlier and shown an affinity for non-A, non-B viral hepatitis. The broad aim of this research is to extend the initial studies by characterisation of M2Ag and to determine the significance of associated serological markers for diagnosis and possible prevention of disease transmission.

Specific lines of research include:

I. Development and application of enzyme immunoassays for detection of M2Ag and specific antibodies of the IgM and IgG type.

2. Use of the above assays to assess the significance of M2 markers for non-A, non-B viral hepatitis.

3. Characterisation of M2Ag and the immune response it promotes.

4. A survey of the occurrence (prevalence) of M2 markers in the serum of normal blood donors and groups at high risk of exposure to human heptitis viruses in general.

PART TWO

DEVELOPMENT OF ENZYME IMMUNOASSAYS FOR DETECTION OF SEROLOGICAL MARKERS.

2.I INTRODUCTION

Detailed characterisation of HAV and HBV has enabled development of sensitive and specific assays for both diagnostic and in the case of HBV, for screening purposes. The techniques employed have evolved from immunodiffusion (Kim and Tilles I97I) through haemagglutination(Cayzer et al I974) and enzyme immunoassay (EIA) (Wolters et al I977, Duermeyer et al I978).

There is now an urgent need for both screening and diagnostic assays to detect the causative agents of non-A, non-B hepatitis. The M2 antigen-antibody system was first discovered using immunodiffusion (Hopkins, I98Ia). Although this technique has the advantage of establishing reactions of non, partial and complete identity and allowing precipitin lines to be excised from the gel for examination by electron microscopy, the technique is relatively insensitive (detecting I-IOμg/ml protein), time consuming, wasteful of scarce reagents and cannot be automated for use in the routine laboratory.

In the Edinburgh Blood Transfusion Service, screening for HBsAg is performed using a solid phase sandwich RIA (Kane et al I983). This method is very sensitive (detecting approximately 0.5ng/ml) and has been (semi-) automated. However, RIA reagents present an additional biological hazard requiring adherence to a strict protocol for storage, use and waste disposal. They also

have a short shelf-life. The alternative, EIA, although lacking slightly in sensitivity and precision, have a longer shelf-life, are easily stored and, if certain substrates are avoided present minimal additional health hazards. As with RIA, EIA also has the potential for automation. These reasons, combined with existing equipment and expertise within the Blood Transfusion Service for the application of a solid phase sandwich immunoassay, led to the development of EIA's, rather than RIA's for the detection of M2Ag, anti-M2 IgM and anti-M2 IgG. The reproducibility and specificity of these EIA's for a form of parenterally transmitted non-A, non-B hepatitis were investigated.

2.2 ASSAY DEVELOPMENT, MODIFICATIONS AND PROTOCOLS

ORIGINAL ENZYME IMMUNOASSAYS.

Reagents:

M2Ag purification:

Serum was obtained from a blood donor twice implicated as the possible cause of post-transfusion non-A, non-B hepatitis with an incubation period of approximately four weeks on both occasions. One hundred millilitres of serum were clarified by centrifugation at 20,000g for 20 minutes prior to dilution with an equal volume of physiological saline and centrifugation at I20,000g for 5 hours at room temperature on an MSE Superspeed 75 ultra-centrifuge using an 8 x 25ml angle head rotor. Pellets were resuspended in I:20 volume 0.0I M Tris/HCl pH 7.4 containing 0.005 M EDTA and 0.5 percent (w/v) neutral protease, the latter to remove any masking antibody. Following incubation for I hour at 37°C and at 4°C overnight the protease mixture was layered onto a 30-64 percent w/v sucrose gradient (Appendix 2) and centrifuged at I00,000g for I8 hours at room temperature in a 3 x 20ml swing out rotor. The interface of the two sucrose concentrations was transferred to a 20ml preformed I.I to I.5 g/ml caesium chloride gradient (Appendix 3) and centrifuged at I20,000g for 24 hours at room temperature. One ml fractions were collected and their refractive index determined by a refractometer (Precision Instruments Ltd). Buoyant density was estimated from tables (Appendix 4). Fractions reactive for M2Ag by EIA were

pooled and dialysed against 0.I5 M PBS pH 7.2. Protein concentration was determined spectrophotometrically at 280nm (E_{280} = 35.7). The gradient purified concentrate was aliquoted and stored at -20°C in the presence of Img/ml sodium azide.

Preparation of anti-M2:alkaline phosphatase (M2Ab:AP) conjugate: Antibody to M2Ag (anti-M2) was obtained from the convalescent serum of a haemophilia B patient who had experienced an episode of non-A, non-B viral hepatitis 4 weeks after administration of a commercial Factor IX concentrate. Prior to this episode the patients serum contained both anti-HBs and anti-HAV, whose titres remained unaltered. The immunoglobulin fraction was obtained by addition of 50 percent saturated ammonium sulphate solution. One milligram of this immunoglobulin was conjugated to 5mg of alkaline phosphatase (Sigma Type VII S) using the one step glutaraldehyde method of Avramaes and Ternynck (I969 and Appendix 5). Most of the unconjugated enzyme was removed by precipitation of enzyme:antibody conjugate with an equal volume of saturated (4g in I0ml distilled water) sodium sulphate solution. The conjugate was resuspended in 2ml 0.05 M Tris/HCl pH 8.0 containing 0.00I M $MgCl_2$ and stored at 4°C. Titration of the conjugate revealed the optimum dilution for use to be I:I00 in physiological saline containing I percent bovine serum albumin (BSA) and 0.05 percent Tween 20 (saline - BSA - Tween).

Coating of the solid phase:

i). With anti-M2: 6.5mm diameter polystyrene beads

(Northumbria Biologicals) with a specular finish (to increase surface area) were coated by passive absorption with $(NH_4)_2\ SO_4$ precipitated anti-M2, at a concentration of I00µg/ml in 0.05 M carbonate buffer pH 9.6 (2 beads per ml), by mixing for 48 hours at 4°C, then stabilised with I percent BSA in coating solution at 37°C for I hour, dried and stored in an airtight container at 4°C.

ii). With antibody to human IgM: The method was essentially the same as described above, except that the coating antibody was a commercial (Seward Laboratories) sheep antibody, specific for the mu-chain of human IgM, and used at a dilution of I00µg/ml in coating buffer (2 beads per ml).

iii). With M2Ag: The procedure was essentially the same as above except that beads were coated with 500ng/ml of the gradient purified antigen, using 5 beads per ml of coating solution.

Assay protocols.

M2Ag:

Two hundred microlitres of test serum were incubated overnight at room temperature with anti-M2 coated beads in 25 well interlocking trays (Northumbria Biologicals), the wells being sealed to prevent evaporation. The beads were then washed 6 times with tap water pH 7.9 using a Pentawash gun and Filamatic dispenser (semi-automated bead washing equipment available from Abbott Laboratories) before 200µl anti-M2:AP conjugate was added to each well. The plates were sealed and incubated as before. Following

a further wash the beads were transferred to clean wells to avoid the possibility of false positive readings due to residual enzyme adsorbed non-specifically to the inner surface of each well. Bound enzyme activity was determined by the addition of 200µl substrate (Img/ml p-nitrophenyl-phosphate (Sigma Chemicals) in 0.05 M carbonate buffer pH 9.8). After 30 minutes incubation at room temperature the reaction was stopped by the addition of I00µl N NaOH. Absorbance was read at 4I0nm using a Dynatech ELISA mini-reader. Each assay plate contained 2 negative controls and I positive control. Test sera were presumed positive if their O.D. value exceeded twice that of the negative control mean.

Anti-M2 IgM:

The method is essentially the 'antibody capture' technique of Flehmig et al (I979) as modified by Mortimer et al (I98I) for the detection of anti-HAV IgM. Two hundred microlitres of test sera at a dilution of I:I0,000 (to overcome interference by rheumatoid factor) in saline-BSA-Tween were incubated with an anti-human IgM (µ-chain specific) bead for I hour at 45°C. The beads were washed as described previously and 200µl of M2Ag at a concentration of 50-I00 ng/ml protein was added and incubated for I hour at 45°C. After a further wash 200µl anti-M2:AP conjugate was added and incubated for I hour at 45°C. The beads were then re-washed and transferred to clean wells prior to the addition of substrate. Each assay contained two negative controls and one positive control. Sera were

presumed positive if the O.D. exceeded twice the negative control mean.

Anti-M2 IgG:

Two hundred microlitres of test sera at a dilution of I:I,000 in saline-BSA-Tween were incubated with an M2Ag coated bead for 3 hours at 45°C. After the washing procedure, 200µl of goat anti-human IgG:AP conjugate (Miles-Yeda) at a dilution of I:500 in saline-BSA-Tween was added to each bead and incubated for a further 2 hours at 45°C. The beads were washed as before and transferred to clean wells prior to the addition of substrate. Each assay contained two negative controls and I positive control. Sera were presumed positive if the O.D. value exceeded twice the mean of the 2 negative controls.

SUBSEQUENT MODIFICATIONS

Confirmation assay for M2Ag positive samples:

One hundred microlitres of test sera were incubated in duplicate with an equal volume of anti-M2 positive serum or an equal volume of serum negative for anti-M2 for I hour at 37°C. Anti-M2 coated beads were added to each well and incubated overnight at room temperature. After washing, 200µl of anti-M2:AP conjugate was added, followed by a further overnight incubation at room temperature. The beads were washed as before and transferred to clean wells prior to the addition of substrate. The presence of M2Ag was considered to be confirmed if the O.D. of the test well showed 50 percent inhibition compared with that of the control well.

Detection of complexes of M2Ag and anti-M2 IgM:
Selected sera, reactive for both M2Ag and anti-M2 IgM were tested for the presence of immune complexes at a dilution of I:I0,000 in saline-BSA-Tween. The method was essentially the 'anti-mu' technique described earlier, with the omission of incubation with gradient purified M2Ag. Sera were considered positive when the O.D. value exceeded twice the mean of the two negative controls.

Reagent supply and purification:
A hyperimmune rabbit serum (for details of preparation see Part 3, Section 2) was used as a source of coating antibody for the M2Ag assay and in confirmation of positive sera. Although this did not significantly improve the intra or inter-assay c.v.'s, it eased the demand for antibody and (theoretically) improved specificity by replacing the human-human reagent system with a human-rabbit combination. One percent normal rabbit serum was included in the conjugate diluent to avoid non-specific human-rabbit cross reactions.

Normal serum immune globulin (ϑ 28I) with a high titre of anti-M2 IgG was also used as a coating antibody at a concentration of 500µg/ml. This improved both the intra and inter-assay c.v.'s (Table 2.2a) and enhanced sensitivity by lowering the O.D. of the negative controls while increasing the O.D. of the positive control sera.

Preparation of an M2Ag affinity column (Appendix 6)

TABLE 2.2a

IMPROVEMENTS IN COEFFICIENT OF VARIATION FOR EACH ASSAY SYSTEM

	ENZYMEIMMUNOASSAYS FOR DETECTION OF:		
	M2Ag	Anti-M2 IgM	Anti-M2 IgG
INTERASSAY CV*			
Original	23%	26.5%	39.0%
Current	17.0%	20.2%	14.0%
INTRAASSAY CV**			
Original	41.0%	47.0%	32.0%
Current	18.0%	10.0%	8.0%

* Based on testing the 'in-house' positive control on 50 separate occasions.

** Based on testing 25 replicates of the 'in-house' positive control.

allowed the use of affinity purified antibody (ϑ 28I) for bead coating and use in the confirmatory neutralisation assays. Storage of beads in the stabilising solution at 4°C increased their shelf-life from approximately 2 weeks to 6 weeks.

Addition of 5 percent normal human serum (NHS) to the conjugate diluent further reduced background noise and allowed the anti-M2:AP conjugate to be used at a dilution of I:200 in assays for M2Ag and anti-M2 IgM.

Increasing the final wash to I0 times circumvented the need to transfer the beads to clean wells prior to the addition of substrate.

CURRENT ASSAY PROTOCOLS.

M2Ag:

Two hundred microlitres of test serum are incubated overnight at room temperature with anti-M2 (ϑ 28I) coated beads (500µg/ml) in 25 well interlocking trays (Northumbria Biologicals) sealed to prevent evaporation. The beads were then washed 6 times with tap water (pH 7.9) using a Pentawash gun and Filamatic dispenser, and 200ul anti-M2:AP conjugate at a dilution of I:200 in saline-Tween-BSA containing 5 percent NHS was added to each well and the plates sealed and incubated as before. The beads were then washed I0 times with tap water and bound enzyme activity was determined by the addition of 200ul substrate (Img/ml p-nitrophenyl phosphate in 0.05 M carbonate

buffer pH 9.8). After 30 minutes incubation at room temperature the reaction was stopped by the addition of I00µl NaOH. Absorbance was read at 4I0nm using a Dynatech ELISA minireader. Each assay plate contained two negative controls and one positive control. Test sera are considered positive when their O.D. value exceeded twice that of the negative control mean.

Anti-M2 IgM:

The assay is identical to that described earlier except that the anti-M2:AP conjugate is used at a dilution of I:200.

Anti-M2 IgG:

The assay is identical to that described earlier except that the beads were coated at a concentration of 500µg/ml.

2.3 CHARACTERISTICS OF THE ENZYME IMMUNOASSAYS

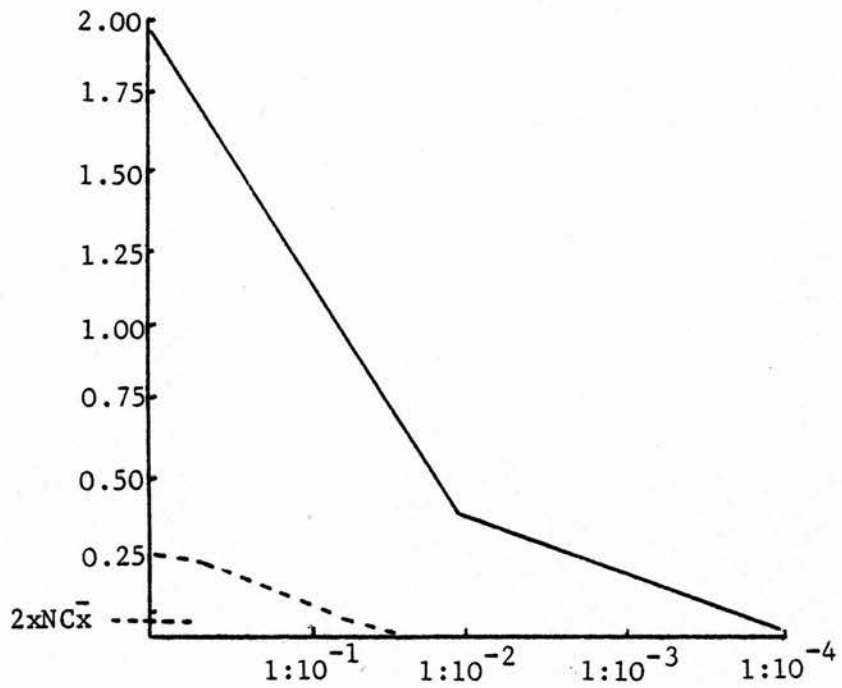
Figures 2.3i, 2.3ii and 2.3iii represent typical titration curves obtained for M2Ag, anti-M2 IgM and anti-M2 IgG respectively. A strongly reactive M2Ag (T:N = 10-50+) will titrate up to approximately 1:10,000 whereas a weak M2Ag (T:N = 2-10) titrates to approximately 1:64. A similar situation is observed with anti-M2 IgM and anti-M2 IgG where strongly reactive samples will still be reactive at a dilution of 1:100,000. It may be feasible to employ these curves for quantitation purposes.

Figures 2.3iv, 2.3v and 2.3vii illustrate the range of values obtained when 100 replicates of the appropriate negative controls and 10 replicates of appropriate positive controls were assayed. In each case little variation was observed among negative controls while the positive controls for anti-M2 IgM and anti-M2 IgG show a greater variation, a phenomenon observed with other well established RIA's or EIA's (Appendix 7).

Figures 2.3vii, 2.3viii and 2.3ix demonstrate a narrowing of the spread of O.D. values obtained when 100 random blood donor sera were tested by the current assay protocols and compared with values obtained from blood donor screening two years previously. Although improvement was evident in all three assays, it was most noticeable in the test for M2Ag. Based upon the most recent population spread information a cutoff value of twice the negative control

FIGURE 2.3i

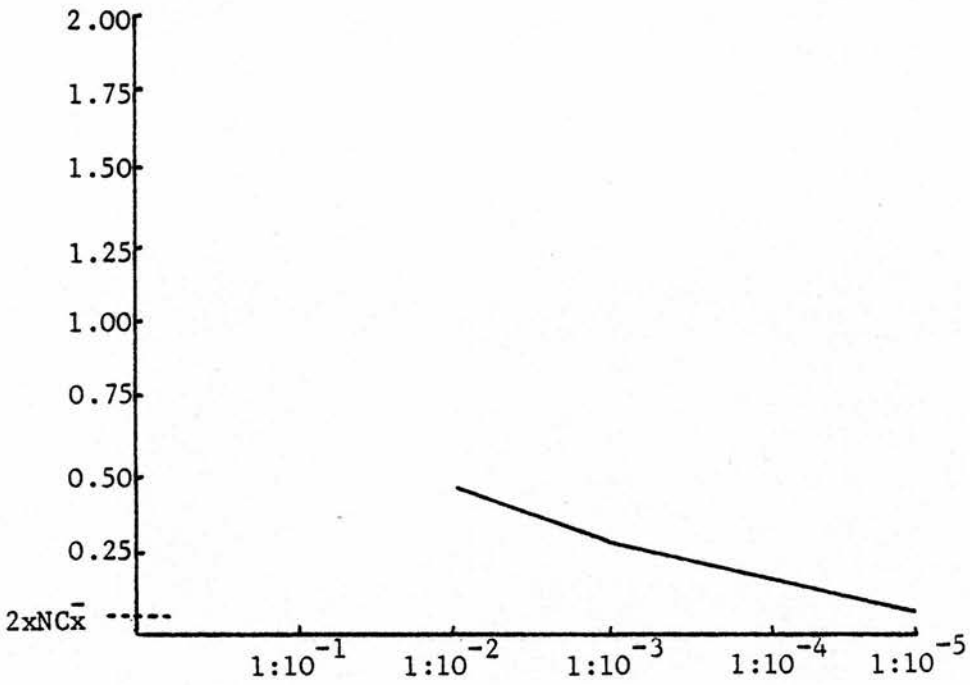
TITRATION CURVES OF M2Ag USING A STRONGLY REACTIVE FACTOR VIII AND A WEAK POSITIVE SERUM.



NC$\bar{x}$ - NEGATIVE CONTROL MEAN

FIGURE 2.3ii

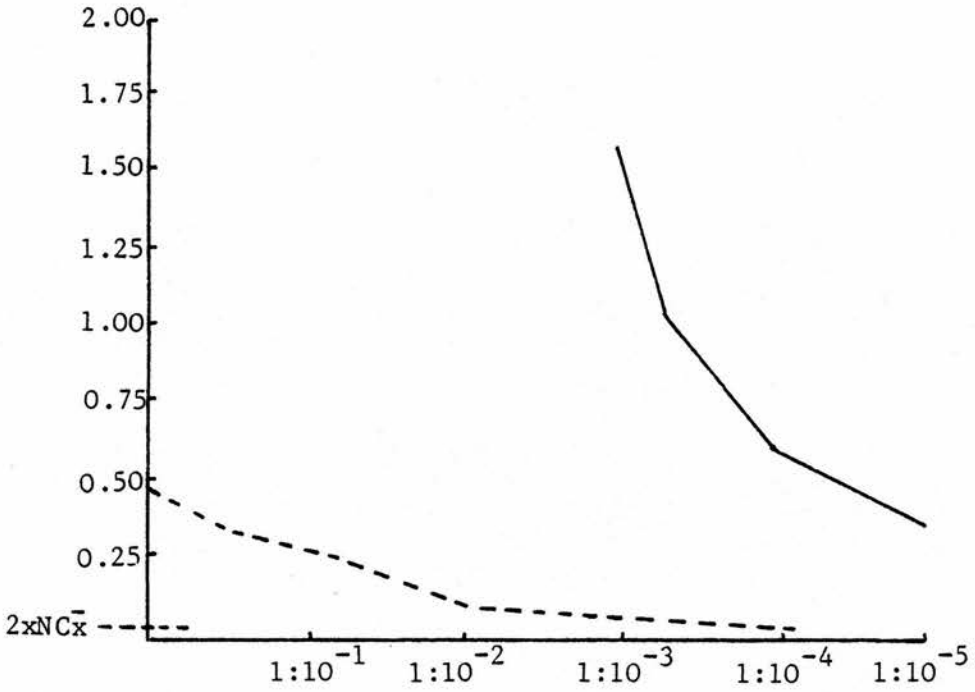
TITRATION CURVE OF ANTI-M21gM USING A RHEUMATOID FACTOR NEGATIVE ANTI-M21gM POSITIVE SERUM.



NC$\bar{x}$ - NEGATIVE CONTROL MEAN

FIGURE 2.3iii

TITRATION CURVES OF ANTI-M21gG USING NORMAL SERUM IMMUNE GLOBULIN AND AFFINITY PURIFIED ANTI-M21gG



- - - - AFFINITY PURIFIED ANTI-M21gG

——— NORMAL IMMUNE SERUM GLOBULIN WITH HIGH LEVEL OF ANTI-M2 ACTIVITY.

NC$\bar{x}$ - NEGATIVE CONTROL MEAN

FIGURE 2.3iv

HISTOGRAM SHOWING O.D. VALUES OBTAINED FOR 100 REPLICATES OF A M2Ag NEGATIVE SERUM AND 10 REPLICATES OF A M2Ag POSITIVE SERUM.

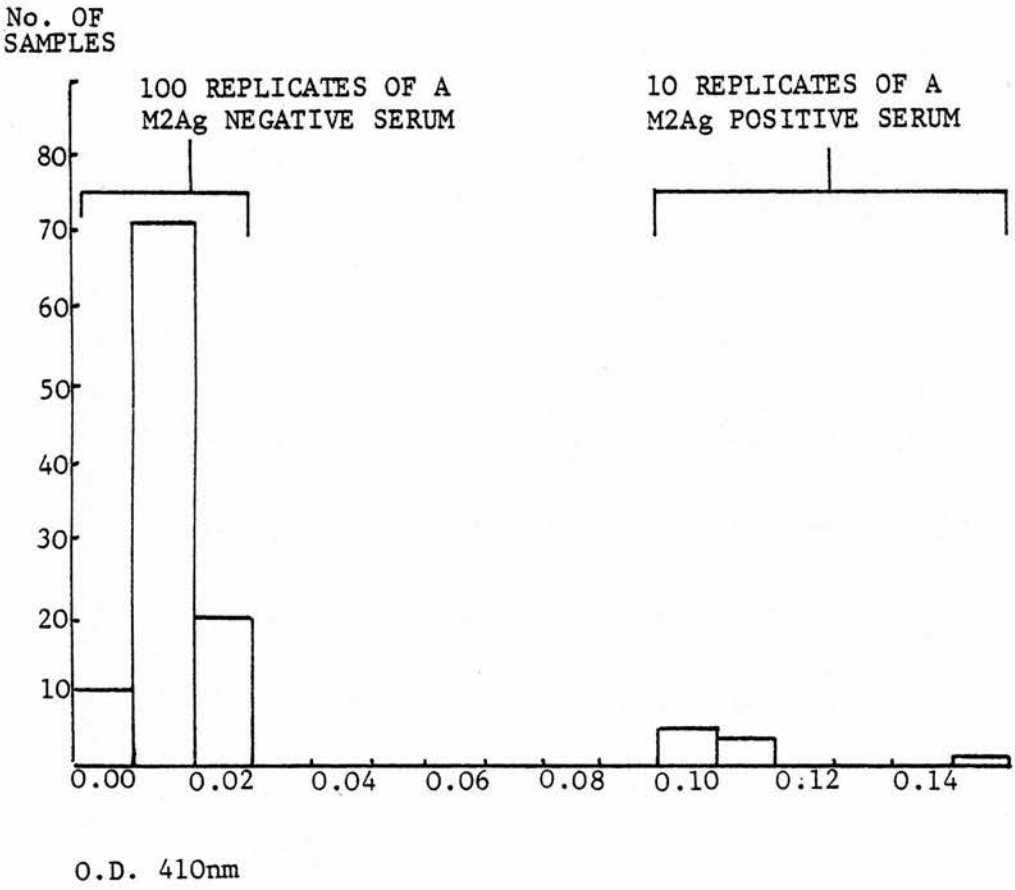


FIGURE 2.3v

HISTOGRAM SHOWING O.D. VALUES OBTAINED FOR 100 REPLICATES OF AN ANTI-M21gM NEGATIVE SERUM AND 10 REPLICATES OF AN ANTI-M21gM POSITIVE SERUM.

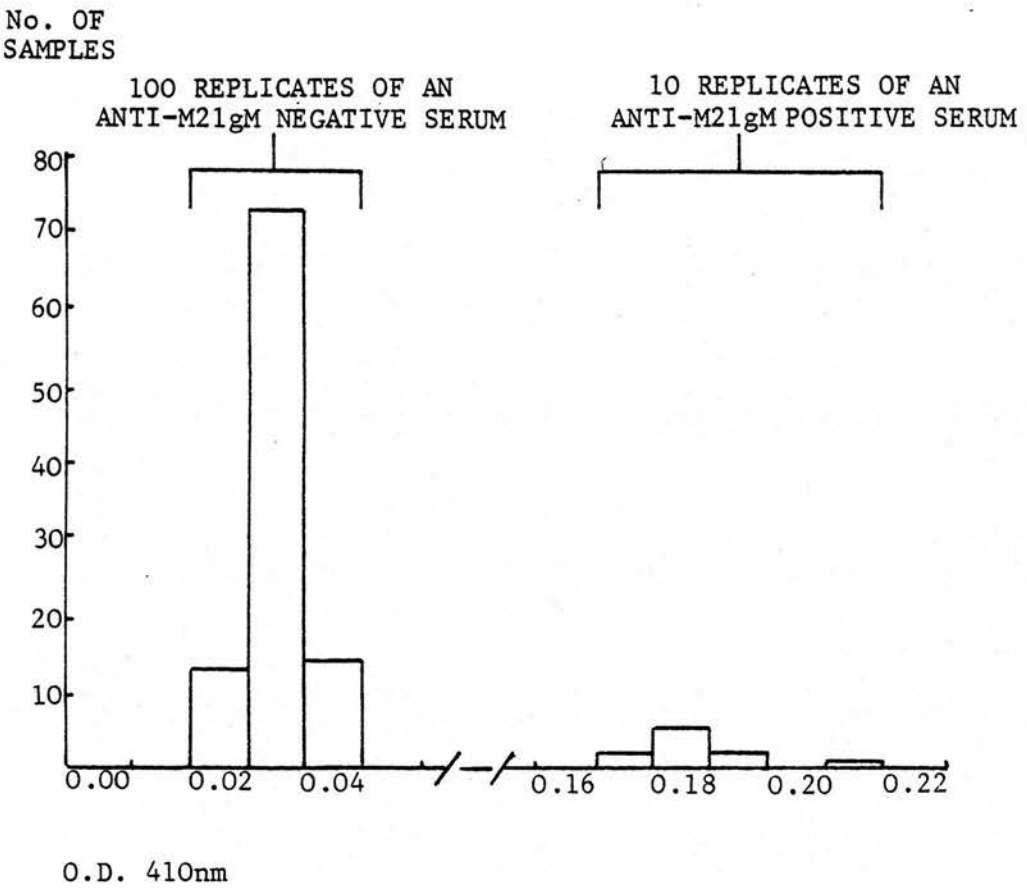


FIGURE 2.3vi

HISTOGRAM SHOWING O.D. VALUES OBTAINED FOR 100 REPLICATES OF AN ANTI-M21gG NEGATIVE SERUM AND 10 REPLICATES OF AN ANTI-M21gG POSITIVE SERUM.

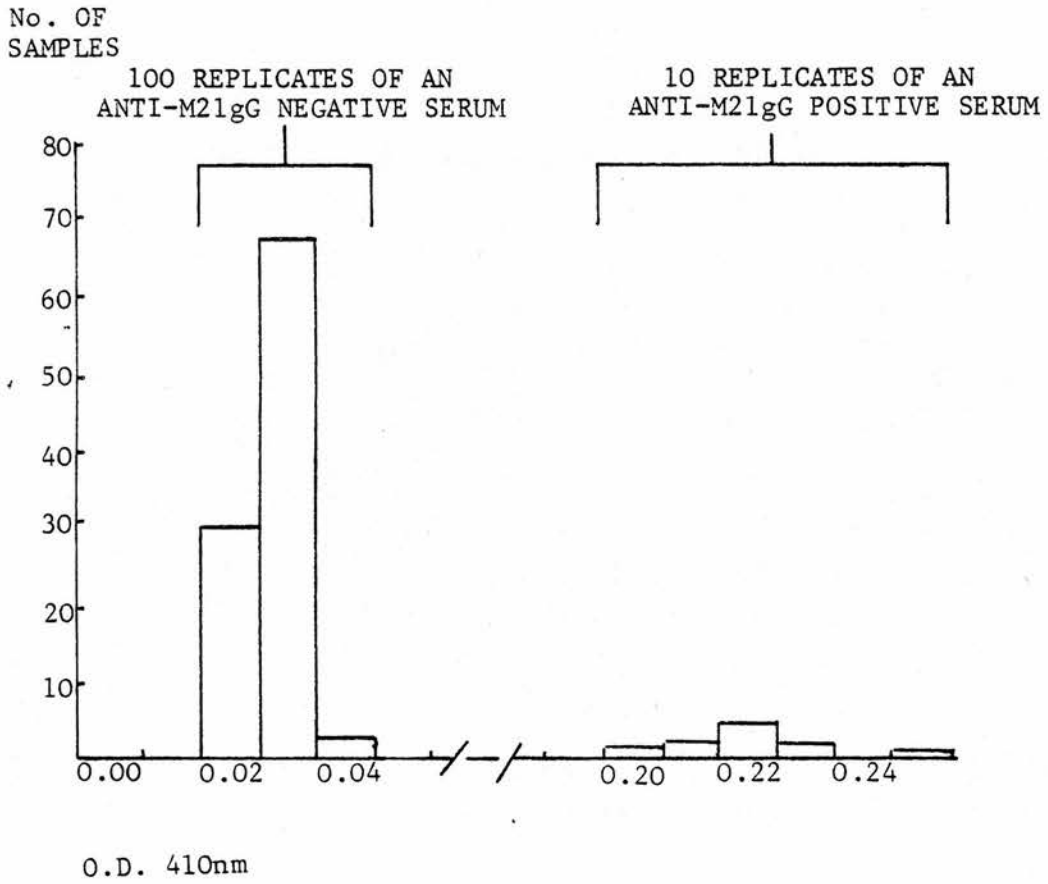


FIGURE 2.3vii

M2Ag E.I.A. : COMPARISON OF O.D. RANGE OBTAINED BY TESTING SERA FROM 100 BLOOD DONORS WITH THE CURRENT AND ORIGINAL E.I.A.

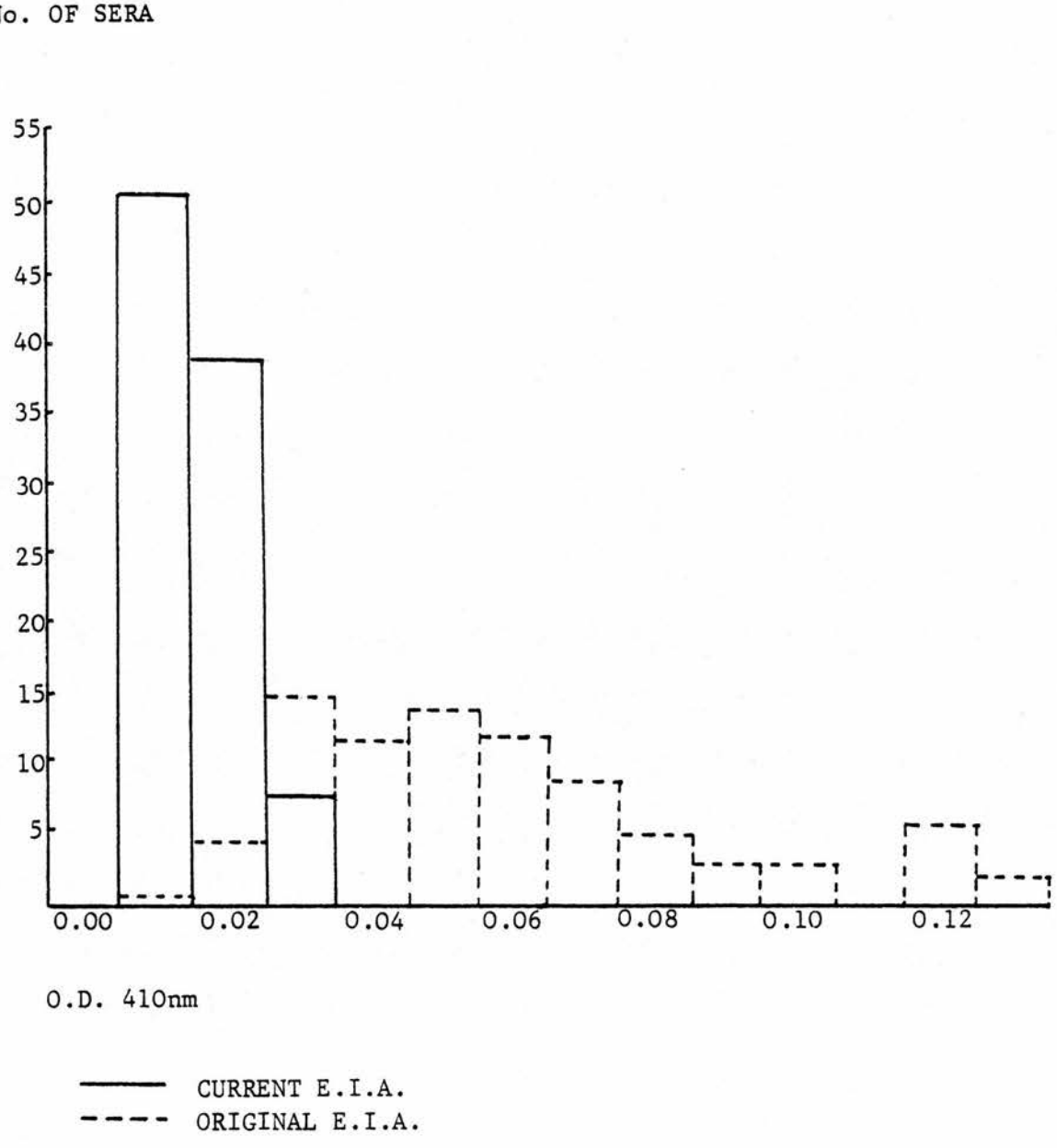


FIGURE 2.3viii

ANTI-M21gM E.I.A. : COMPARISON OF O.D. RANGE OBTAINED BY TESTING SERA FROM 100 BLOOD DONORS WITH THE CURRENT AND ORIGINAL E.I.A.

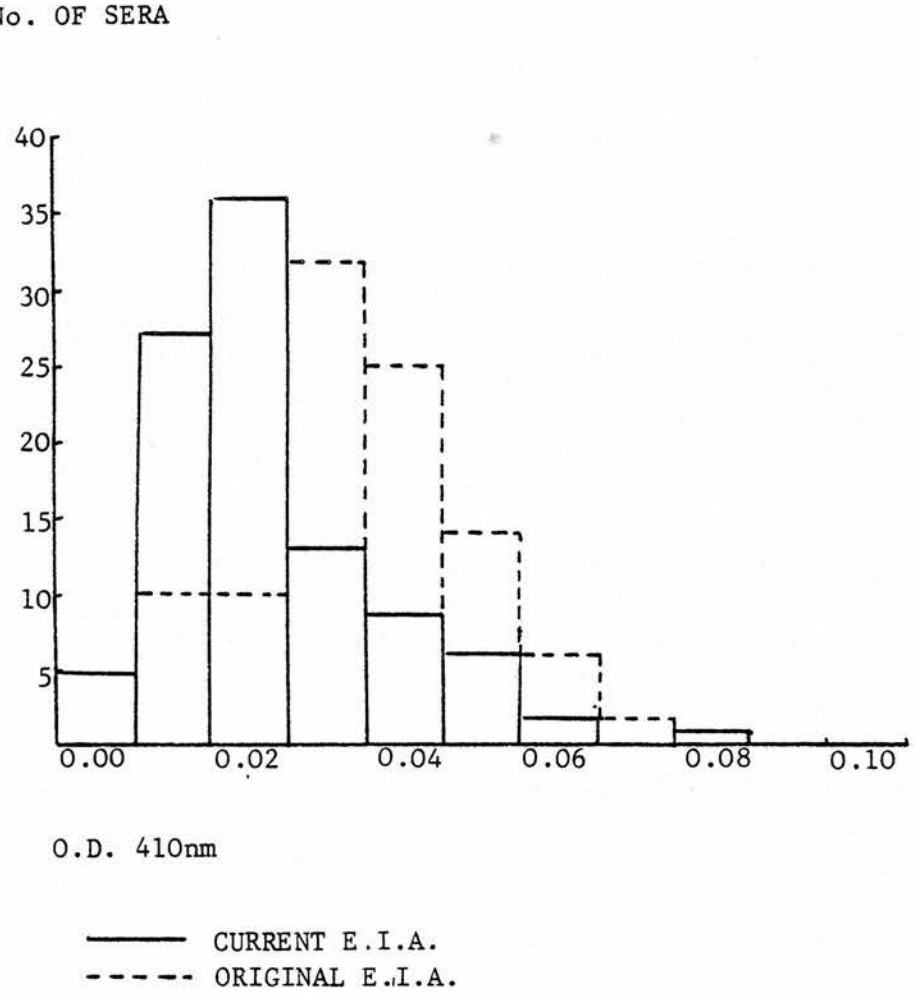
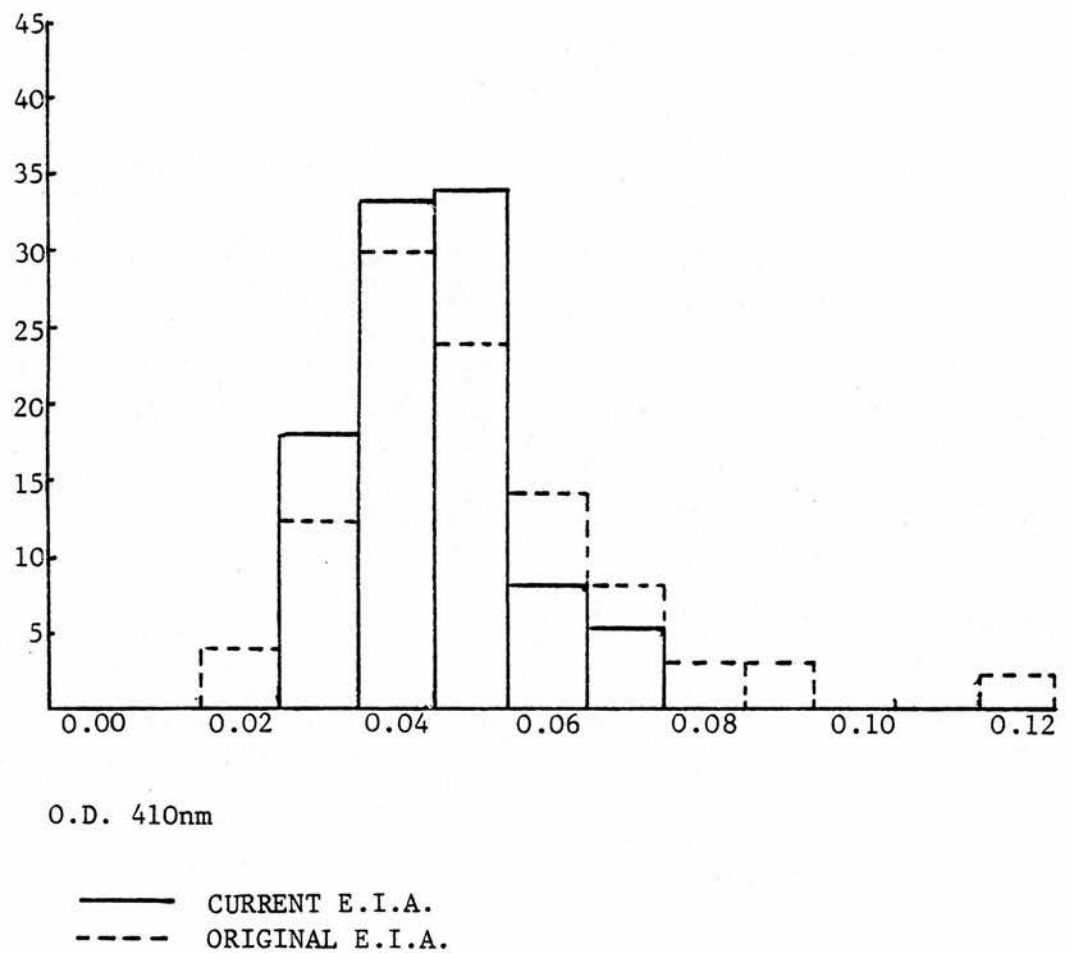


FIGURE 2.3ix

ANTI-M21gG E.I.A. : COMPARISON OF O.D. RANGE OBTAINED BY TESTING SERA FROM 100 BLOOD DONORS WITH THE CURRENT AND ORIGINAL E.I.A.



mean seems a practical criteria for presumption of positivity. Statistical analysis has revealed that sera giving this value or greater are behaving in a significantly different manner. For example if the negative control mean (NC $\bar{x}$) is 0.023 then the negative control mean plus 5 standard deviations is 0.0458 making test sera with an O.D. value of greater than 0.04 significantly different from the rest of the population ($p > 0.001$).

2.4 SPECIFICITY OF THE ENZYME IMMUNOASSAYS FOR NON-A, NON-B VIRAL HEPATITIS.

Having established the existence of a relatively low prevalence of M2 markers in local blood donors (approximately 2 percent of each marker) during the initial phase of assay development the next step was to compare these 'background' findings with the occurrence of M2 markers likely to indicate the specificity of M2 markers for non-A, non-B viral hepatitis.

CODED PANELS.

Panels examined were (I) the second NIH* panel, kindly provided by Dr. H. Alter, and (2) a panel of chimpanzee sera, kindly provided by Dr. D. Bradley, C.D.C. **. Both panels were supplied and tested under code. The NIH panel was obtained from cardiac surgery patients who developed non-B hepatitis 8 weeks or more after transfusion ie. 'long incubation' disease. The C.D.C. panel was composed of chimpanzee sera representing normal, acute HAV, acute HBV, acute and chronic non-A, non-B infected animals. Prior to testing for M2Ag, the chimpanzee panel was tested for anti-human activity, using normal human serum coated latex beads. These sera that were reactive were then incubated with normal human serum coated latex beads to remove any anti-human activity. Due to the small sample size sera could only be tested for M2Ag. The N.I.H. panel was tested for all M2 markers.

* National Institute of Health, Bethseda, Maryland, U.S.A.

** Centre for Disease Control, Atlanta, Georgia, U.S.A.

Results of tests for both panels of sera are shown in Tables 2.4a and 2.4b. The completely negative findings in the N.I.H. panel emphasize the lack of non-specificity of the assays. The panel design selected only cases of post-transfusion hepatitis with a long (8-I2 week) incubation period (Alter, H., I983 personal communication). It is worth recalling that M2Ag was isolated from a donor twice implicated in the transmission of 'short (3-7 week) incubation' form of non-A, non-B hepatitis. M2Ag was detected in 2 chimpanzees who were given the same inoculum (containing the tubule forming non-A, non-B hepatitis agent) but was absent in chimpanzees infected with HAV, HBV and in uninfected controls. Of interest is the finding of M2Ag in the serum at or near the peak of ALT. This probably reflects a situation in which liver disruption is at its greatest and therefore release of antigen into the serum is at its maximum. However, M2Ag does not appear to be a component of the normal chimpanzee liver as there are no markers present when serum ALT is elevated due to HAV or HBV infection.

ACUTE PHASE HAV AND HBV SERA.

Ten acute phase sera positive for anti-HAV IgM and I0 acute phase sera positive for HBsAg and anti-HBc IgM were kindly provided by Dr. E.A.C. Follett (Hepatitis Laboratories, Ruchill Hospital, Glasgow). All were negative when tested for the presence of M2-related serological markers.

TABLE 2.4a

THE SECOND N.I.H. PANEL : O.D. VALUES FOR M2 MARKERS

CODE	M2Ag	ANTI-M2IgM	ANTI-M2IgG
1	0.03	0.02	0.05
2	0.03	0.02	0.04
3	0.04	0.01	0.04
4	0.03	0.01	0.04
5	0.03	0.05	0.05
6	0.03	0.04	0.06
7	0.01	0.04	0.05
8	0.04	0.03	0.05
9	0.02	0.04	0.04
10	0.02	0.02	0.03
11	0.02	0.02	0.05
12	0.02	0.04	0.05
13	0.06	0.03	0.03
14	0.03	0.03	0.05
15	0.04	0.05	0.05
16	0.04	0.02	0.05
17	0.04	0.02	0.03
18	0.01	0.03	0.04
19	0.03	0.01	0.04
20	0.03	0.03	0.04
21	0.02	0.03	0.02
22	0.01	0.02	0.06
23	0.05	0.02	0.06
24	0.02	0.02	0.06
25	0.02	0.05	0.03
26	0.01	0.03	0.03
27	0.02	0.03	0.04
28	0.03	0.03	0.04
29	0.03	0.03	0.04
30	0.01	0.03	0.05
31	0.05	0.03	0.04
32	0.05	0.03	0.05
33	0.05	0.03	0.03
34	0.05	0.03	0.05
35	0.05	0.03	0.04
36	0.05	0.04	0.03
37	0.03	0.01	0.03
38	0.06	0.03	0.05
39	0.03	0.04	0.03
40	0.03	0.03	0.02
41	0.03	0.03	0.04
42	0.03	0.04	0.05
43	0.05	0.02	0.05
44	0.03	0.04	0.04
'In House' control			
-	0.04	0.03	0.05
-	0.05	0.03	0.05
-	0.04	0.04	0.06
+	0.19	0.16	0.43
+	0.21	0.15	0.39

Sera were considered positive if the O.D. values were greater than: (1) M2Ag 0.05;

(2) Anti M2IgM 0.04;

(3) Anti M2IgG 0.06.

TABLE 2.4b

RESULTS OF TESTING THE CODED PANEL OF CHIMPANZEE SERA PROVIDED BY DR. D. BRADLEY (C.D.C.).

Name & Date of serum	Innoculum	Illness & day post-innoculation	ALT (mu/ml)	M2Ag O.D.(410nm)
CAM 4.5.82	None	Normal	12	0.01
JULIE 14.10.82	None	Normal	7	0.01
LORIE 3.1.80	None	Normal	12	0.01
LYN 24.8.82	None	Normal	8	0.05
VIN 26.8.82	None	Normal	7	0.01
BIL 6.11.78	HAV	Acute HAV	47	0.01
NATE 2.11.78	HAV	Acute HAV	187	0.01
SPARKEY 21.12.80	HAV	Acute HAV	128	0.01
BROWN 3.12.81	HBV	Acute HBV	11	0.01
LORI 15.7.81	HBV	Acute HBV	43	0.01
AJ 23.2.82]		Acute NANB 18	8	0.02
AJ 23.4.82]	Chronic-	Acute NANB 76	15	0.19
AJ 18.5.82]	phase chimp	Acute NANB 101	48	0.29
HAL 2.2.82]	NANB plasma	Acute NANB 47	22	0.01
HAL 19.2.82]		Acute NANB 63	78 (peak)	0.15
HAL 23.2.82]		Acute NANB 67	71	0.27
JACKIE 16.3.78]	FVIII (lot C)	Acute NANB 28	10	0.01
JACKIE 12.4.78]		Acute NANB 55	71 (peak)	0.01
SPARKEY 21.4.82]	Acute phase	Acute NANB 7	13	0.01
SPARKEY 23.4.82]	chimp NANB	Acute NANB 9	60	0.01
SPARKEY 4.5.82]	liver	Acute NANB 18	24	0.01
]	homogenate			
DASH 28.9.82]	FVIII (lot C)	Chronic NANB	5	0.03
DAREL 22.3.82]	Chronic-phase	Chronic NANB	8	0.05
]	chimp NANB			
]	plasma			
DON 20.11.81]	FVIII (lot A)	Chronic NANB	35	0.04
DON 23.4.82]		Chronic NANB	20	0.01
RAY 3.2.82]	FVIII (lot C)	Chronic NANB	10	0.01
RON 19.3.79]	CsCl fraction	Chronic NANB	12	0.01
RON 26.3.79]	of chimp acute	Chronic NANB	20	0.02
RON 29.3.79]	NANB liver	Chronic NANB	11	0.05
RODNEY]	Chronic-phase chimp NANB plasma	Chronic NANB	-	0.01
'In-house control' Normal chimp sera				
1	None			0.05
2	None			0.03
3	None			0.05
4	None			0.05
5	None			

Sera were considered positive if the O.D. value exceeded 0.05.

RETROSPECTIVE INVESTIGATION OF NON-B POST TRANSFUSION HEPATITIS CASES.

The application of sensitive 'third' generation assays for screening blood donors for HBsAg has led to the majority of post-transfusion viral hepatitis cases falling into the non-A, non-B viral hepatitis category. During the course of this thesis 7 cases of post-transfusion non-A, non-B hepatitis were studied. Only HAV (HAVAB-M, Abbott Laboratories) HBV (HBsAg by RIA, Kane et al I983, anti-HBc IgM by EIA, Field I983), CMV (complement fixation test, Cremner et al I975) and EBV (by immuno fluorescence, Edwards I982) negative hepatitis diagnosed as being of a viral aetiology in which stored serum from all the original implicated donations were available were included. All patients had received isolated transfusion therapy and none had experienced obvious parenteral exposure to hepatitis viruses during the preceeding six months. Case histories are presented below. Table 2.4c summarises information relating to exposure and incubation period for each of the 7 patients studied.

Patient S.H.

Patient S.H. is a 35 year old anti-Rh (D) plasmapheresis donor whose serum antibody level was boosted by infusion of appropriate cells from a single donor on I0.3.80. Liver function was normal prior to and on the day of transfusion. Four weeks later S.H. was found to have raised serum ALT levels which exhibited a reducing multi-phasic pattern over the ensuing months. Further

TABLE 2.4c

EXPOSURE TO DONATED BLOOD, APPROXIMATE INCUBATION PERIOD AND M2 INVOLVEMENT IN SEVEN PATIENTS WITH NON-A, NON-B HEPATITIS FOLLOWING BLOOD TRANSFUSION.

Patient	Number of donations transfused	Incubation period (days)	M2 Markers in donation	M2 markers in recipient
SH	1	28	IgM	Yes
MH	4	68 (J)	None	No
PP	8	17-28 (J)	IgM	Yes
HR	16	21 (J)	Ag+IgM	Yes
EA	2	47 (J)	Ag	Yes
AT	3	25 (J)	Ag	Yes
HG	2	31 (J)	Ag	No*

*Single blood specimen taken 11 days after appearance of clinical symptoms.

(J) = Jaundiced.

monitoring revealed that having returned to normal by week 65 at least two subsequent albeit milder ALT elevation occurred at weeks 78 and I76 after transfusion. The patient was never jaundiced and only complained of feeling unwell at week 35 coincidental with the third peak of ALT elevation.

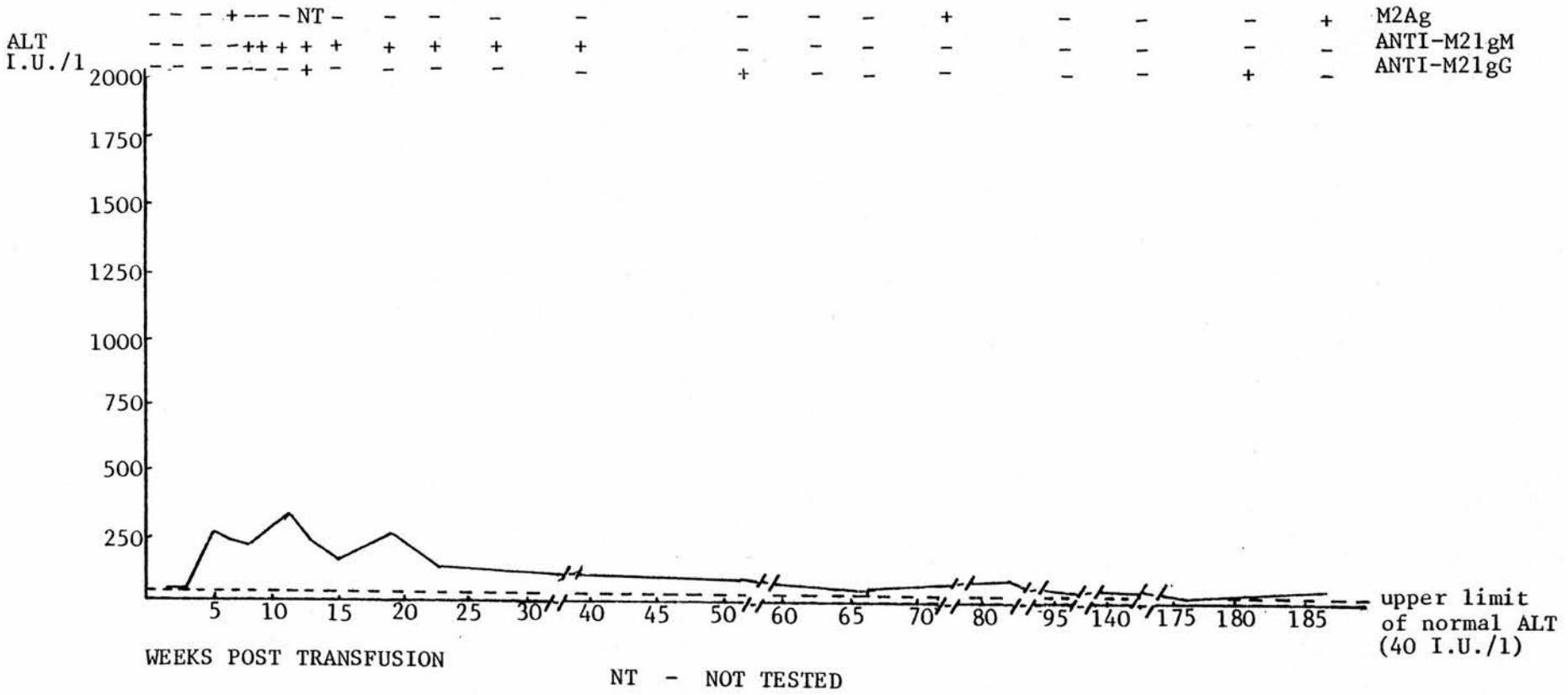
Although serum from the implicated donation was available the donor subsequently moved and was lost to follow-up.

Stored serum from a blood sample taken on the day of infusion revealed the absence of any detectable markers associated with the M2 system. Figure 2.4i illustrates the course of serum ALT and M2 markers. Although M2Ag was detected at the observed peak of serum ALT, M2Ag:IgM complexes were present between weeks I0-I5 followed by loss of antigen at week I7 and the transient appearance of anti-M2 IgG at 20 weeks after transfusion. By week 25 no M2 markers were present and ALT had dropped to just above the upper limit of normal. At week 27 anti-M2 IgM had reappeared, overlapping a reappearance of anti-M2 IgG. However, M2Ag was detected at week 82 coincident with a minor peak of ALT. Specific IgG was found to be present at week 52 (ALT 88 IU/l) and at week 177 (ALT 24 IU/l). In view of this recrudescence of liver dysfunction a further blood sample was obtained on week I87 when ALT had returned to normal but M2Ag was once again detectable in the serum.

FIGURE 2.4i

M2 MARKERS AND SERUM ALT VALUES FOR PATIENT S.H.

WEEKS POST TRANSFUSION	0	1	3	5	7	8	9	11	13	15	19	23	27	39	52	62	66	82	97	142	177	187
ALT I.U./l	50	39	42	268	237	218	N.T.	310	211	151	252	122	109	92	88	N.T	47	68	45	55	24	42
M2Ag	-	-	-	-	+	-	-	-	N.T.	-	-	-	-	-	-	-	-	+	-	-	-	+
ANTI-M21gM	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
ANTI-M21gG	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-



NT - NOT TESTED

Although there was no evidence of current infection with HAV or HBV, the implicated donor did possess specific IgG to both markers, in addition to anti-M2 IgM and a slightly raised ALT.

Patient M.H.

Patient M.H. is a 49 year old woman who underwent a hysterectomy on 23.I0.80 following diagnosis of cervical carcinoma. She was transfused with 4 units of blood. On 30.I2.80 she presented with jaundice, dark urine and nausea. Over I6 months of follow-up (Figure 2.4ii) during which 20 specimens of blood were taken, her jaundice subsided but serum ALT fluctuated peaking at 830 IU/l in January I98I and 9I2 IU/l in July I98I. A liver biopsy taken in August I98I showed 'chronic hepatitis of uncertain aetiology'. From January I982 onwards ALT levels returned to normal and a further biopsy in October I982 revealed a normal liver architecture with normal histology and no evidence of cirrhosis. M.H. failed to exhibit any detectable evidence of M2 involvement. All stored serum specimens from the implicated donations were also unreactive for M2 markers.

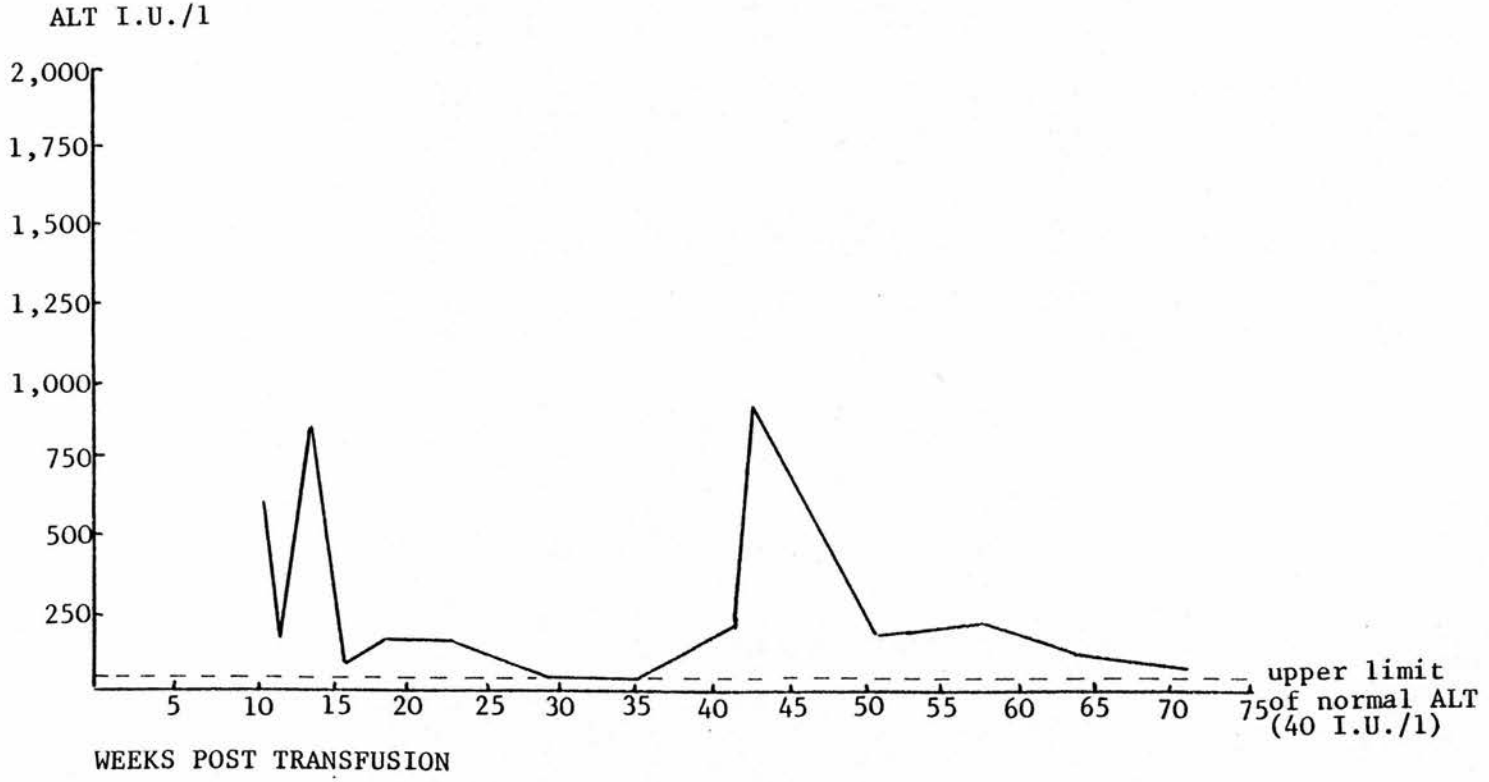
Patient P.P.

Patient P.P. is a 3I year old airforce navigator who received blood from a total of 8 donors between 9.5.8I and 20.5.8I as a consequence of surgery relating to duodenal ulceration. Four weeks after the first transf-

FIGURE 2.4ii

M2 MARKERS AND SERUM ALT VALUES FOR PATIENT M.H.

WEEKS POST TRANSFUSION	11	12	14	15	16	18	23	25	30	36	42	43	51	57	63	72
ALT I.U./l	600	167	830	415	96	124	124	105	22	51	235	912	162	218	111	60
M2Ag	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ANTI-M2lgM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ANTI-M2lgG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-



usion he presented with jaundice and raised ALT, the latter persisting for at least 4 months at which point he was lost to follow-up as a result of being transferred overseas . Although jaundice developed 28 days after transfusion, the patient only came to my attention when abnormal liver function persisted. A blood specimen taken I26 days after transfusion was positive for M2Ag. Stored serum from all implicated donations were tested for M2 markers. Among 4 units transfused on 9.5.8I one was positive for anti-M2 IgM. (The reactive serum came from a I9 year old 'first-time' male donor who did not respond to a subsequent request for a further blood specimen). Three days later, P.P. received two further blood units, one of which was also reactive for anti-M2 IgM. (This donor, a 25 year old male had donated on 6 previous occasions and a repeat specimen 7 months later was positive for anti-M2 IgM). The two remaining units transfused on 20.5.8I were unreactive for M2 markers.

Patient H.R.

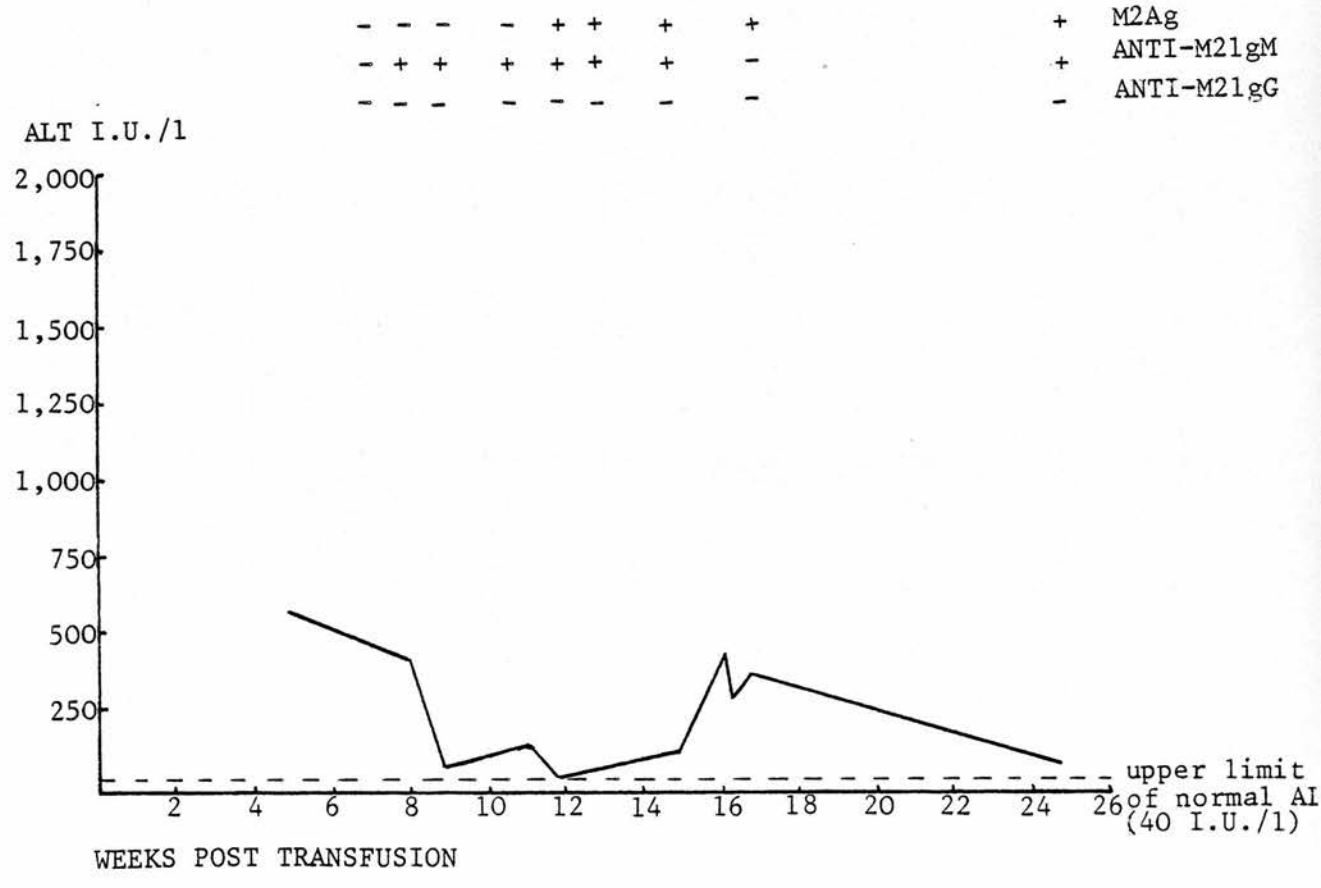
Patient H.R.is a 50 year old woman who underwent cardiac by-pass surgery on 3I.3.82, being exposed to red cells and platelet concentrate from a total of I6 donors. Although the patient became jaundiced within a few hours of surgery, 3 weeks later she developed nausea, anorexia, pruritis, dark urine and pale stools and was readmitted on 7.5.82 with a bilirubin of 63 μmol/l and an ALT of 565 IU/l. Figure 2.4iii illustrates the course of ALT and M2 markers. A blood sample taken on readmission,

FIGURE 2.4iii

M2 MARKERS AND SERUM ALT VALUES FOR PATIENT H.R.

WEEKS POST TRANSFUSION	5	7	8	9	11	12	13	15	16	16	17	25
ALT I.U./l	565	NT	466	67	165	20	50	134	354	289	366	78
M2Ag	NT	-	-	-	-	+	+	+	NT	NT	+	+
ANTI-M21gM	NT	-	+	+	+	+	+	+	NT	NT	-	+
ANTI-M21gG	NT	-	-	-	-	-	-	-	NT	NT	-	-

NT - NOT TESTED



37 days following transfusion and I6 days after the appearance of symptoms suggestive of viral hepatitis showed no evidence of M2 markers. However, subsequent bleeds revealed the development of anti-M2 IgM from 27.5.82 until 22.6.82, disappearing coincident with the apparent return to normal of serum ALT level. Within a month ALT had risen once again and M2Ag could be detected in the patient's serum.

Two of the sixteen implicated blood donors were reactive for M2 markers, one for M2Ag and the other for anti-M2 IgM.

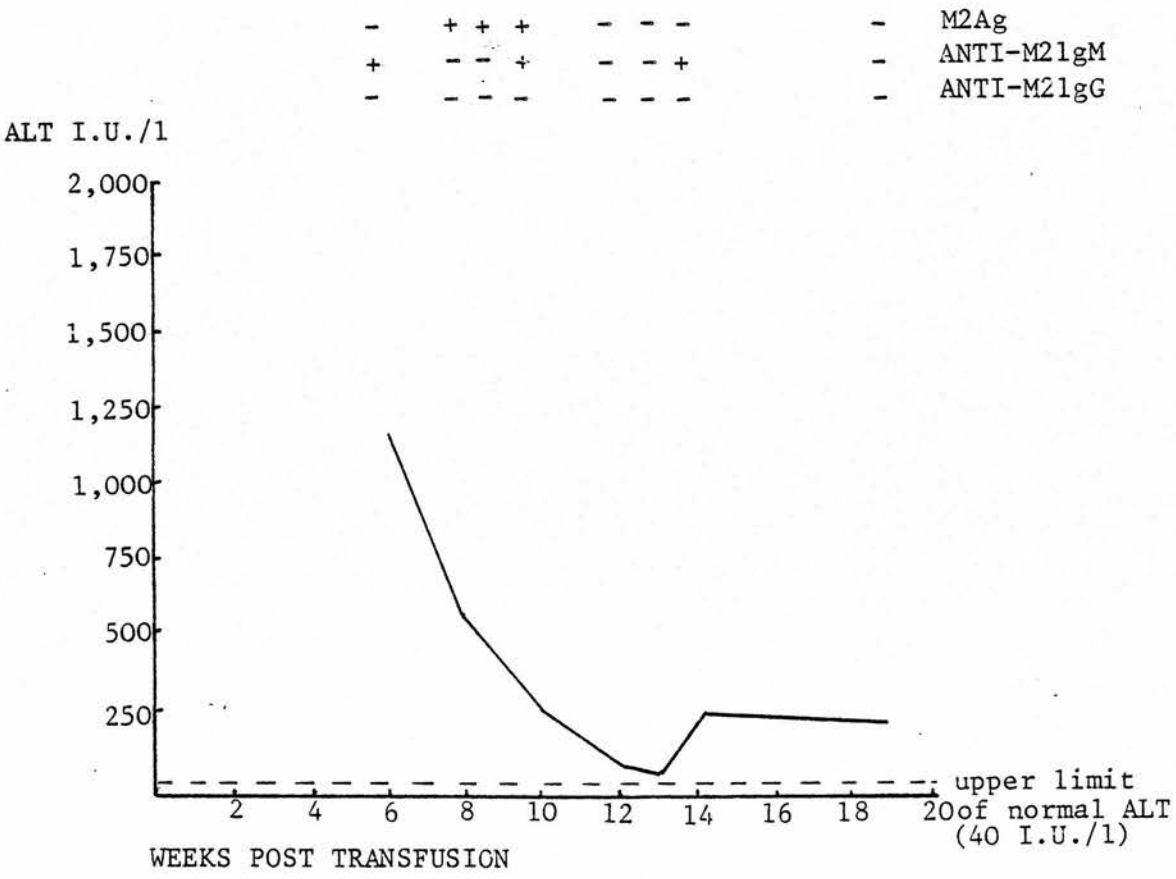
Patient E.A.

Patient E.A. is a 75 year old female polycythaemic who received 2 units of blood on 22.I2.82. She had no known exposure to blood or blood products for a period of years prior to this transfusion, and her serum ALT level was within I0-20 IU/l during the 2 months prior to this episode. Between 6 and 7 weeks after transfusion E.A. presented with jaundice and an ALT of II95 IU/l. A high level of anti-M2 IgM was observed in this serum. (Figure 2.4iv). Follow-up sera obtained on 7.2.82 and 3.3.82 were positive for M2Ag, the latter also possessing detectable anti-M2 IgM inferring the presence of immune complexes. No further samples were available for study until weeks 55 and 57 after transfusion, when ALT values were found to be I49 and 80 IU/l respectively, although no M2 related markers were detectable.

FIGURE 2.4iv

M2 MARKERS AND SERUM ALT VALUES FOR PATIENT E.A.

WEEKS POST TRANSFUSION	7	8	9	10	12	13	14	19
ALT I.U./1	1195	560	>500	249	77	49	225	209
M2Ag	-	+	+	+	-	-	-	-
ANTI-M21gM	+	-	-	+	-	-	+	-
ANTI-M21gG	-	-	-	-	-	-	-	-



Serum from both implicated donors were M2Ag positive at the time of donation. Specimens obtained from both donors on 7 occasions over the ensuing I2 months revealed a serological pattern in which M2Ag and anti-M2 were alternately detectable despite apparently normal liver function (Table 2.4d).

Patient A.T.

Patient A.T. is a 66 year old woman who underwent mastectomy and subsequent skin grafting on 9.8.83 and I3.8.83 respectively. During the latter operation she was transfused with three units of blood. Acute viral hepatitis was diagnosed 25 days later, when a serum specimen was weakly reactive for M2Ag. One of the implicated donations possessed M2Ag.

Patient H.G.

Patient H.G. is a 42 year old woman who was transfused with 2 units of packed red cells on I.II.83 following cholecystectomy. She was discharged from hospital on 8.II.83 but re-admitted 36 days after transfusion with a 5 day history of pain in the upper right quadrant and a 24 hour history of jaundice. Upon re-admittance bilirubin was 90 µmol/l and AST over I0 times the upper limit of normal. Although this patient had an underlying bile duct defect, a viral hepatitis, possibly transfusion related, was diagnosed. Although the only acute phase serum available was negative when tested for M2 markers, one of the two implicated donations possessed M2Ag. Sixteen weeks later this donor had seroconverted to anti-M2 IgG.

TABLE 2.4d

M2 MARKERS AND ALT VALUES FOR TWO OF THE IMPLICATED DONORS OVER A ONE YEAR PERIOD.

DONOR HS

Weeks after donation	0	13	14	16	20	30	52
ALT IU/l	15	25	19	35	24	31	19
M2Ag	+	-	-	+	-	+	-
Anti-M2 IgM	-	+	-	-	-	-	-
Anti-M2 IgG	-	-	-	-	-	-	+

DONOR DC

Weeks after donation	0	12	14	16	20	30	52
ALT IU/l	18	14	15	14	15	38	31
M2Ag	+	+	+	+	-	+	+
Anti-M2 IgM	-	+	-	-	+	-	-
Anti-M2 IgG	-	-	-	-	-	-	-

Implicated M2-reactive donations.

Information relating to age, sex, ALT level at time of donation (where available) and number of previous donations for each implicated M2 reactive donor is presented in Table 2.4e. Most were below the average donor age of 35 years and had either donated for the first time or had previously donated on less occassions than the average repeat donor (approximately I0 donations). Three of the four implicated donations available for ALT testing exhibited values within the normal range.

DISCUSSION.

The acute phase of transfusion related non-A, non-B hepatitis is generally less severe than that associated with HBV (Prince et al I974, Seeff et al I978). Six of the seven events investigated were only brought to the attention of the Blood Transfusion Service because the patient had developed jaundice. The remaining patient (S.H.) was only identified as a post-transfusion hepatitis case because she was a plasmapheresis donor whose liver function was monitored routinely.

It is possible to divide the patients into two groups on the basis of exposure to and/or expression of M2 markers and incubation period. Patient M.H. received blood negative for M2 markers. She exhibited an incubation period twice as long as the mean for that of the other six patients all of whom received at least one transfusion of blood possessing M2Ag or anti-M2 IgM.

TABLE 2.4e

INFORMATION RELATING TO IMPLICATED BLOOD DONORS WITH SEROLOGICAL EVIDENCE OF M2 INVOLVEMENT.

RECIPIENT	DONOR				
	Age (yrs)	Sex	M2 marker	ALT (IU/l)	Number of previous donations
SH	50	M	IgM	63	None
PP	19	M	IgM	NA	None
	26	M	IgM	NA	6
HR	19	F	IgM	NA	1
	NA	M	IgM	NA	None
EA	34	M	Ag	18	4
	22	F	Ag	17	6
AT	20	F	Ag	NA	None
HG	23	F	Ag	25	8

NA: Information not available

The possibility exists that at least one of the units to which M.H. was exposed contained a marker below the level of detection, although this seems unlikely as no serological evidence of M2 markers was seen during follow-up which involved the testing of 20 blood samples over an 80 week period. This lends support to the theory that M2 markers are more likely to occur in association with non-A, non-B hepatitis developing 3-7 weeks after transfusion rather than a later date. It also suggests that in this region the shorter incubation form of the disease is more likely to result in jaundice.

It appears that transfusion of either M2Ag or anti-M2 IgM positive blood could result in post-transfusion hepatitis, inferring that infectious immune complexes may be involved. Modification of the 'anti-μ' capture technique in which addition of M2Ag is omitted suggests that this is the case.

Biochemical evidence of persisting liver dysfunction was apparent in three patients (S.H., M.H., E.H.) whose follow-up extended beyond six months. Patient S.H. has now been studied for over three years and while remaining clinically well, continues to exhibit unexplained intermittent episodes of mild liver dysfunction, parallelling previously reported observations in infected chimpanzees. (Bradley et al I983a). The reappearance of M2Ag in the serum of this patient could provide a clue to the cause of these transient liver enzyme elevations. Similarly, the recrudescence of hepatitis in patient H.R. I6 weeks after

transfusion was associated with the reappearance of serum M2Ag following several weeks during which liver function had almost returned to normal in conjunction with a detectable anti-M2 IgM response.

Analysis of available information concerning implicated blood donors (positive for M2Ag or anti-M2 IgM) indicates an approximately equal distribution of males and females with an average age of 26.6 years compared to the regions average age of 34.6 years. Four were first time donors, one had donated once before and the remainder had all donated on less than the average (for repeat donors in this region) number of occasions. Review of the donation records of 5 implicated donors who were marker positive and had donated between I and 8 times previously revealed no evidence of prior implication in icteric transfusion related hepatitis. Even if one assumes that each was carrying a transmissible non-A, non-B infectious agent on the occasion of each previous donation, lack of association with a hepatitis event may be due to either the predominantly anicteric nature of the illness in the recipient and/or the possible protective/attenuating influence of co-transfused anti-M2 IgG. Alternatively these donors may have been exposed to M2Ag since their previous donation. Analysis of HBsAg screening data shows that the proportion of HBsAg carriers detected among repeat blood donors in the South-East Scotland has risen from 0% in I979 to account for 33% and 3I% of carriers detected in I982 and I983 respectively. This

phenomenon parallels a dramatic increase in the diagnosis of acute HBV infection among the local drug addict community who presumably also significantly facilitate the spread of non-A, non-B hepatitis agents (Francis et al I984).

Although limited, findings concerning serum ALT levels in implicated donations suggests that while exclusion of donors with raised ALT may reduce disease transmission, this approach is unlikely to totally prevent it since both donations transfused to patient E.A. exhibited normal ALT. Follow-up of these two M2Ag positive donors revealed persistance of M2 markers over a 40 week period during which serum ALT levels remained within the normal range. This is in keeping with the findings of Alter (I984) who reported that the majority of donors implicated in the transmission of non-A, non-B post-transfusion hepatitis had normal liver function.

ASSOCIATION OF THE MARKERS WITH ACUTE, CHRONIC AND NON-VIRAL HEPATITIS.

Sera from patients with a final clinical diagnosis of acute HBsAg/CMV/EBV negative hepatitis (without any known history of parenteral exposure) were made available from the following areas:

i) I22 sera from the Edinburgh area, kindly supplied by Dr. J.M. Inglis, Regional Virus Laboratory, City Hospital, Edinburgh.

ii) I38 sera from the Dundee area, kindly supplied by Dr. D. Green, Regional Virus Laboratory, Ninewells Hospital, Dundee.

(iii) I20 sera from the Aberdeen area, kindly supplied by Dr. T. Brown, Regional Virus Laboratory, Forresterhill, Aberdeen.

(iv) 50 sera from the Cardiff area, kindly supplied by Dr. J. Munro, Public Health Laboratory, University Hospital of Wales, Heath Park, Cardiff.

(v) I35 sera from the Dublin area, kindly supplied by Dr. A. Shattock, Department of Microbiology, University of Dublin, Eire.

Forty-nine sera from patients with a final diagnosis (clinical and/or histological) of chronic hepatitis were kindly made available by Dr. N.D.C. Finlayson, Liver Unit, Royal Infirmary, Edinburgh.

Sixty-nine sera from patients with a final clinical diagnosis of non-viral hepatitis obtained from patients with paracetamol poisoning, obstructive jaundice, alcoholic cirrhosis, Gilbert's Syndrome, Sjögrents Syndrome, Wilsons Disease or hepatoma were tested for M2 markers.

Tables 2.4f and 2.4g summarize the prevalence of M2 markers in patients with acute, chronic and non-viral

TABLE 2.4f

PREVALENCE OF M2, HAV, HBV IN SPORADIC HEPATITIS OF VIRAL AETIOLOGY AND IN PATIENTS WITH HEPATITIS OF A NON-VIRAL AETIOLOGY.

CENTRE	NUMBER TESTED	M2[a] only		HAV[b] only		HBV[c] only		HBV, HAV + M2		HBV+M2		HAV+M2		HAV+HBV		TOTAL[d] M2 (%)
		No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	
EDINBURGH	122	21	(17.2)	14	(11.5)	3	(2.5)	3	(2.5)	6	(5.0)	10	(8.2)	1	(0.8)	32.9
DUNDEE	138	29	(21.0)	4	(3.6)	0	(0.0)	0	(0.0)	0	(0.0)	4	(2.9)	0	(0.0)	23.9
ABERDEEN	120	25	(20.8)	3	(2.5)	3	(2.5)	1	(0.8)	1	(0.8)	5	(4.2)	1	(0.8)	26.6
DUBLIN	135	36	(26.7)	0	(0.0)	4	(3.0)	0	(0.0)	3	(2.2)	0	(0.0)	0	(0.0)	28.9
CARDIFF	28	14	(50.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	50.0
NON-VIRAL HEPATITIS	69	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	(0.0)

a = positive for M2Ag or anti-M2 IgM

b = positive for anti-HAV IgM

c = positive for anti-HBc IgM (all sera were HBsAg negative)

d = sum of all sera positive for M2 markers

TABLE 2.4g

PREVALENCE OF M2 MARKERS IN CHRONIC HEPATITIS

DIAGNOSIS	NUMBER TESTED	M2Ag only	(%)	Anti-M2IgM only	(%)	M2 IgG only	(%)	M2Ag+M2 IgM	(%)	M2IgM+ M2IgG	(%)	%M2 involvement
Chronic Viral Hepatitis	23	2	(8.7)	3	(13.0)	4	(13.8)	1	(3.5)	1	(3.5)	47.8
Chronic Non-Viral Hepatitis	26	0		0		0		0		0		0

hepatitis. Markers of M2 do not appear to be associated with general (non-specific) liver dysfunction, being absent in the sera of 69 patients with hepatitis of a non-viral aetiology but present as either M2Ag or anti-M2 IgM (or both) in 29.I percent of HBsAg negative viral hepatitis patients, occurring with a similar frequency in Edinburgh (32.9%), Dundee (23.9%), Aberdeen (26.6%) and Dublin (28.5%). The prevalence of Cardiff was 50 percent. As criteria for selection of the sera tested were left to clinical and laboratory diagnosis, it was not possible to judge precisely what stage of the illness that the sera had been collected. It is also possible that some patients have a different form of non-A, non-B hepatitis acquired by other routes.

The prevalence of markers of current M2 infection was approximately 4 times that of the anti-HAV IgM in acute HBsAg negative hepatitis patients in the East of Scotland. Serological evidence of current CMV infection was found in 0.002 percent of such patients, while markers of current EBV infection were absent in all 80 of the sera tested. The finding of 6.I percent reactivity for more than one marker (HAV, HBV or M2) is of interest and could be explained by concurrent dual infection, or infection with one agent while carrying or recovering from infection with another. In the context of anti-HAV IgM these findings may emphasize the need to consider the amount of specific IgM present before inferring a diagnostic significance. It is

perhaps of some relevance that Bucens et al (I983) have reported a false positive rate of 6 percent using the same diagnostic kit.

The presence of M2 markers in 47.8 percent of patients with a final diagnosis of chronic liver dysfunction of a viral aetiology lends support to the theory that M2 may be associated with a transmissible agent, more ubiquitous than HBV (markers of HBV were present in 20.3 percent of patients). It is interesting to note reports of progression of non-A, non-B hepatitis to chronic liver disease is more frequent than chronic liver disease following an acute HBV infection. It has also been suggested by Alter (I984) that non-A, non-B hepatitis may be the cause of hitherto unexplained cirrhosis. However, it must also be remembered that chronic hepatitis patients possess a large amount of auto-antibodies which could result in false positive reactions.

FULMINANT NON-A, NON-B HEPATITIS.

Sera from two patients who died of fulminant HBsAg negative viral hepatitis were kindly provided by Dr. W.N. MacLeod, Institute of Neurological Sciences, Southern General Hospital, Glasgow. Case histories are presented below:

Patient J.W.

Patient J.W. was a 70 year old white male Jehovah's Witness and was admitted to hospital on 5.I.8I. He had felt generally unwell for 3 weeks and had nausea, anorexia and

constipation. Immediately prior to admission he became intermittently confused and agitated, having been jaundiced for 3 days. There was a 50 year history of rheumatoid arthritis for which he had taken Ibuprofen (400mg t.i.d.). The following were excluded: exposure to hepatotoxins (notably paracetamol and anaesthetic agents), needlestick, blood transfusion, tattooing, drug abuse, excess alcohol, known chronic liver disease.

Over the following 26 days his condition progressively deteriorated with confusion, drowsiness and then Grade IV coma in the last week. His haemoglobin fell from II.5g/dl to 8g/dl but transfusion was refused on religious grounds. He died with bronchopneumonia. Treatment was conservative (no exchange transfusion or steroids).

The initial ALT was elevated at I,050 IU/l, fell rapidly in the first 5 days and more slowly thereafter to 75 IU/l. The bilirubin was modestly elevated and remained around 300 µmol/l while the alkaline phosphatase was normal. As the illness evolved the albumin fell from 32g/l to I9g/l and there was a preterminal rise in serum creatinine. Fibrinogen, fibrinogen degradation products and platelets remained normal.

Anti-HAV was present although specific IgM was not. There was no evidence of infection with HBV, CMV or EBV. Post mortem examination confirmed acute massive

hepatic necrosis with no obvious cause for hepatic failure or any evidence of chronic liver disease.

Patient A.R.

Patient A.R. a 20 year old white woman, was admitted to hospital on 26.3.8I. Over the preceeding weeks she felt 'fluish', Icterus appeared 3 days before admission following which her mental condition very rapidly deteriorated. On examination she was icteric, confused and agitated. There was normal hepatic dullness on percussion and no signs of haemorrhage. Within I2 hours she became deeply unconscious with no response to painful stimuli. The following day she had a cardiorespiritory arrest. Following this she was ventilated for 3 days at which time brain death was confirmed.

The initial ALT was very high at 3,650 IU/l, falling steeply over the next 48 hours. Bilirubin and alkaline phosphatase were only modestly elevated and rose slightly over 3 days to 264μmol/l and 326μmol/l respectively. The creatine and albumin concentrations remained within the normal range. There was gross disturbance of the clotting mechanism, with a prothrombin time ratio of 9.I, despite attempted corrections. Haemoglobin, fibrinogen and platelet levels remained normal. Hypoglycaemia was initially severe. There was no serological evidence of infection with HAV, HBV, CMV or EBV.

Both patients J.W. and A.R. developed a late anti-M2 IgM

response. Fulminant hepatitis as a result non-A, non-B hepatitis is a feature which has been reported by Mathieson et al (I980b) and Bamber et al (I98Ia). It is possible that infection with M2Ag was the cause of the fulminant hepatitis episode, or the patient could have been a carrier of M2Ag and infected with another agent, similar to the situation seen with superinfection by delta agent in a hepatitis B virus carrier.

INFLUENCE OF RHEUMATOID FACTOR.

Rheumatoid factors (RF) are defined as antibodies directed against determinants on the Fc fragment of IgG (Stage and Mennik I973). The majority of RF's are of an IgM nature but antibody activity against IgG may also be found in both IgG and IgA classes (Torrigiani and Roitt I967). Rheumatoid factors possess the potential for giving false positive results in certain types of immunoassays. Although it has been suggested that interference by rheumatoid factor can be circumvented by the use of Fab́ fragments for labelling (Duermeyer et al I979, Kato et al I979), the problems encountered by van der Waart et al (I980) of non-specificity in patients with liver disorders discouraged use of Fab́ fragments in this thesis.

To establish the relative interference of RF in M2 immunoassays 350 sera from patients with a final diagnosis of viral hepatitis of unknown aetiology were tested for RF by latex agglutination (RheumaWellcotest, Wellcome

Diagnostics). Rheumatoid factor was absorbed from positive samples by incubation with latex beads coated with aggregated human IgG. The sera were then retested for M2 markers. (Table 2.4h). Sera positive for M2 markers and rheumatoid factor retained their M2 marker positivity after absorption of rheumatoid factor. Although RheumaWellcotest is designed to detect at least I5 IU/l (clinically significant of rheumatoid arthritis) absorption of RF positive sera without interferring with M2 markers suggests that RF is not a source of false positives in these assays. (Cayzer, personal communication, I984). This is supported by statistical analysis of the data as the Chi^2 test with Yates correction indicates no significant difference for RF in M2- or M2+ groups. Furthermore, physical characterisation of M2Ag (Part 3) supports the view that M2Ag is unlike RF.

TABLE 2.4h (i)

DISTRIBUTION OF RHEUMATOID FACTOR AND M2 MARKERS IN 350 SERA FROM PATIENTS WITH SPORADIC ACUTE NON-A, NON-B HEPATITIS SERA.

Number tested	M2+ RF+	M2+ Rf-	M2- RF+	M2- RF-
350	11	87	35	217

M2 = either M2Ag or anti-M2

+/- = indicates marker present/absent

TABLE 2.4h (ii)

DISTRIBUTION OF M2 MARKERS IN M2 AND RHEUMATOID FACTOR POSITIVE SERA AND M2 POSITIVE, RHEUMATOID FACTOR NEGATIVE SERA.

	M2Ag only	Anti-M2IgM only	Anti-M2IgG only	M2Ag+ Anti-M2IgM	M2Ag+ Anti-M2IgG
M2+ RF+	4	1	1	2	3
M2+ RF-	35	27	20	4	1

Using the chi^2 test with Yates correction:

		RF +	RF -		%RF+
	+	11	87	98	11%
M2	-	35	217	252	14%
		46	304	350	

χ^2 = 0.23 indicating there is no significant difference between the occurrence of RF and M2 among the patients tested.

2.5 DISCUSSION

Development and application of enzyme immunoassays for the detection of M2Ag, anti-M2 IgM and anti-M2 IgG has led to the discovery of an apparent association between M2 markers and a form of non-A, non-B viral hepatitis. The assays are reproducible and production of hyperimmune rabbit and guinea pig antibody for use in M2Ag EIA and anti-M2 IgM has eased the demand for reagent supply from a human source. This has also increased the specificity of the assays by replacing a human-human system with a human-animal combination. Sensitivity has been improved by the use of an M2Ag affinity column for purification of anti-M2, and by the addition of 5 percent normal human serum to the conjugate diluent for use in M2Ag EIA and anti-M2 IgM EIA.

The specificity of M2 markers for non-A, non-B viral hepatitis was examined by testing appropriately selected sera. Results of two coded panels indicate that M2 markers are not associated with a non-A, non-B hepatitis exhibiting a 'long' (greater than 8 weeks) incubation period, and has revealed the association of M2Ag in chimpanzees injected with the 'tubule forming' agent of Bradley *et al* I983b. However, M2 markers do not appear to be a component of normal human or chimpanzee sera, or associated with HAV or HBV as confirmed by testing sera possessing serological markers indicative of acute infection with these agents.

Results from retrospective investigation of non-B post-transfusion events revealed an association between M2 markers and a 'short' (3-7 weeks) incubation form of hepatitis. In each case, at least one implicated donation was positive for M2 markers (M2Ag or anti-M2 IgM). Of interest is the absence of anti-M2 IgG in any of the implicated donations particularly as donor screening indicates that all three markers occur with a similar frequency in the local donor population.

Examination of non-parenterally transmitted 'sporadic' non-A, non-B hepatitis revealed M2 markers in approximately 33 percent of acute phase sera. M2 markers were present in 48 percent of chronic viral hepatitis sera, but absent in controls of non-viral hepatitis. The finding of anti-M2 IgM in 2 patients who died of fulminant non-A, non-B viral hepatitis may reflect a possible cause or merely an unrelated carrier state.

A common source of false positive reactions in EIA is rheumatoid factor. Study of rheumatoid factor positive serum has shown that M2 marker reactivity is retained after absorption of rheumatoid factor. There appears to be no significant association between rheumatoid factor and the appearance of M2Ag.

The apparent specificity of these assays for M2 markers and their occurrence in serum of non-A, non-B hepatitis is encouraging and prompts further characterisation of M2Ag and its epidemiology.

PART THREE

CHARACTERISATION OF M2Ag

3.I INTRODUCTION

Biochemical and biophysical characterisation of HAV and HBV have been established using such techniques as sucrose and caesium density gradient centrifugation (Bradley et al I978, Siegl et al I98I) and polyacrylamide gel electrophoresis (Gerin and Sher I978, Peterson I98I, Siegl et al I98I). In addition the nucleic acids of both these viruses have been purified and analysed (Charney et al I979, Coulepis et al I98I). Similar techniques were employed in an attempt to characterise M2Ag. In addition, the antigenic stability to heat, pH, chloroform, ether and formalin were investigated.

Establishment of the chimpanzee as an animal model for non-A, non-B viral hepatitis has proved invaluable since these primates can be used to demonstrate the infectious nature of the causative agent(s) (Alter et al I978b, Tabor et al I978); their lack of immunological relatedness to HAV, HBV, or the GB agent (Alter et al I978b, Tabor et al I978, Tabor et al I980b) and the fact that they can be inactivated by heat and formalin (Tabor and Gerety I980). In addition many putative serological markers and virus like structures have been reported in infected chimpanzee serum and liver (Part I:4) although their specificity for non-A, non-B viral hepatitis has yet to be confirmed.

As chimpanzees are both expensive and scarce, the marmoset has been investigated for use as a potential animal model.

Marmosets are relatively more abundant and cheaper to house and maintain. Unfortunately they are not easily bred or maintained in captivity. Reports concerning the transmission of human non-A, non-B viral hepatitis agents to marmosets are conflicting. Feinstone et al (I98I) suggested that marmosets are susceptible to non-A, non-B hepatitis agents although not as readily as chimpanzees, a finding supported by Karayannis et al (I983). However, Tabor et al (I979b) failed to induce any response to a non-A, non-B inoculum in marmosets over a I2 weeks period. The situation is further confused by the finding that normal human serum can induce a toxic liver dysfunction in marmosets if administered in sufficient volumes. (Appleton, H., I983 personal communication). The transmission of M2Ag to six colony bred marmosets was investigated in an attempt to establish the infectivity of this agent.

Finally, M2Ag was studied as an immunogen, using a rabbit, six guinea-pigs and several mice. The mice were immunised with the intention of producing monoclonal anti-M2.

3.2 MATERIALS AND METHODS

DETERMINATION OF MOLECULAR WEIGHT.

(a) Polyacrylamide gel electrophoresis (PAGE).

Three micrograms of gradient (sucrose/CsCl) purified M2Ag, containing 25 percent w/v sucrose were electrophoresed using Pharmacia Electrophoresis apparatus and polyacrylamide gradient gels PAA 4/30 in parallel with high molecular weight (HMW) standards (Pharmacia). The electrophoresis buffer contained 0.09 M Tris/HCl pH 8.4; 0.08 M boric acid and 0.93 g/l EDTA. Gels were pre-electrophoresed for 20 minutes at 70 V, after sample addition electrophoresis continued for I6 hours at I50 V. Gels were fixed in I0 percent sulphosalicyclic acid for 30 minutes, stained with 0.02 percent Coomassie Blue in 7 percent acetic acid and destained with several changes of 7 percent acetic acid. The distance that the HMW standards had migrated were measured and the relative migration distance (Rf) calculated. (Rf = distance migrated/gel length). Rf was plotted against log molecular weight of the standards, enabling the molecular weight of unknown proteins to be determined graphically.

(b) Precipitation by polyethylene glycol (PEG) 6,000.

0.6g PEG 6,000 was dissolved in I0ml M2Ag positive serum to give a final concentration of 6 percent PEG. After incubation overnight at $4^{o}C$ and centrifugation (I,000g I0 minutes) the precipitate was dissolved in Iml saline, dialysed against saline overnight before testing for M2Ag.

A further 0.4g PEG 6,000 was added to the supernatent and incubated overnight at $4^{o}C$. Following centrifugation the precipitate was dissolved in Iml saline and dialysed against saline overnight before testing for M2Ag. An identical procedure was followed for M2Ag negative sera.

(c) Sephacryl S I,000 profile.

An 80cm column with a diameter of I.5cm was packed with Sephacryl S I,000 (Pharmacia) and equilibrated in 0.05M Tris/HCl pH 8.0. The column was calibrated using ^{I25}I HBsAg 22nm particles (approximate molecular weight of $3.5 - 4.5 \times I0^{6}$ daltons). 0.5ml of ^{I25}I HBsAg was added to the column and run at a constant speed overnight. One hundred I.6ml fractions were collected, assayed for HBsAg (Hepatest 3, Wellcome Diagnostics) and radioactivity (cpm). Following calibration 0.4ml M2Ag (PEG 6,000 precipitate) and 0.Iml ^{I25}I HBsAg were mixed, added to the column and run under calibration conditions. Fractions were assayed for M2Ag (EIA), HBsAg (Hepatest 3, Wellcome Diagnostics) and radioactivity (cpm). A third profile was obtained using 0.5ml of M2Ag. Fractions were collected and assayed for M2Ag (EIA). The column was washed thoroughly with 0.05M Tris/HCl pH 8.0, to remove residual protein, between each sample application.

RATE ZONAL BANDING OF M2Ag IN CAESIUM CHLORIDE GRADIENTS.

Five hundred microlitres of M2Ag positive serum (previously concentrated by pelleting were layered onto a preformed

I.I - I.5g/ml CsCl gradient (Appendix 3) and centrifuged for I8 hours at I20,000g at 5°C. One ml. fractions were collected and their refractive index and density determined. (Appendix 4). Fractions were dialysed against 0.05M PBS pH 7.2 and assayed for M2Ag (EIA).

ESTIMATION OF SEDIMENTATION COEFFICIENTS.

Four hundred microlitres of gradient purified M2Ag containing I.5mg/ml protein was mixed with I00ul ^{I25}I HBsAg and layered onto a 6ml 5 - 30 percent preformed linear sucrose gradient prepared in 0.05 M PBS pH 7.2. Gradients were centrifuged at 270,000g in a 3 x 6ml titanium swing-out rotor (MSE Superspeed 75 ultra centrifuge) for I20 minutes at 5°C (w^2t = I.972 IO^{II}). Twelve 0.5ml gradient fractions were collected and weight percent sucrose of each fraction determined in a refractometer (Precision Instruments). Fractions were dialysed against 0.05M PBS pH 7.2, assayed for M2Ag (EIA) HBsAg (cpm) and the protein concentration determined spectrophotometrically at 280nm.

The sedimentation coefficients for HBsAg and M2Ag were determined using the method of McEwen (I967) and Appendix 5.

POLYPEPTIDE ANALYSIS.

Gradient purified M2Ag, similarly purified normal human serum (NHS), HMW and low molecular weight (LMW) standards were electrophoresed as for PAGE but with the following modifications. The electrophoresis buffer was 0.04M Tris/HCl pH 7.4 containing 0.02 M sodium acetate 0.002M

EDTA and 0.2 percent sodium dodecyl sulphate (SDS). Samples were prepared in 0.0I M Tris/HCl pH 8.0 containing 0.00I M EDTA and for the HMW standards I percent SDS and I percent β-mercaptoethanol. The HMW standards were heated at 60°C for I5 minutes. LMW standards and M2Ag were prepared using 2.5 percent SDS and 5 percent β-mercaptoethanol and heated for 5 minutes at I00°C. Gels were pre-electrophoresed for I hour at 70 V. After addition of samples (6µl HMW standard, 3µl LMW standard, 3µg M2Ag) electrophoresis continued at 300 V for I0 minutes followed by I50 V for 2.5 hours. The gels were fixed and SDS eluted by an overnight incubation with 25 percent isopropanol and I0 percent acetic acid followed by staining with 0.02 percent Coomassie Blue and destaining with 7 percent acetic acid. The molecular weights of the polypeptides were determined as for PAGE.

STABILITY OF M2 ANTIGENICITY.

(a) Heat: Aliquots of sera, positive and negative for M2Ag, at a dilution of I:5 in 0.I5 M PBS pH 7.2 were heated to 50°C for I hour, 60°C for I hour, 60°C for I0 hours and I00°C for 5 minutes. The sera were then tested for M2Ag.

(b) pH: Aliquots of sera, positive and negative for M2Ag, were dialysed against buffers of pH 2, 4, I0 and I2 overnight followed by an overnight dialysis against 0.I5 M PBS pH 7.2 prior to testing for M2Ag.

(c) Formalin: Aliquots of sera, positive and negative for M2Ag were incubated with equal volumes of either I:4,000 formalin at 37°C for 72 hours or I:2,000 formalin at 37°C for 96 hours prior to testing for M2Ag.

(d) Chloroform extraction: Aliquots of sera, positive and negative for M2Ag were pelleted at I20,000g for 5 hours and the pellets resuspended in 2ml 0.I5M PBS pH 7.2. 0.5ml of chloroform was added to each of the resuspended pellets and vortexed for I0 minutes. A 'sham' extraction was performed in an identical way, substituting 0.5ml 0.I5M PBS pH 7.2 for chloroform./ Bradley et al 1983b The aqueous layers were extracted, dialysed against 0.I5M PBS pH 7.2 overnight and tested for M2Ag.

(e) Ether: Aliquots of sera, positive and negative for M2Ag were pelleted at I20,000g for 5 hours and the pellets resuspended in 2ml. 0.I5M PBS pH 7.2. Fifty percent ether v/v was added to each pellet, vortexed for 20 minutes and the ether removed by evaporation at room temperature. A 'sham' extraction was performed using 50 percent PBS pH 7.2 v/v.

ELECTRON MICROSCOPY.

(a) Direct electron microscopy: Ten microlitres of a M2Ag preparation (concentrated by pelleting at I20,000g for 5 hours and purified on a caesium

chloride density gradient) were floated on to 400 mesh formvar coated copper grids (Agar Aids) for I hour, followed by a saline wash for I hour. Grids were stained with 3 percent phosphotungstic acid (PTA) pH 7 for 2 minutes, washed with saline for 30 seconds and stored in a dessicator prior to viewing in the electron microscope. The procedure was repeated for unprotease treated, PEG precipitated M2Ag after elution through a Sephacryl S I,000 column.

(b) Immune electron microscopy. One hundred microlitres of a PEG precipitated, gradient purified M2Ag or normal human serum, unreactive for M2 markers were added to I00µl of anti-M2 (either guinea pig anti-M2, affinity purified anti-M2, a hyperimmune gammaglobulin or a hepatitis A immune globulin) and incubated at 37°C for I hour prior to an overnight incubation at 4°C. The samples were then processed as for direct electron microscopy prior to viewing in the electron microscope. M2Ag:anti-M2 precipitin lines were excised from an agarose gel, washed and macerated prior to processing and viewing in the electron microscope.

NUCLEIC ACID EXTRACTION FROM PURIFIED M2Ag.

As RNase are ubiquitous and not easily degraded, the following precautions were taken. All glassware was

heat treated at I80°C overnight, plasticware and buffers were autoclaved. Where possible plastic and glassware were coated with 'Sigmacote' (Sigma Chemicals) to prevent any nucleic acid adsorbing to these surfaces. 2ml of an M2Ag preparation with a protein concentration of 300µg/ml was digested with 40µl Proteinase K (Boehringer-Mannheim) at a concentration of 200µg/ml for I hour at 37°C. Following two extractions with phenol-chloroform in TNE buffer (0.0I M Tris/HCl pH 7.4, 0.I M NaCl, 0.00I M EDTA containing I percent SDS) the aqueous phase was removed, combined and extracted 3 times with ether to remove traces of phenol. Ether was removed by evaporation at 37°C for 30 minutes. Three volumes of ethanol and 0.I volume 3 M sodium acetate pH 6.0 were added and the nucleic acid precipitated overnight at -20°C. The precipitate was pelleted at I,000g for 5 minutes and washed twice with 75 percent ethanol. The remaining precipitate was dried and resuspended in I00ul of sterile water. The absorbance was scanned between 260nm (nucleic acid) and 280nm (protein). SV 40 virus was used as a control.

INFECTIVITY STUDIES IN MARMOSETS.

(a) Infectious inocula: The materials studied for infectivity in marmosets were derived from several different sources.

i. Serum obtained from a donor twice implicated in non-A, non-B post-transfusion hepatitis from which M2Ag was originally isolated.

ii. Factor VIII originating from the American National Institute of Health that caused 'H' strain like alterations with an infectivity titre of 10^6 marmoset infectious doses per ml. (Feinstone et al I98I).

iii. Factor VIII concentrate (Armour) believed to be the cause of non-A, non-B hepatitis in three Edinburgh haemophiliacs.

(b) Marmosets: All six marmosets (two S. oedipus and four S. labiatus) were colony bred at the Central Public Health Laboratory, Colindale. The age of the animals ranged from 2I months to 3 years at the start of the inoculation schedule. The weight of the animals ranged between 500 and 600kg at the start of the study. Marmosets were inoculated intravenously or intramuscularly with 0.5ml of the inocula and then with Iml of the same inocula 4 months later. (Table 3.2a). Venous blood samples were collected three weeks before inoculation and at weekly intervals thereafter for approximately six months. Liver biopsies were only planned during the period of elevated ALT levels because of the risk of the procedure for such small animals.

(c) Serological studies: Sera from the marmosets were assayed for M2 markers using the standard EIA for M2Ag with the addition of I percent normal (M2Ag and anti-M2 negative) marmoset serum to the conjugate

TABLE 3.2a

INOCULATION SCHEDULE FOR MARMOSETS

Number	Species	Sex	Inoculum	Volume
B33	S. Oedipus	F	Normal blood donor	0.5ml 1.0ml*
B34	S. oedipus	F	Agent 'H'	0.5ml 1.0ml*
HB21	S. labiatus	M	Implicated blood donor	0.5ml 1.0ml*
HB24	S. labiatus	M	Implicated blood donor	0.5ml 1.0ml*
HB30	S. labiatus	F	Commercial Factor VIII	0.5ml 1.0ml*
HB32	S. labiatus	M	Commercial Factor VIII	0.5ml 1.0ml*

* Second inoculum administered 4 months after the first inoculum.

diluent. Antibody to M2Ag was assayed using an inhibition system in which 50ul of test serum was added to 50ul of gradient purified M2Ag, and incubated at 45°C for 2 hours. As a negative control 50ul of test serum was added to 50ul of gradient purified normal human serum negative for M2Ag and anti-M2. Following incubation, I00ul of saline-Tween 20- BSA and an antibody coated bead were added to each well and followed by an overnight incubation at room temperature. After washing, 200ul of anti-M2:AP conjugate containing I percent normal marmoset serum was added and incubated overnight at room temperature. Following the washing procedure 200ul substrate was added as described for the standard EIA's.

Alanine aminotransferase values were estimated on fresh sera at the Central Public Health Laboratory, Colindale using a commercial assay purchased from Sigma Chemicals, the normal level for marmosets being < 40 I.U./l.

IMMUNOGENICITY IN ANIMALS.

The immunogenic properties of M2Ag were investigated using several mice, one rabbit and 6 guinea pigs as models. Animals were inoculated and bled according to the schedules in Tables 3.3a and 3.3b. Sera were absorbed with normal human serum coupled, gluteraldehyde-activated affinity absorbant columns (BCL) until no anti human activity was

TABLE 3.3a

INOCULATION SCHEDULE AND ANTIBODY TITRE FOR ONE RABBIT

	WEEKS AFTER INITIAL INOCULATION+													
	7	12	24	25	32	42	45	46	48	50	59	61	63	64
Boost (ug M2Ag)	500ug in FCA	500ug in FCA	200ug in FCA	-	200ug in Alum	200ug in Alum	100ug in Alum	-	160ug in Alum	160ug in Alum	160ug in Alum	-	160ug	-
Anti-M2	-	-	-	1/100	-	-	-	1/1280	-	-	-	1/200	-	1/2000

+ Initial inoculation of 500ug M2Ag in FCA.

FCA - Freunds Complete Adjuvant

Alum - Alum Adjuvant

TABLE 3.3b

INOCULATION SCHEDULES AND ANTIBODY TITRES FOR SIX GUINEA PIGS

		WEEKS AFTER INITIAL* INOCULATION								
		4	8	10	11	12	14	16	18	21
GUINEA PIG 1	Boost (ug M2Ag)	50ug in FCA	50ug in FCA	-	-	50ug in FCA	-	50ug in FCA	-	50ug in FCA
		-	-	-	-	-	1/4000	-	1/12800	1/16000
GUINEA PIG 2	Boost (ug M2Ag)	50ug in FCA	50ug in FCA							50ug in FCA
		-	-	-	-	-	1/500	-	-	-
GUINEA PIG 3	Boost (ug M2Ag)	50ug in FCA	50ug in FCA	-	-	-		-	-	50ug in FCA
	anti-M2 titre	-	-	-	1/1280	-	-	-	-	-
GUINEA PIG 4	Boost (ug M2Ag)	50ug in FCA	50ug in FCA	-	-	-	-	-	-	50ug in FCA
	anti-M2 titre	-	-	1/5120	-	-	-	-	-	-
GUINEA PIG 5	Boost (ug M2Ag)	50ug in FCA	50ug in FCA	-	-	-	-	-	-	50ug in FCA
	anti-M2 titre	-	-	-	-	-	1/2000	-	-	-
GUINEA PIG 6	Boost (ug M2Ag)	50ug in FCA	50ug in FCA	-	-	-	1/8000	-	-	50ug in FCA

* Initial inoculation of 75ug M2Ag in FCA per Guinea Pig; FCA - Freunds Complete Adjuvent

detected as determined by lack of agglutination with normal human serum coated latex (Wellcome Diagnostics).

Production of murine monoclonal antibodies were attempted following the protocol of Kohler and Milstein (I975) resulting in a total of I2 fusions.

3.3 RESULTS

MOLECULAR WEIGHT.

Polyacrylamide gel electrophoresis of M2Ag from different donor sources showed that the majority of protein remained in the top of the gel indicating a molecular weight in excess of 2 x 10^6 daltons. M2Ag could be detected by EIA in the supernatent of ground gel tops. M2Ag was precipitated by PEG 6,000 at a concentration of 6 percent w/v. The profile of PEG precipitated M2Ag following gel filtration through a Sephacryl S1,000 column showed a similar molecular weight range to 22nm diameter HBsAg spherical particles of 3.5 to 4.5 x 10^6 daltons. (Figure 3.3i).

DENSITY IN CAESIUM CHLORIDE.

Protease treated M2Ag from several different sources exhibited a buoyant density of I.27 - I.29g/ml and I.3I - I.32g/ml. Untreated material showed a density of I.24 - I.25g/ml.

SEDIMENTATION COEFFICIENTS.

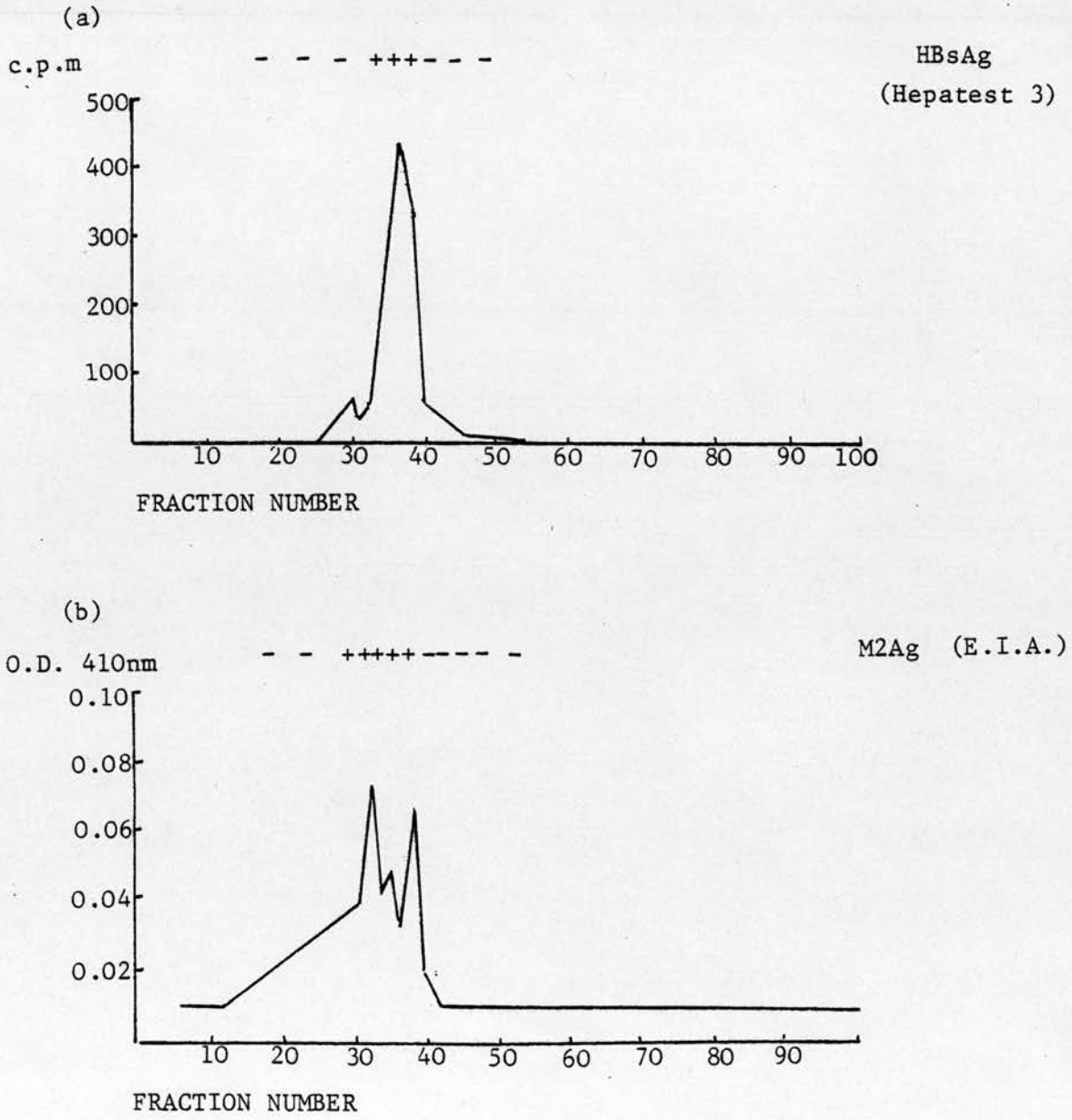
Both protease treated and untreated material exhibited sedimentation coefficients of II8S - I35S irrespective of the antigen source. Identically treated HBsAg exhibited a sedimentation coefficient of II3S with a variation between separate runs of +/- II.5 percent.

POLYPEPTIDE ANALYSIS.

SDS-PAGE revealed four major polypeptides present in

FIGURE 3.3i

SEPHACRYL S1,000 PROFILE OR (a) RADIOLABELLED 22nm HBsAg PARTICLES AND (b) TOTAL PROTEIN IN A PEG PRECIPITATED M2Ag POSITIVE SERUM



protease treated M2Ag positive sera, but absent in similarly processed normal human sera, of molecular weight 2I,000, 32,000, 43,000 and 58,000 daltons. Four major polypeptides of molecular weight 25,000, 63,000, 83,000 and 9I,000 were present in untreated M2Ag positive sera, but absent in similarly processed normal human sera. (Figure 3.3ii).

ELECTRON MICROSCOPY.

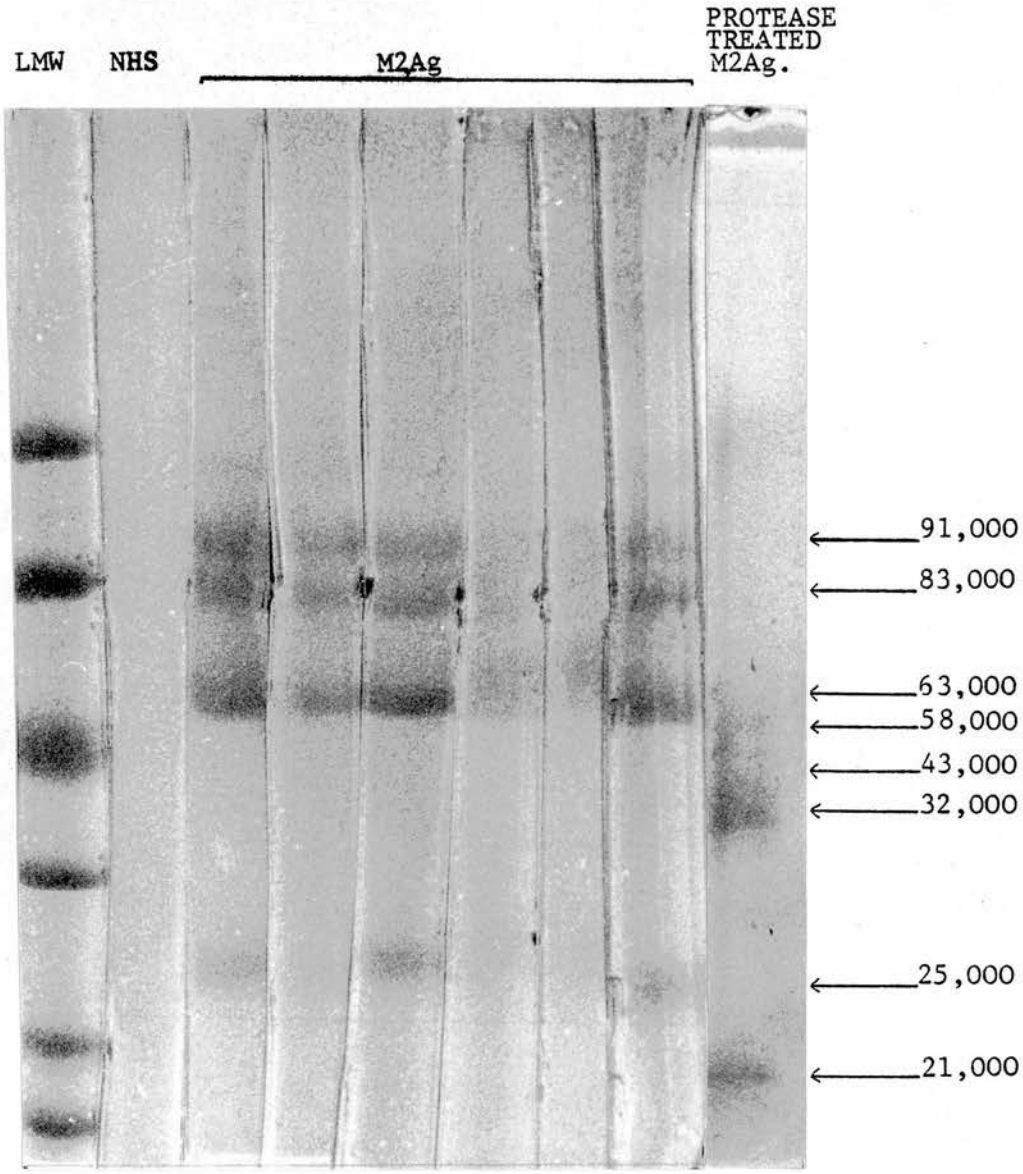
Direct electron microscopy of protease treated M2Ag sera revealed no characteristic 'virus-like' structures, although 20nm-30nm structures were observed in negatively stained M2Ag gradient fractions (Figure 3.3iii). Similar structures ranging in diameter from 20nm-40nm were observed in M2Ag reactive fractions after gel filtration through a Sephacryl SI,000 column. The starting material was not treated with protease. (Figure 3.3iv). Immune electron microscopy showed aggregates of these structures (Figure 3.3v).

STABILITY OF M2Ag ANTIGENICITY.

M2Ag activity was retained after 2-3 years at -20°C, months at 4°C, I hour at 50°C and 60°C, but lost when heat treated at I00°C for 5 minutes. M2Ag activity was also destroyed at pH 2,4 and I2; activity remained but was reduced at pH I0. M2Ag activity was also destroyed by both formalin (I:2,000 and I:4,000) and chloroform (20 percent v/v) treatment, but resistant to 50 percent v/v ether. These results are summarised and properties

FIGURE 3.3ii

S.D.S. - P.A.G.E. OF GRADIENT PURIFIED M2Ag (WITH AND WITHOUT PROTEASE TREATMENT) AND GRADIENT PURIFIED M2Ag NEGATIVE SERUM.



CALIBRATION CURVE FOR CALCULATION OF MOLECULAR WEIGHTS

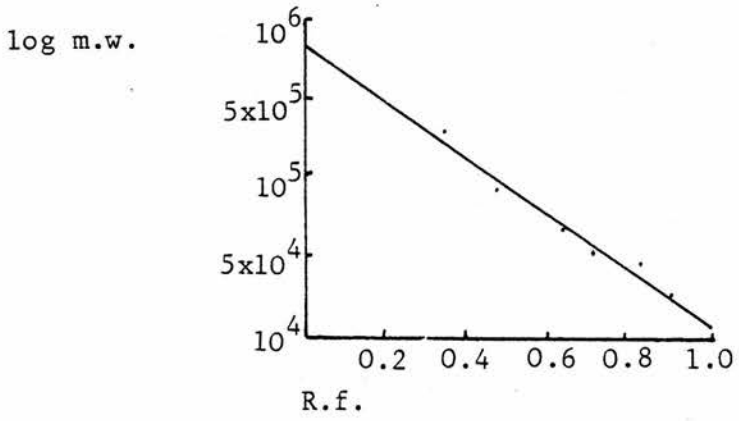
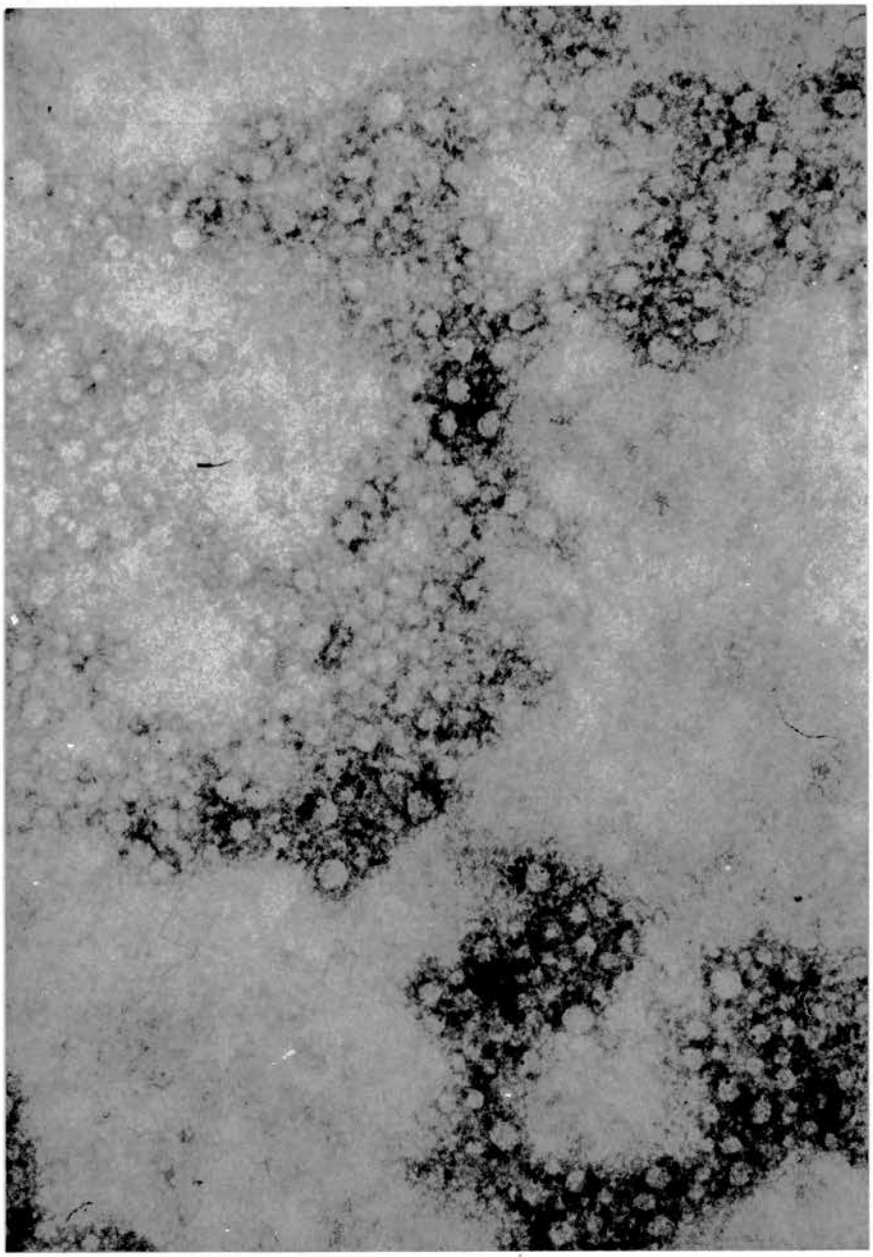


FIGURE 3.3iii

ELECTRON MICROGRAPH OF GRADIENT PURIFIED, PROTEASE TREATED M2Ag POSITIVE SERUM.

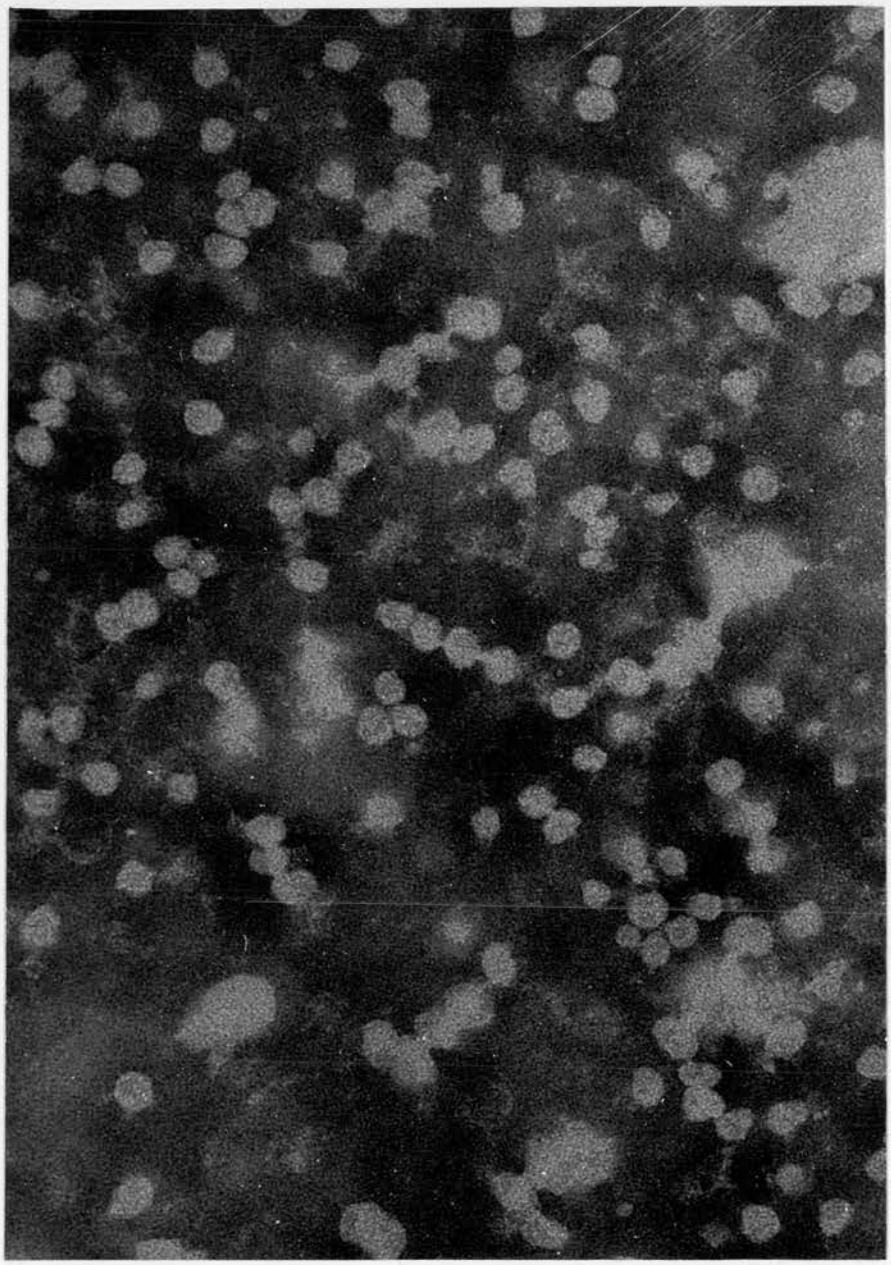


Magnification X 72,000

⊢ represents 20nm

FIGURE 3.3iv

ELECTRON MICROGRAPH OF M2Ag REACTIVE FRACTIONS AFTER SEPARATION ON A SEPHACRYL S1,000 COLUMN

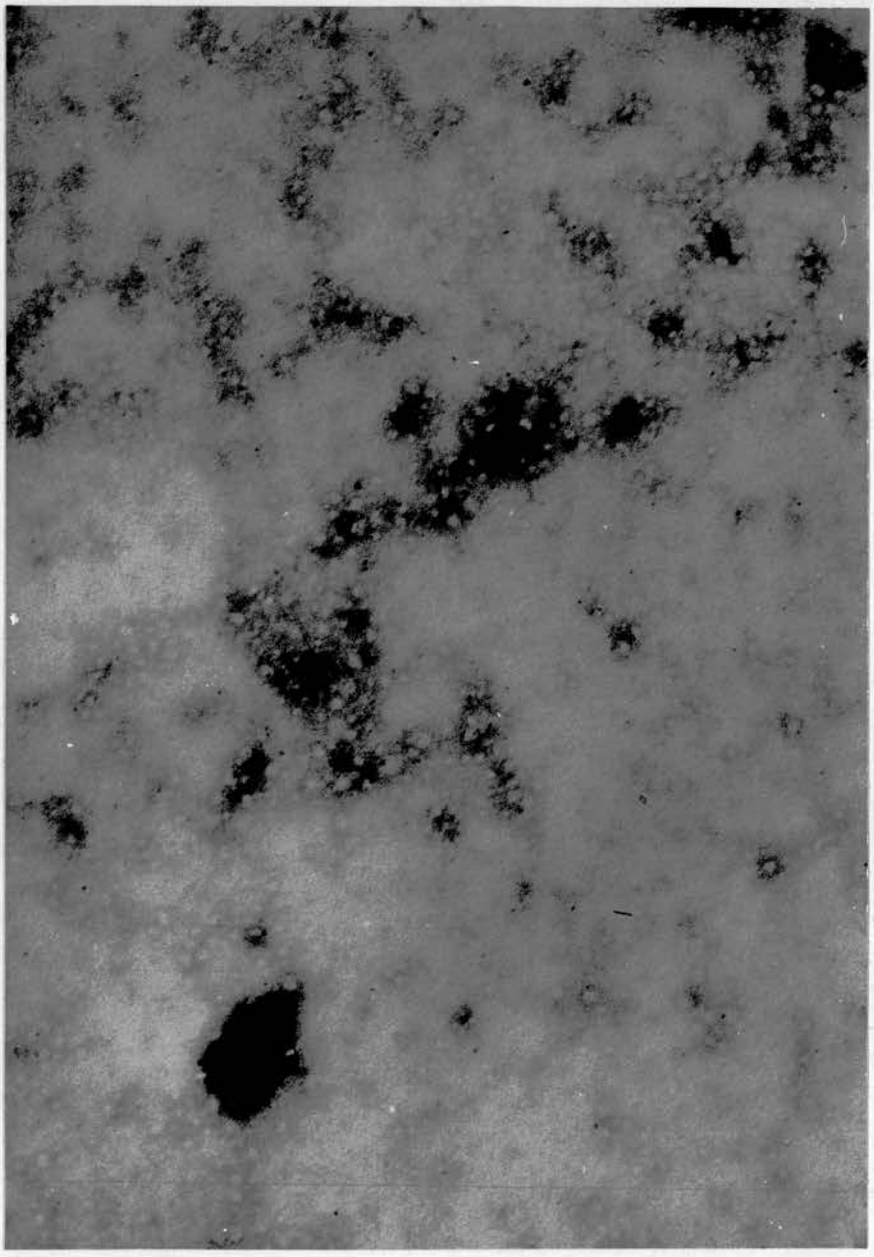


Photography by courtesy of Professor C.R. Madely, Newcastle University.
Magnification X 150,000

⟝ represents 20nm

FIGURE 3.3v

ELECTRON MICROGRAPH OF PROTEASE TREATED M2Ag : ANTI M2 PRECIPITIN LINES



Magnification X 36,000

⊢ represents 20nm

compared with those of HAV and HBV in Tables 3.3c and 3.3d respectively.

INFECTIVITY STUDIES IN MARMOSETS.

Inoculation of six marmosets with either M2Ag or the 'H' strain non-A, non-B inoculum failed to produce any serum ALT elevation over a six month period. Furthermore all sera remained negative for both M2Ag and anti-M2.

EXTRACTION OF NUCLEIC ACID.

There was no evidence of any nucleic acid from M2Ag preparations although DNA was isolated from similarly processed SV40.

IMMUNOGENICITY IN ANIMALS.

Immunisation of mice, guinea pigs and a rabbit produced an anti-M2 IgG response after removal of anti-human activity. (Table 3.3a and Table 3.3b). The overall titre of rabbit anti-M2 following immunisation was approximately six times less than that of guinea pig anti-M2, although the same inoculum was used and both were monitored using the same solid phase. Rabbit anti-M2 proved to be useful for coating of the solid phase and for neutralisation. However, it behaved poorly when it was conjugated with alkaline phosphatase. In contrast guinea pig anti-M2 could not only be used for coating and neutralisation but produced a conjugate which was more sensitive and could be used at a greater dilution than the original human antibody conjugate.

TABLE 3.3c

STABILITY OF M2Ag TO HEAT, pH, FORMALIN, CHLOROFORM AND ETHER

	Treatment	M2Ag activity
Heat	1 hour 50°C	retained
	1 hour 60°C	retained
	10 hours 60°C	retained
	5 minutes 100°C	destroyed
pH	2	destroyed
	4	reduced
	10	reduced
	12	destroyed
Formalin	1,2000 72 hours	destroyed
	1,4000 96 hours	destroyed
Chloroform	25% v/v	destroyed
Ether	50% v/v	retained

TABLE 3.3d

COMPARISON OF BIOCHEMICAL AND BIOPHYSICAL PROPERTIES OF HAV, HBsAg AND M2Ag.

	HBsAg	HAV	M2Ag
Size and morphology	20-25nm spherical particles	25nm unenveloped	20nm and 40nm spherical particles
Molecular weight	3.5 - 4.5 10^6 daltons		3.5 - 4.5 10^6 daltons
Density in CsCl	1.19-1.21g/ml	1.34-1.36g/ml	1.24-1.25g/ml 1.27-1.31g/ml*
Sedimentation coefficient	110 'S'	160 'S'	118-135 'S'
Polypeptides	22-26,000 25-32,000 38,000 49,000 69,000 97,000	6-14,000 21-23,000 26-27,000 31-33,000	21,000* 25,000 32,000* 63,000 43,000* 83,000 58,000* 91,000
Stability			
100 C 5 minutes		inactivated	inactivated
60 C 10 hours	inactivated	mostly inactivated	resistant
60 C 1 hour	resistant	mostly resistant	resistant
50 C 1 hour	resistant	resistant	resistant
4 C months	resistant	resistant	resistant
-20 C years	resistant	resistant	resistant
pH 2			inactivated
4		resistant	inactivated
10			partially inactivated
12			inactivated
Ether	resistant	resistant	resistant
Chloroform			inactivated
Formalin	inactivated		inactivated

* indicates protease treated M2Ag

Although a total of I2 fusions were attempted to produce monoclonal anti-M2, only 2 anti-M2 secreting hybridomas were obtained. However, these proved to be unstable and were subsequently lost.

3.4 DISCUSSION

Preliminary biochemical and biophysical characterisation of M2Ag has indicated that it is distinct from other reported serological markers for non-A, non-B hepatitis. The estimated molecular weight of 3.5 - 4.5×10^6 daltons, precipitation by 6 percent PEG 6,000 and a sedimentation coefficient of II8 S- I35S all suggest the possiblity of an association with a particulate structure. Electron microscopy has revealed the presence of 20nm-40nm particles similar to 22nm HBsAg spherical particles. There is limited evidence to indicate that these structures are aggregated by anti-M2 in I.E.M.

Polypeptide analysis revealed four polypeptides which were undetectable in normal human serum, but consistently present in M2Ag positive sera regardless of the antigen source. The molecular weight range is similar to polypeptides reported for HAV and HBV. Likewise, the stability of M2Ag to heat and pH resembled that of HAV and HBsAg. Of interest is the difference in buoyant densities in CsCl between protease treated and untreated material, whereas the sedimentation coefficients for both preparations were similar. The reason for this is not clear.

M2Ag was no longer detectable following formalin treatment under similar conditions to those used by Tabor and Gerety (I980) who reported that a I:2,000 dilution of formalin both inactivated and possibly destroyed antigenic activity

(as evidenced by lack of subsequent immunity) of an agent of non-A, non-B hepatitis which was known to be infectious for chimpanzees.

Treatment with ether did not affect M2Ag activity whereas chloroform destroyed M2Ag activity. Although both chemicals are lipid solvents, chloroform also denatures proteins and it is possible that this treatment destroyed the antigenic site. Chloroform treatment has been reported to destroy the infectivity of the tubule forming agent of non-A, non-B hepatitis (Bradley et al I983b).

The lack of nucleic acid in the gradient purified fractions studied may indicate that infective particles, if present, are present in very low concentrations. Alternatively they may band at a different buoyant density to the M2Ag bearing particles. If M2Ag is an antigenic determinant on the infective particles it is likely that their concentration is too low to be detected by the current M2Ag EIA.

Accumulating evidence suggests that if M2Ag is associated with a particulate proteinaceous structure of viral origin, it may represent excess non-infectious coat material being a similar phenomenon to that exhibited by 22nm HBsAg spheres and filaments. M2Ag is immunogenic in animals. Activity is retained after removal of anti-human activity confirming that M2Ag is not a component of normal human serum.

Failure to infect marmosets with the 'H' strain inoculum or M2Ag positive sera obtained from acute phase patients and an implicated donor lends support to the findings of Tabor *et al* (I979b) that marmosets are not a suitable animal model for infectivity studies, at least for the 'H' strain (and it would appear M2Ag associated) non-A, non-B disease. The finding that all sera were negative for M2Ag and anti-M2 also supports the lack of non-specificity of the M2 marker assays. If M2Ag is related to an agent of non-A, non-B hepatitis then it appears that the marmoset is also an unsuitable animal model for transmission studies of this agent.

PART FOUR

EPIDEMIOLOGICAL STUDIES

4.I INTRODUCTION

Non-A, non-B viral hepatitis was first recognised as a transfusion complication (Prince et al I974), but is now known to occur worldwide, apparently transmitted by both parenteral and non-parenteral routes (Alter et al I975, Knodell et al I975, Villarejos et al I975, Dienstag et al (I977a). The prevalence of M2 markers in the normal donor population in South-East Scotland was established and compared with apparently normal donors from Tanzania, Greece and Taiwan, where the prevalence of HBV and presumably non-A, non-B hepatitis agents are higher (Bagshawe et al I97I, Hadziyannis et al I972 and Simons et al I972).

There have been reports of presumed non-A, non-B hepatitis (determined by elevated serum ALT levels) among patients undergoing regular plasmapheresis (Guyer et al I979, Martini I979). Mijović et al (I983) have suggested that ALT elevations in North London plasmapheresis donors could be influenced by factors such as alcohol intake and obesity. During the course of this research some plasmapheresis donors at the Edinburgh Centre exhibited mildly elevated serum ALT values and were investigated for evidence of M2 markers, the prevalence being compared to other plasmapheresis donors (at the Edinburgh Centre) whose ALT values remained within the normal range.

HBsAg negative blood donors with a history of jaundice within the previous ten years in East Scotland exhibit on average a ten-fold higher titre of specific anti-HAV activity (Hopkins I98Ib). From I977, persons negative for HBsAg with a history of jaundice (not within the previous I2 months) were accepted as blood donors in South-East Scotland. This group were studied for the presence of M2 markers, as they could represent a sub-population of blood donors at high risk of past exposure to one of the agents of non-A, non-B hepatitis.

An isolated outbreak of viral hepatitis amongst employees on a North Sea Oilrig was investigated, and serum from the patients studied for the presence of M2 markers.

Hepatitis B virus and at least one of the non-A, non-B agents are primarily transmitted by parenteral routes. The prevalence of M2 markers were assessed in various local patient groups (haemophiliacs, drug addicts, HBsAg carriers, prisoners, patients attending a clinic for sexually transmitted diseases, patients with chronic renal failure and institutionalised mentally retarded patients) considered to be at high risk of exposure to HBV compared to the local donor population.

Haemophiliacs are exposed to large numbers of blood donations by virtue of receiving multiple intravenous infusions of products containing either Factor VIII or Factor IX plasma proteins of which they are genetically

deficient. Frequent prophylactic infusions of these proteins are essential to treat or prevent 'bleeding' episodes. Currently two products are available to provide necessary Factor VIII replacement therapy to patients with Haemophilia A (classic haemophilia). These are:-

(I) Cryoprecipitate antihaemophiliac factor (CRYO); a product rich in Factor VIII that is prepared from individual donations of blood or plasma. One unit of CRYO will represent a pool of 2-3 donors.

(2) Factor VIII concentrate, a lyophilised concentrate manufactured from 200-500 individual donations in the National Health Service. Commercial preparations may represent up to 2,000 donations.

Factor IX complex is a lyophilised concentrate manufactured from the supernatent during cryoprecipitation of fresh frozen plasma. This may represent a total of 200-500 in clinical donations in the National Health Service products, whereas commercial preparations may represent up to 2,000 donations.

The high risk of hepatitis B associated with intravenous replacement therapy using CRYO, Factor VIII or Factor IX complex is well documented among haemophiliacs (Hoofnagle et al I975, Gerety and Barker I976). Non-A, non-B hepatitis has been transmitted by both Factor VIII (Hruby and Schauff I978) and Factor IX complex (Wyke et al

I979). There is some suggestion that Factor VIII may harbour a 'short' incubation agent, while the disease associated with Factor IX complex tends to exhibit a longer incubation period.

Persistantly mild elevations in liver enzyme, particularly alanine aminotransferase (ALT) are common in haemophiliacs (Gerety and Barker I976, Gerety et al I980b, Hasiba et al I980, Cederbaum et al I98I). There is controversy regarding the aetiology of these elevated ALT. Among the proposed causes are HBV, non-A, non-B hepatitis agent(s) and possibly an immune response to antigenic proteins in therapeutic infusions (Spero et al I978, Gerety et al I980b, Myers et al I980). Several groups have studied liver biopsy sections from haemophiliacs with chronically elevated ALT (Mannucci et al I975, Lesesne et al I977, Schimpf et al I977, Preston et al I978, Spero et al I978). Results of these studies indicate a significant degree of histologically confirmed liver disease is common in this group.

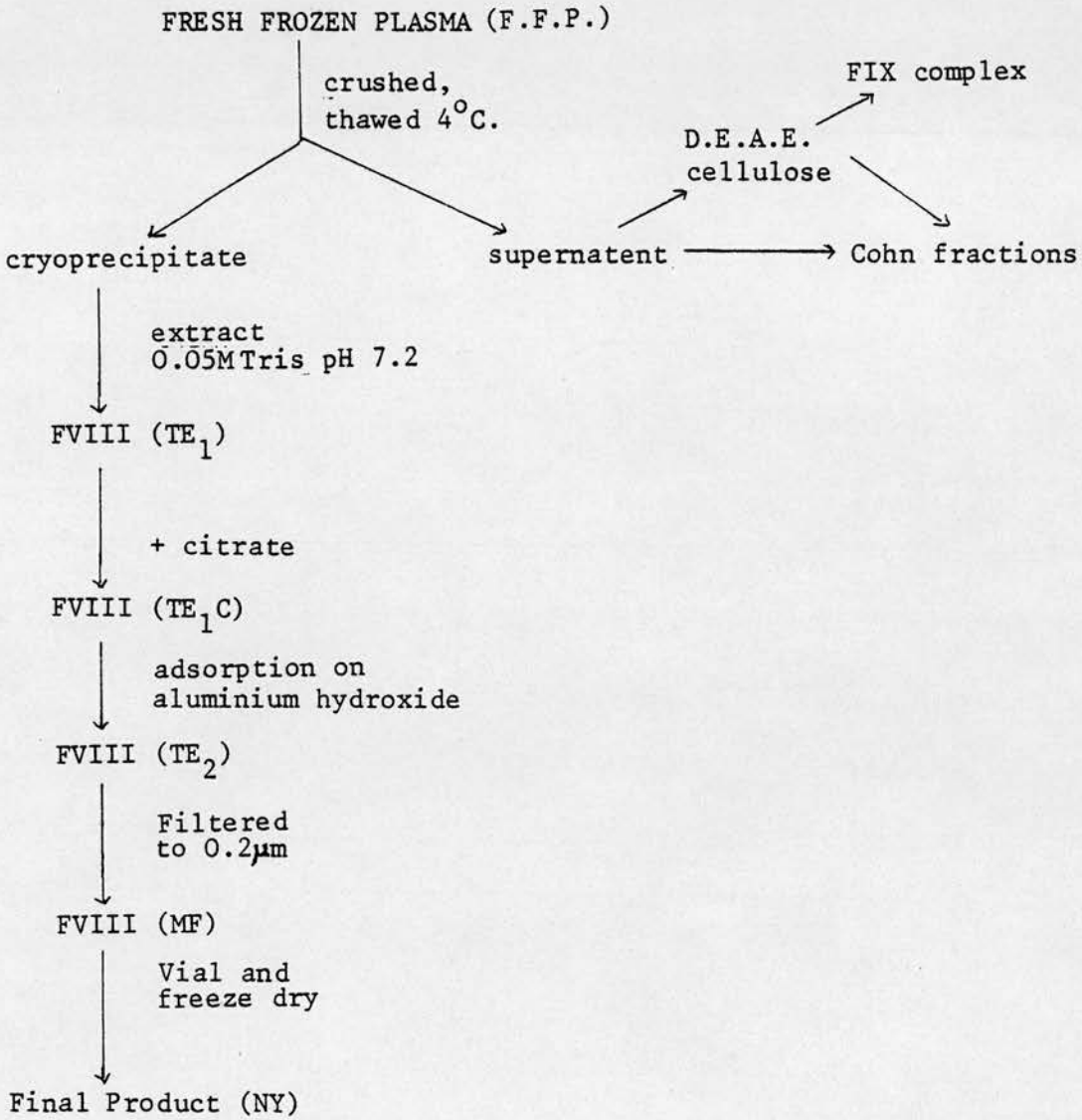
The introduction of blood donor screening for HBsAg has effectively reduced the number of HBV infections resulting from therapy. However, there is an urgent requirement for a means of protecting these patients from exposure to non-A, non-B hepatitis agent(s). The most effective means would be to screen donations for markers of non-A, non-B infectivity. In the absence of such tests prevention by passive immunisation with

normal immune globulin has been shown to be effective in some studies. However, to obtain maximum efficiency from such a policy it is necessary to identify specific protective antibody in blood donors, analogous to the donor selection process that presently exists for production of hepatitis B immune globulin. If the disease occurs sufficiently often to ensure a high frequency of specific antibody in the general population who also contribute to plasma, analogous to HAV, donor screening for specific antibody would be unnecessary.

The prevalence of M2 markers in blood products, including immune serum globulin, was estimated and an attempt made to relate this to occurrence of ALT elevations and M2 markers observed in the Edinburgh haemophiliac population. Figure 4.Ii provides a diagrammatic representation of the process involved in the manufacture of Scottish blood products.

FIGURE 4.1i

SCHEMATIC REPRESENTATION OF THE PREPARATION OF BLOOD PRODUCTS



Yield ≡ 200 U/kg F.F.P.

FVIII - Factor VIII
FIX - Factor IX

4.2 MATERIALS AND METHODS

Assays for M2Ag, anti-M2 IgM, anti-M2 IgG and immune complexes were performed as described in Part 2. HBsAg status was determined by RIA (Kane et al I983), anti-HBc IgM by EIA (Field I983), HAV status by an anti-HAV IgM RIA (HAVAB-M Abbott Laboratories) cytomegalovirus infection by complement fixation (Cremer et al I975) and anti-EBV IgM by immunofluorescence (Edwards I982).

SERA TESTED

Healthy HBsAg negative blood donors.

(a) I097 sera from blood donors resident in South-East Scotland. In this region approximately 60 percent of donors are female and approximately 70 percent of donors are between 25 and 40 years of age.

(b) I00 sera from blood donors resident in South-East London, kindly supplied by Mr. I. Cayzer, Wellcome Research Laboratories, Beckenham, Kent.

(c) 54 sera from blood donors resident in Tanzania, kindly supplied by Dr. M. Steele, M.R.C. Unit, Western General Hospital, Edinburgh.

(d) 79 sera from blood donors in Greece, kindly supplied by Dr. M. Sotiropoulou, Pireas, Greece.

(e) I00 sera from blood donors in Taiwan, kindly supplied by Mr. I. Cayzer, Wellcome Research Laboratories, Beckenham, Kent.

Other categories of blood donors.

(a) I00 sera from donors with a history of jaundice resident in South-East Scotland.

(b) 23 sera from plasmapheresis donors with raised serum ALT elevations and 8 sera from plasmapheresis donors who exhibited normal serum ALT values throughout this period.

(c) 20 sera from HBsAg positive donors resident in South-East Scotland.

Outbreak of viral hepatitis amongst staff on a North-Sea oilrig.

During the Autumn of I982 27 employees of a North Sea oilrig developed signs of viral hepatitis ranging from overt jaundice, with raised alanine aminotransferase (ALT) serum levels to mild flu-like illness. Sera from a further 4I asymptomatic employees on the same rig were used as 'controls'. All patients recovered uneventfully and appeared clinically well.

Populations with an increased risk of exposure to hepatitis B virus.

(a) I83 sera from short-term, young adult prisoners

attending H.M.P. Saughton, Edinburgh. As the sera were obtained at a routine blood donation session information about intravenous drug abuse or homosexuality was not available.

(b) 67 sera from patients with chronic renal failure treated by dialysis and transplantation, kindly provided by Dr. R. Winney, Consultant Physician, Medical Renal Unit, Royal Infirmary, Edinburgh.

(c) 87 sera from patients attending a clinic for sexually transmitted diseases kindly provided by Dr. J. Peutherer, Bacteriology Department, Edinburgh University Medical School. Data regarding sex and/or sexual proclivity of the patients were unobtainable.

(d) I00 sera from institutionalised mentally retarded patients, kindly provided by Dr. J. Peutherer, Bacteriology Department, Edinburgh University Medical School. Of these patients 29 percent were male, 7I percent female. The majority (72 percent) were admitted between I965 and I980, while the remainder(II percent)were day patients.

(e) 60 sera from patients with 'bleeding' disorders (Haemophilia A, Christmas Disease, von Willebrands Disease) kindly provided by Dr. C.A. Ludlam, Consultant Physician, Department of Haematology,

Royal Infirmary, Edinburgh.

(f) 50 sera from intravenous drug abusers in Edinburgh seeking medical advice for conditions other than acute hepatitis, kindly provided by Dr. J. Peutherer, Bacteriology Department, Edinburgh University Medical School. Fifty-eight percent of the patients were male, 34 percent female. No details were available on the remaining 8 percent.

Blood products and components.

(a) 52 batches of cryoprecipitate prepared at the Edinburgh Blood Transfusion Centre.

(b) 6I batches of Factor VIII, manufactured at the Scottish National Protein Fractionation Centre, Liberton, Scotland, between I982 and March I984.

(c) 9 batches of commercial Factor VIII manufactured during I983.

(d) 56 batches of Factor IX, manufactured at the Scottish National Protein Fractionation Centre, Liberton, Scotland, between I982 and I984.

(e) 5I batches of normal serum immune globulin manufactured at the Scottish National Protein Fractionation Centre, Liberton, Scotland, between I970 and I984.

(f) IO batches of hepatitis B immune globulin manufactured at the Scottish National Fractionation Centre, Liberton, Scotland, between I974 and I984.

Studies of haemophiliacs.

Following the initial testing of haemophiliacs for markers of M2, serial serum samples from 7 haemophiliacs were studied for evidence of M2 markers and serum ALT for periods of up to 6 months. The study included 6 patients with haemophilia A, all positive for anti-HBs and anti-HBc, and I patient with Christmas Disease negative for markers of HBV at the start of the study. The treatment each patient underwent during the course of the study is listed below:

Patient ARC. No treatment.

Patient ALL. February, April, June, August, September, November, December I983 (Scottish Factor VIII).

Patient McG. January, February, September, October, November I983 (Scottish Factor VIII).

Patient WAR. No treatment.

Patient LYO. August to November I983 (Scottish Factor VIII).

Patient McF. No treatment.

Patient MAL 29.9.83 to I2.IO.83 (Scottish Factor IX).

During the course of this study 6 haemophiliacs with a well-defined hepatitis episode were documented. Where possible, serial serum samples were obtained from these patients and implicated products tested for M2 markers. Individual case histories are presented below:-

Patient D.H.

Patient D.H. is a I7 year old male suffering from mild haemophilia A. He received his first treatment of Scottish Factor VIII at 5 years of age and is treated approximately twice a year. He received 5,500 units of Scottish Factor VIII in January I983 and II weeks later became mildly jaundiced (no previous history of jaundice), with dark urine and malaise. Previous ALT estimations had been normal apart from one episode in I979. Serum from this episode was unavailable.

Patient W.M.

Patient W.M. was a 36 year old male suffering from mild haemophilia A. He received an isolated course of Scottish Factor VIII of I,000 units twice a day for 4 days for haematuria, involving 20 vials NHS Intermediate Factor VIII Batch 704 and I vial NHS Intermediate Factor VIII Batch 70I. He became jaundiced I5 days later with serum ALT levels of greater than 5,000 IU/l.

Patient D.M.

Patient D.M. is a 56 year old male suffering from von Willebrands Disease. He had received regular Scottish Factor VIII therapy from childhood. In July I982 he exhibited a mild increase in serum ALT and alkaline phosphatase levels. Two months previously liver function tests had been normal.

Patients J.A., T.T., and P.W.

All three patients are males suffering from haemophilia A and receive regular Factor VIII therapy. They were exposed to the same batch of commercial Factor VIII in November I980.

4.3 RESULTS

Healthy negative blood donors.

Comparative prevalence values for M2 markers are presented in Table 4.3a. The combined prevalence of M2Ag and/or specific IgM was least (4.7 percent) in donors from South-East Scotland, 2 percent possessed anti-M2 IgG. Markers indicative of recent involvement with M2Ag doubled in donors from South-East London, at 9 percent while anti-M2 IgG increased four-fold to 8 percent.

In donors from both Greece and Tanzania the prevalence of M2Ag and/or anti-M2 IgM was 43.6 percent and 37.I percent respectively; significantly higher than in the U.K. Both areas had a low prevalence of anti-M2 IgG (I.3 percent and 0.0 percent respectively). The prevalence of M2Ag and/or anti-M2 IgM among the I00 HBsAg negative donors from Taiwan was 24.0 percent, I5.0 percent possessed anti-M2 IgG.

Other categories of blood donors.

Comparative prevalence values for M2 markers are presented in Table 4.3b. Donors with a history of jaundice showed a high prevalence (II.0 percent) of anti-M2 IgM compared with the local donor population despite the absence of M2Ag and anti-M2 IgG in the I00 sera tested.

Among the plasmapheresis donors, there was no significant

TABLE 4.3a

PREVALENCE OF M2 IN HEALTHY HBsAg NEGATIVE BLOOD DONORS

REGION	NUMBER TESTED	NUMBER OF SERA POSITIVE FOR							
		M2Ag ONLY	(%)	ANTI-M2IgM ONLY	(%)	ANTI-M2IgG ONLY	(%)	M2Ag + ANTI-M2	(%)
SOUTH-EAST SCOTLAND	1097	21	(1.9)	29	(2.6)	22	(2.0)	2	(0.2)
SOUTH-EAST LONDON	100	7	(7.0)	1	(1.0)	8	(8.0)	1	(1.0)
TANZANIA	54	19	(35.2)	1	(1.9)	0	(0.0)	0	(0.0)
TAIWAN	100	23	(23.0)	1	(1.0)	15	(15.0)	0	(0.0)
GREECE	79	32	(41.0)	2	(2.6)	1	(1.3)	0	(0.0)

TABLE 4.3b

PREVALENCE OF M2 MARKERS IN OTHER CATEGORIES OF BLOOD DONORS.

DONORS	NUMBER TESTED	NUMBER OF SERA POSITIVE FOR: M2Ag only	(%)	Anti-M2 IgM only	(%)	Anti-M2 IgG only	(%)
HBsAg Positive	20	5	(25.0)	1	(5.0)	0	(0.0)
History of Jaundice	100	0	(0.0)	11	(11.0)	0	(0.0)
Plasma-pheresis donors with fluctuating ALT.	25	1	(4.0)	0	(0.0)	1	(0.0)
Plasma-pheresis donors with normal ALT.	8	0	(0.0)	0	(0.0)	3	(37.5)

increase in M2Ag and/or anti-M2 Ig Anti-M2IgG was present in donors with fluctuating (doubled) and those with normal serum ALT levels (approximately I8 times the background). Thirty percent of HBsAg positive donors showed evidence of M2Ag and/or anti-M2 IgM, the majority (25 percent) being reactive for M2Ag.

Outbreak of viral hepatitis among staff on a North Sea Oilrig.

Table 4.3c shows the presence of M2 markers in patient and control groups (a) before and (b) after sub-division based upon possession of normal or raised serum ALT levels at the time of sampling. It may be significant that of the 5 positive sera from the 6 'controls' with raised ALT 35 were involved with catering on the rig.

Populations with an increased risk of exposure to hepatitis B virus.

Evidence for a significantly greater exposure to M2Ag was found in all patient groups studied (Table 4.3d). Prisoners and renal patients and intravenous drug abusers showed a predominance of M2Ag and/or specific IgM, whereas patients attending a clinic for sexually transmitted disease, institutionalised mentally retarded patients and haemophiliacs, upon initial sampling, exhibited an increased prevalence of anti-M2 IgG compared to the local donor population.

Blood products and components.

Prevalence of M2 markers in blood components and blood

TABLE 4.3c

DETECTABLE M2 MARKERS IN PATIENT AND CONTROL GROUP FROM AN OUTBREAK OF VIRAL HEPATITIS ON A NORTH SEA OILRIG.

			Number of sera positive for				
		Number tested	M2Ag only	Anti-M2-IgM only	M2Ag +IgM	Anti-M2-IgG	Total M2 Involvement
(a)	Patient group	27	1	7	1	0	33%
	Control group	41	1	6	0	0	17%

(b) Following sub-division based upon possession of normal or raised serum ALT levels.

		Number of sera positive for				
	Number tested	M2Ag only	Anti-M2-IgM only	M2Ag +IgM	Anti-M2-IgG	Total M2 Involvement
Patients raised ALT	7	0	2	1	0	43%
Patients normal ALT	20	1	5	0	0	30%
Controls raised ALT	6	1	4	0	0	83%
Controls normal ALT	35	0	2	0	0	5.7%

TABLE 4.3d

DISTRIBUTION OF M2 SEROLOGICAL MARKERS IN GROUPS AT HIGH RISK OF HBV INFECTION.

Group	Number tested	M2Ag only (%)	Anti-M2 IgM (%)	Anti-M2 IgG (%)	M2Ag+ M2Ab	Anti HBV (%)
Prisoners	183	13 (7.0)	21 (11.4)	0 (0.0)	3 (1.6)	5 (2.7)
Renal Patients	67	18 (26.9)	4 (6.0)	2 (3.0)	4 (6.0)	
I.V. Drug Abusers	50	30 (60.0)	0 (0.0)	3 (6.0)	3 (6.0)	
STD Clinic Patients	87	2 (2.3)	2 (2.3)	34 (39.1)	3 (3.4)	0 (0.0)
Institution-alized mentally retarded patients	100	5 (5.0)	2 (2.0)	46 (46.0)	2 (2.0)	20 (20.0)
Haemophiliacs	60	1 (1.7)	0 (0.0)	34 (56.7)	10 (16.7)	54 (90.0)

I.V. = Intravenous (drug abusers)

S.T.D. = Sexually Transmitted Disease.

products is presented in Table 4.3e. Testing of 52 batches of cryoprecipitate manufactured at the Edinburgh Blood Transfusion Centre revealed 9.6 percent of the batches were positive for M2Ag, I.9 percent reactive for anti-M2 IgM, and II.5 percent reactive for anti-M2 IgG compared with approximately 4.7 percent of donations positive for M2Ag and/or anti-M2 IgM with 2 percent reactive for anti-M2 IgG. Since each unit of cryoprecipitate is manufactured from 2 to 3 donations these figures are compatable with the expected prevalence rates.

The majority of Factor VIII or Factor IX manufactured at the Scottish National Protein Fractionation Centre possessed detectable M2Ag, present in 8I.9 percent of Factor VIII batches tested and 78.6 percent of Factor IX batches tested. However, analysis of T:N ratios for the positive batches showed a difference in the distribution with the Factor VIII batches being skewed towards the strongly reactive (T:N of II or greater), while the distribution within the Factor IX batches was more or less even (Table 4.3f and Figure 4.3i). Testing of 9 batches of commercial Factor VIII manufactured in I983 showed an absence of M2Ag. However, anti-M2 IgG was detected in 7 of 9 batches tested (77.7 percent). This contrasted markedly with I00 percent positivity for M2Ag among 5 batches tested in I980. (Hopkins, R. personal communication I983).

Anti-M2 IgG was present in 72.5 percent of normal serum

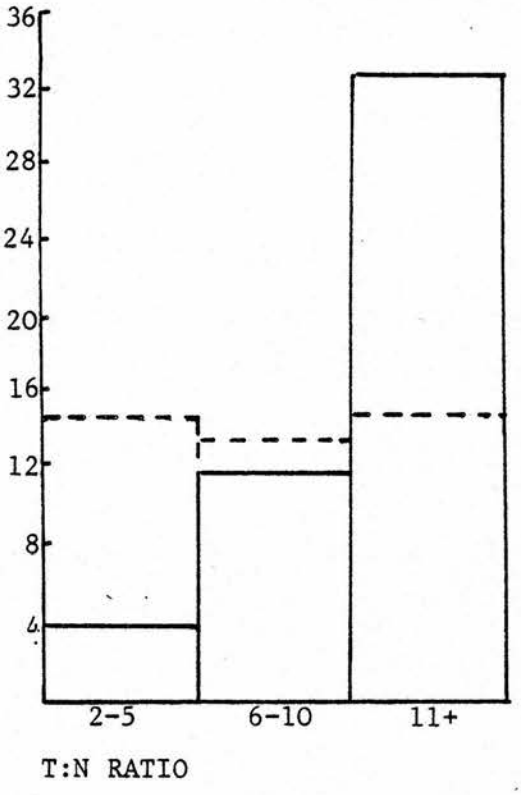
TABLE 4.3e

PREVALENCE OF M2 MARKERS IN BLOOD COMPONENTS AND BLOOD PRODUCTS

COMPONENT/ PRODUCT	NUMBER TESTED	Number of samples positive for M2Ag	(%)	M2IgM	(%)	M2IgG	(%)
Cryoprecipitate	52	5	(9.6)	1	(1.9)	6	(11.5)
NHS FVIII	61	50	(81.9)	0	(0.0)	0	(0.0)
NHS FIX	56	44	(78.6)	0	(0.0)	0	(0.0)
NSIG	51	0	(0.0)	0	(0.0)	37	(72.5)
HBSIG	10	0	(0.0)	0	(0.0)	6	(60.0)
Commercial FVIII 1983	9	0	(0.0)	0	(0.0)	7	(77.7)

FIGURE 4.3i

T:N DISTRIBUTION FOR M2Ag IN SCOTTISH FACTOR VIII AND FACTOR IX CONCENTRATES



——— FACTOR VIII

- - - - FACTOR IX

T:N $\frac{\text{O.D. (410nm) of test sample}}{\text{O.D. (410nm) of negative control}}$

TABLE 4.3f

T:N DISTRIBUTION OF M2AG IN BLOOD PRODUCTS

Product	Number tested	T:N ratios		
		2-5	6-10	Over 11
NHS Factor VIII	49	4	12	33
NHS Factor IX	44	15	14	15

immune globulin tested. Analysis of P:N ratio and the year showed a fluctuation of the antibody concentration with an estimated 5 year periodicity. A similar trend was seen for anti-M2 IgG in hepatitis B immune serum globulin batches over the same period (Figure 4.3ii, Tables 4.3g and 4.3h).

Studies of haemophiliacs.

Initial screening of sera from Edinburgh haemophiliacs revealed a high positivity of anti-M2 IgG (74 percent). Study of serum samples taken over a period of several months from 7 haemophiliacs confirmed the findings of other authors (Gerety and Barker I976, Gerety et al I980b, Hasiba et al I980, Cederbaum et al I98I) of fluctuating serum ALT with the majority of values above the upper limit of normal. (Figures 4.3iii - ix). The length of each episode varied markedly from one to four months. Markers of M2 were present in all but one haemophiliac studied. M2Ag was detected at or near the peak ALT with apparent 'cycling' between M2Ag and anti-M2 IgM. Anti-M2 IgG when present was transient usually appearing when ALT had declined but absent upon subsequent ALT elevations.

All haemophiliacs were positive for anti-HBs and anti-HBc apart from patient MAL who suffers from Christmas Disease. While the study was in progress he developed an acute HBV infection. Prior to this he had been M2Ag positive for 2 months with a transient seroconversion to anti-M2 IgG.

TABLE 4.3g

T:N RATIOS OF ANTI-M2IgG IN NORMAL SERUM IMMUNE GLOBULIN BETWEEN 1970 AND 1983.

YEAR	NUMBER TESTED	MEAN T:N RATIO
1970	4	3:13
1971	2	3.90
1973	2	1.25
1974	2	2.84
1975	2	10.25
1976	2	10.75
1978	3	5.67
1979	1	7.30
1980	5	2.38
1981	11	10.46
1982	4	4.70
1983	11	5.90

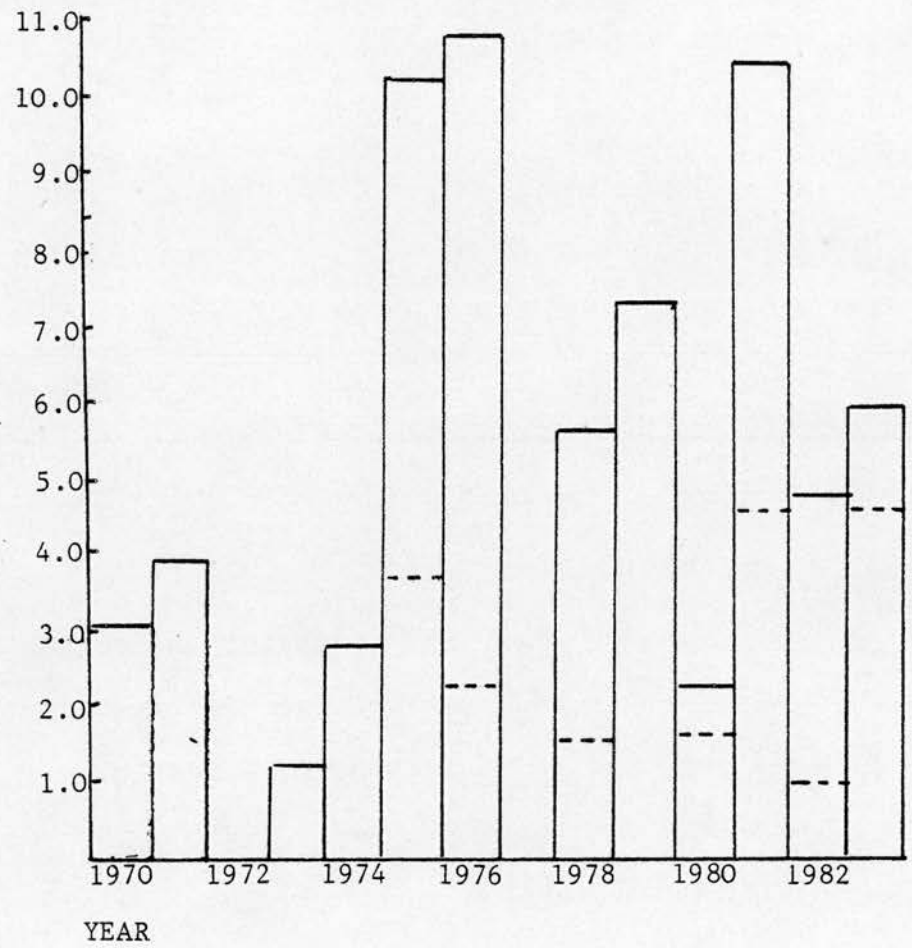
TABLE 4.3h

T:N RATIOS OF ANTI-M2 IgG IN HEPATITIS B IMMUNE GLOBULIN BETWEEN 1975 AND 1983.

YEAR	NUMBER TESTED	MEAN T:N RATIO
1975	1	3.67
1976	1	2.30
1978	1	1.60
1980	1	1.70
1981	5	4.60
1982	1	1.00
1983	2	4.60

FIGURE 4.3ii

ANTI-M21gG IN NORMAL IMMUNE SERUM GLOBULIN (-) AND HEPATITIS B IMMUNE GLOBULIN (-.- -) BETWEEN 1970 AND 1983



$$\text{T:N} \quad \frac{\text{O.D. (410nm) of test sample}}{\text{O.D. (410nm) of negative control}}$$

FIGURE 4.3iii

M2 MARKERS AND SERUM ALT VALUES EXHIBITED BY PATIENT ARC OVER A 5 MONTH PERIOD

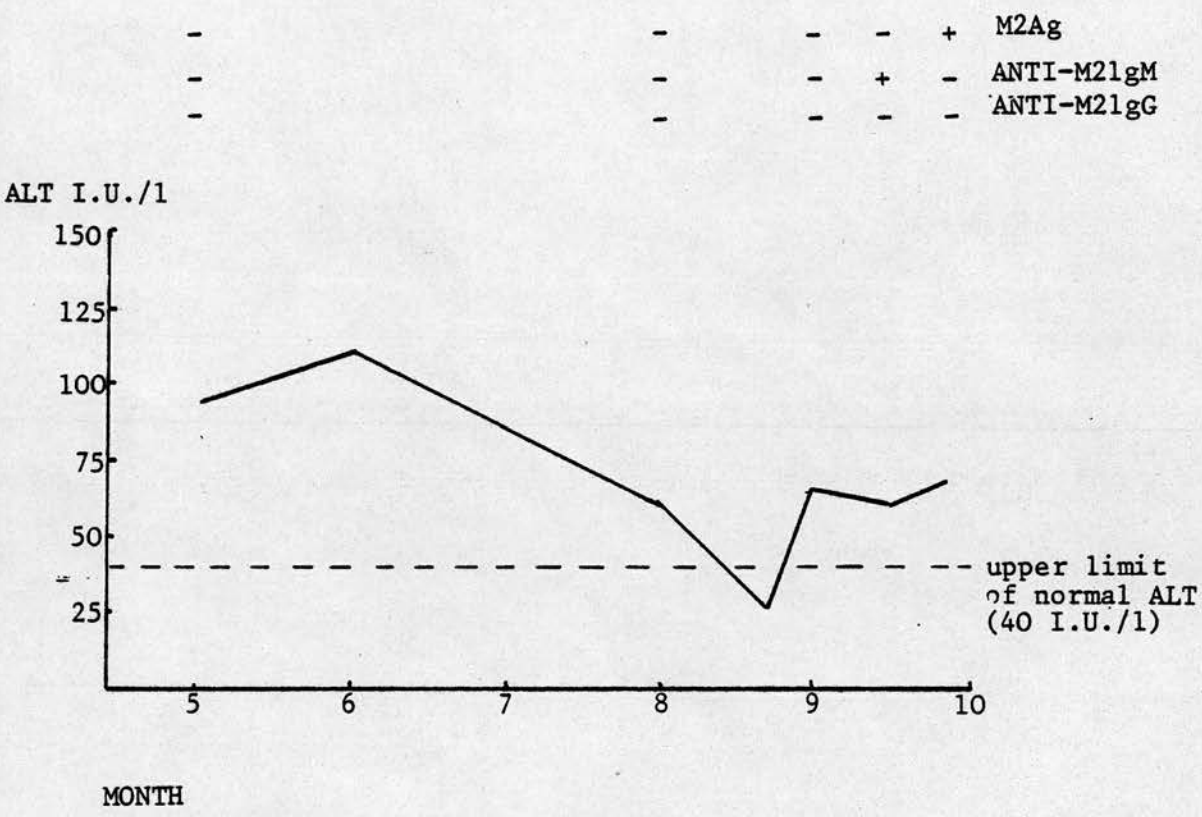


FIGURE 4.3iv

M2 MARKERS AND SERUM ALT VALUES EXHIBITED BY PATIENT ALL.

OVER A 4 MONTH PERIOD

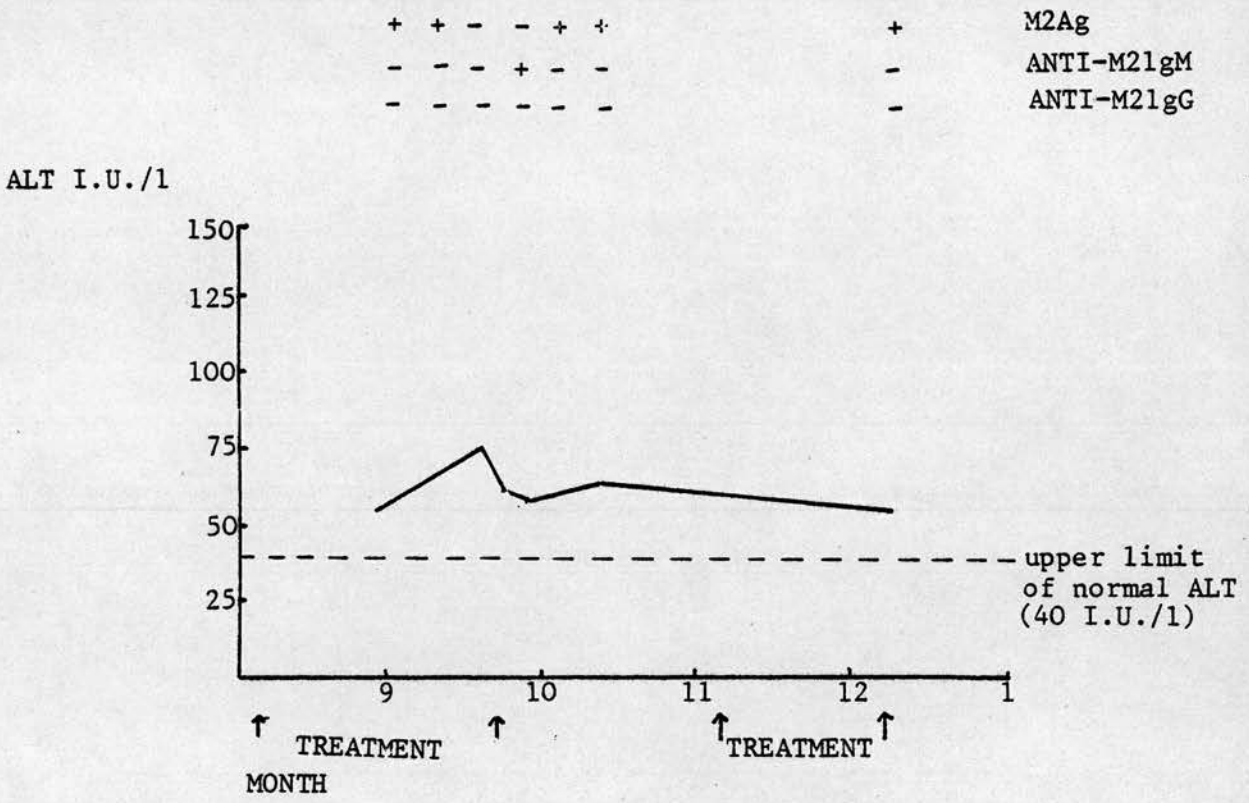


FIGURE 4.3v

M2 MARKERS AND SERUM ALT VALUES EXHIBITED BY PATIENT $M^{C}G$

OVER A 5 MONTH PERIOD

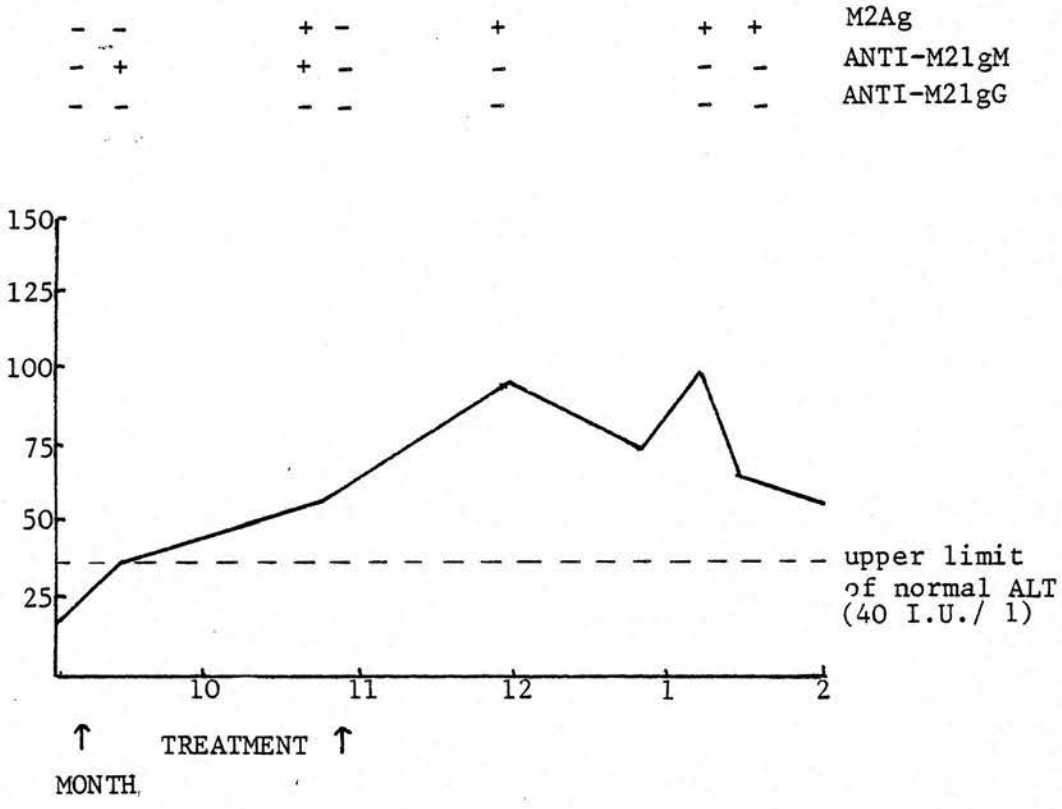


FIGURE 4.3vi

M2 MARKERS AND ALT VALUES EXHIBITED BY PATIENT WAR.

OVER A 3 MONTH PERIOD

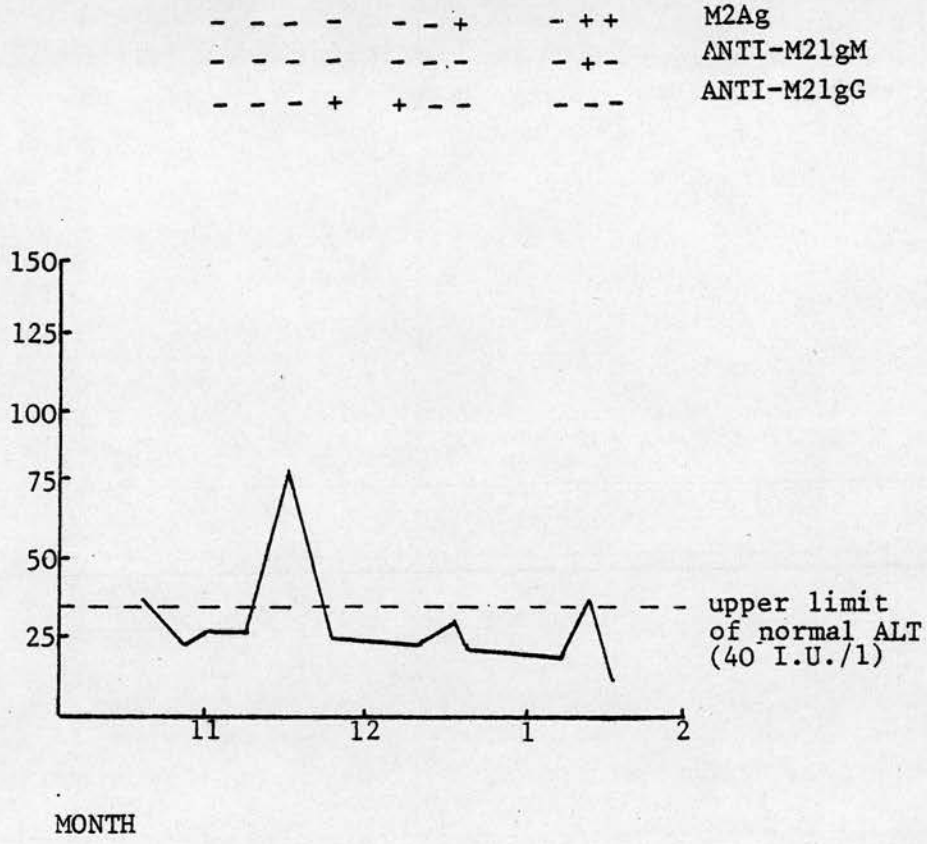


FIGURE 4.3vii

M2 MARKERS AND SERUM ALT VALUES EXHIBITED BY PATIENT LYO.

OVER A 6 MONTH PERIOD

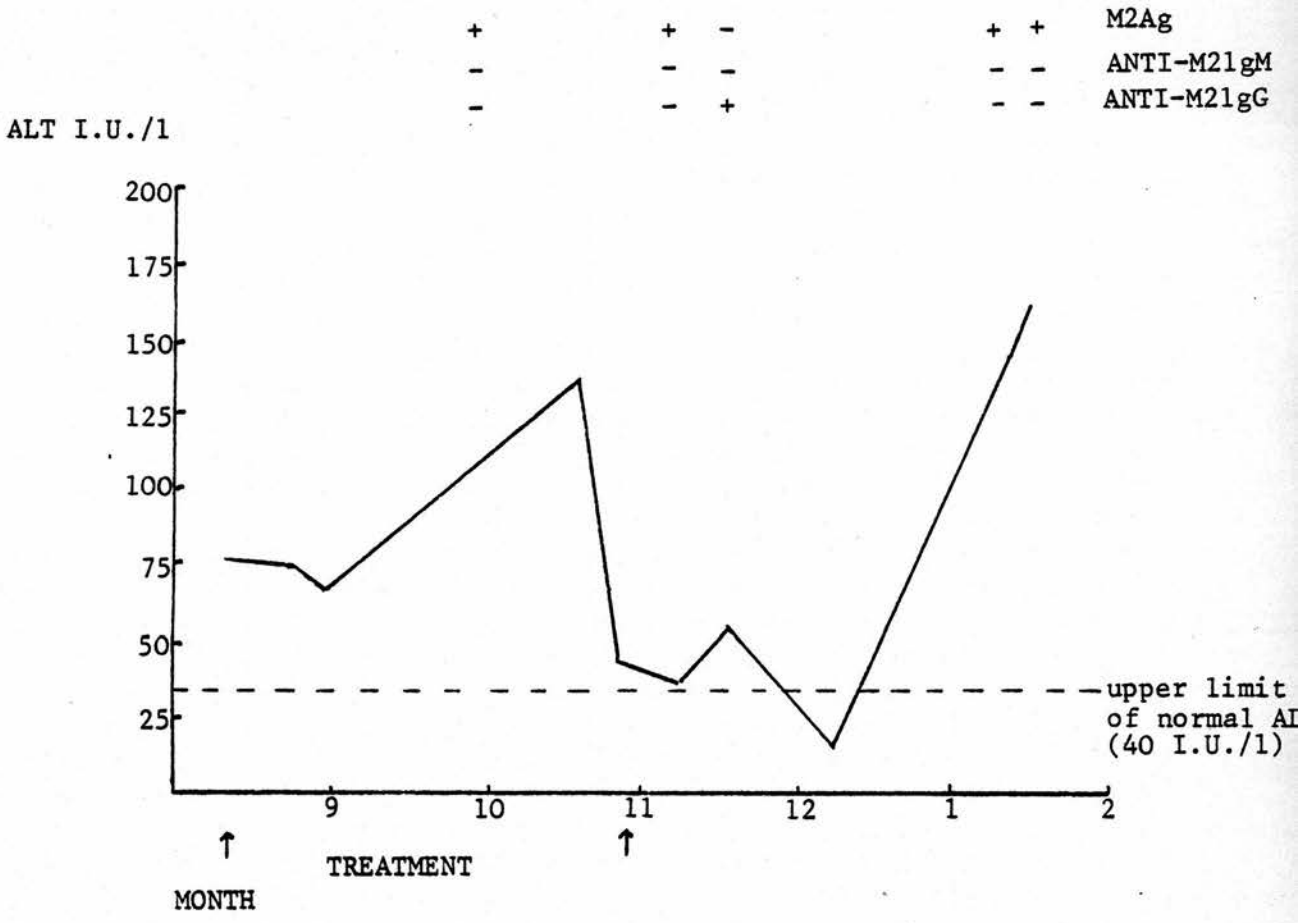


FIGURE 4.3viii

M2 MARKERS AND SERUM ALT VALUES EXHIBITED BY PATIENT McF OVER A 4 MONTH PERIOD

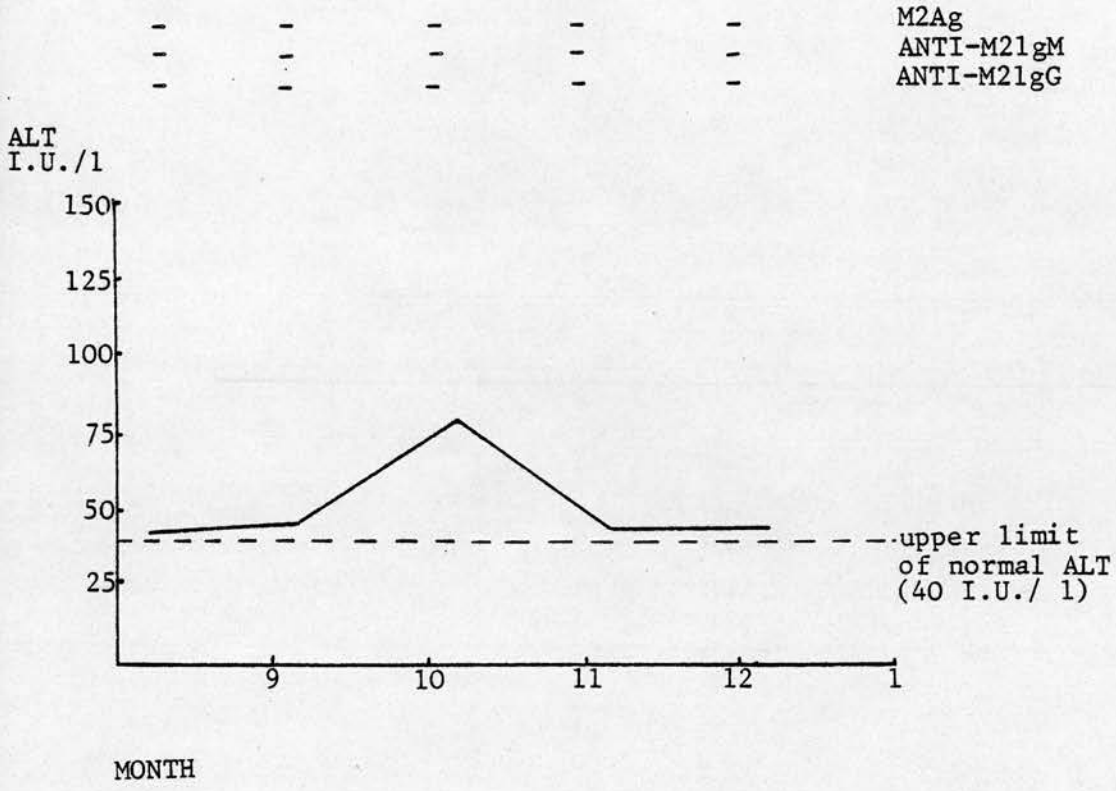
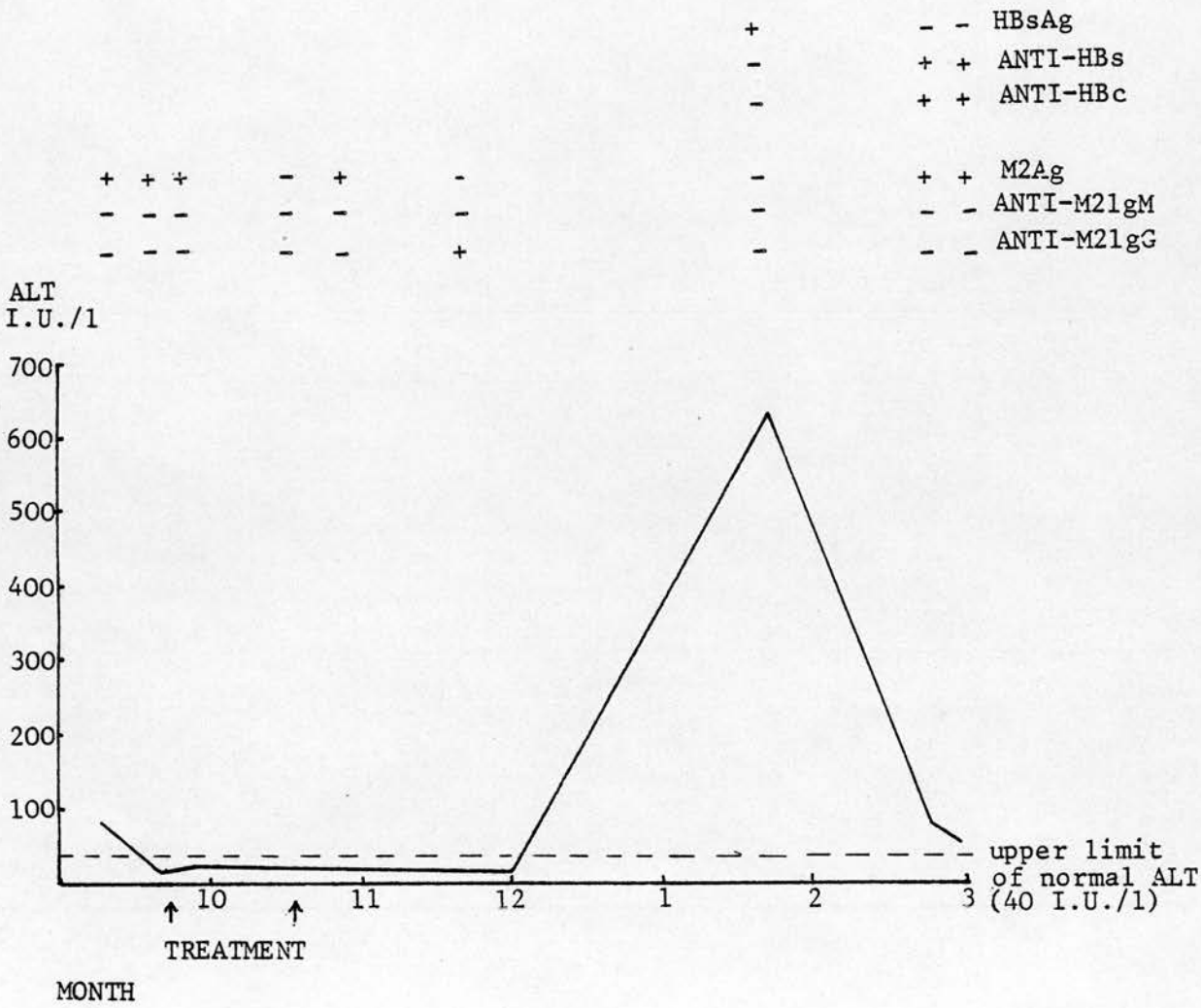


FIGURE 4.3 ix

HBV MARKERS, M2 MARKERS AND SERUM ALT VALUES EXHIBITED BY PATIENT MAL. OVER A 6 MONTH PERIOD.



At the onset of HBV infection M2 markers disappeared. A low level of M2Ag was found two weeks later. This is similar to observations of patient DH.

Studies of 'isolated' hepatitis events are presented below:-

Patient DH.

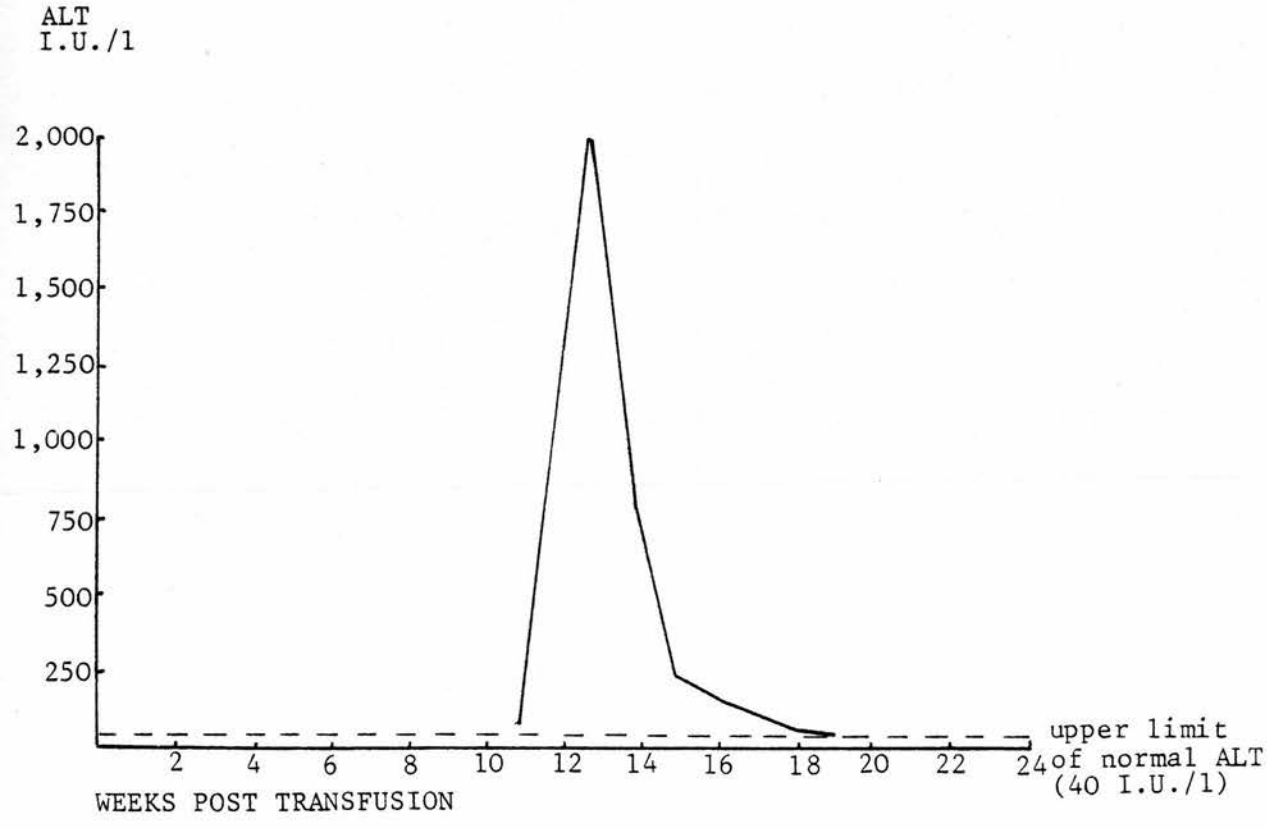
During the current episode (Figure 4.3x) patient DH became positive for anti-HBc IgM indicative of an acute HBV infection. Serum ALT values peaked at 2,055 IU/l at I3 weeks after treatment when he was positive for M2Ag and anti-HBc IgM. With the decline of serum ALT, the patient seroconverted to anti-HBs and transient anti-M2 IgG, with reappearance of M2Ag 3 weeks later. The incubation period is unusually long for that observed in an M2 related transfusion episode. It is probable that the single peak of raised ALT was due to HBV (with a non-A, non-B infection, bi- or multiphasic peaks are usually observed). However, the possibility of interference by HBV of M2 infection cannot be discounted. It is also possible that DH had become a carrier of M2Ag which could have resulted from the episode in I979 when the serum ALT first became elevated. HBV infection could have released M2Ag from the liver into the serum resulting in a reactivation of a latent M2 infection. This is supported by the rapid appearance of anti-M2 IgG 8 weeks after the appearance of M2Ag, indicative of an anamastic immune response. There was no evidence of CMV or EBV infection.

FIGURE 4.3x

HBV MARKERS, M2 MARKERS AND SERUM ALT VALUES FOR PATIENT D.H.

WEEKS POST TRANSFUSION	11	13	14	15	16	17	18	19	21	22
ALT I.U./1	54	2055	760	256	130	99	64	41	27	30
M2Ag	-	+	+	-	-	-	-	-	-	+
ANTI-M21gM	-	-	-	-	-	-	-	-	-	-
ANTI-M21gG	-	-	-	-	-	-	-	-	+	-

HBsAg	+	-	-	-	-	-	-	-	-	-
ANTI-HBs	-	-	+	+	+	+	+	+	+	+
ANTI-HBc	-	+	+	+	+	+	+	+	+	+
(1gM)										
M2Ag	-	+	+	-	-	-	-	-	-	+
ANTI-M21gM	-	-	-	-	-	-	-	-	-	-
ANTI-M21gG	-	-	-	-	-	-	-	-	+	-



Patient WM.

M2Ag was detected in his blood 3 weeks after treatment (Figure 4.3xi) subsequently disappearing with the appearance of anti-M2 IgM and anti-M2 IgG at 6 weeks after treatment. M2Ag reappeared at 9 weeks with the loss of antibody. Sera taken from this patient 3 years previously was reactive for anti-M2 IgG and M2Ag. Having recovered from this hepatitis event, the patient died 22 weeks later after a gastric bleed. During the period of acute hepatitis the patient exhibited anti-HBs with no evidence of infection with HAV, CMV or EBV. He had received no blood or blood products in the 6 months prior to Factor VIII treatment. This patient had shown a rapid appearance of anti-M2 IgG indicative of an anamastic response and retrospective testing of stored sera produced evidence of previous M2Ag exposure as early as three years prior to this event.

Patient DM.

M2Ag was present at I2 weeks after treatment followed by anti-M2 IgM 6 weeks later with the reappearance of M2Ag. (Figure 4.3xii). Anti-M2 IgG was present at week 22 but disappeared with the reappearance of M2Ag and anti-M2 IgM. The patient remained M2Ag positive at 35 weeks after treatment although the ALT had returned to the upper limit of normal. The patient had no previous history of jaundice and was positive for anti-HBs. There was no evidence of infection with CMV or EBV.

FIGURE 4.3xi

M2 MARKERS AND SERUM ALT VALUES FOR PATIENT W.M.

WEEKS POST TRANSFUSION	2	3	5	6	9	22
ALT I.U./1	5,000	2025	105	430	48	121
M2Ag	-	+	-	-	+	NT
ANTI-M21gM	-	-	-	+	-	NT
ANTI-M21gG	-	-	-	+	-	NT

NT- NOT TESTED

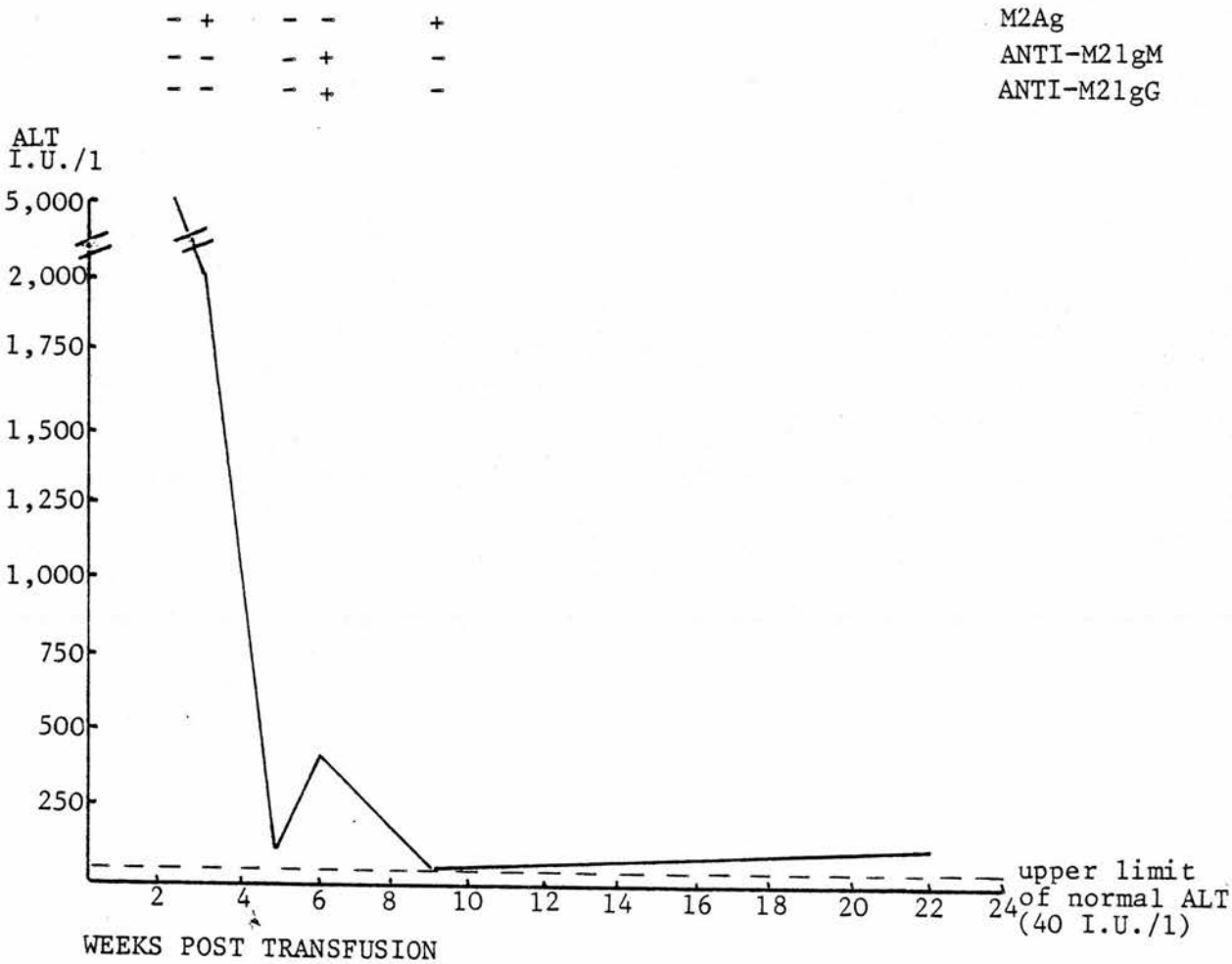
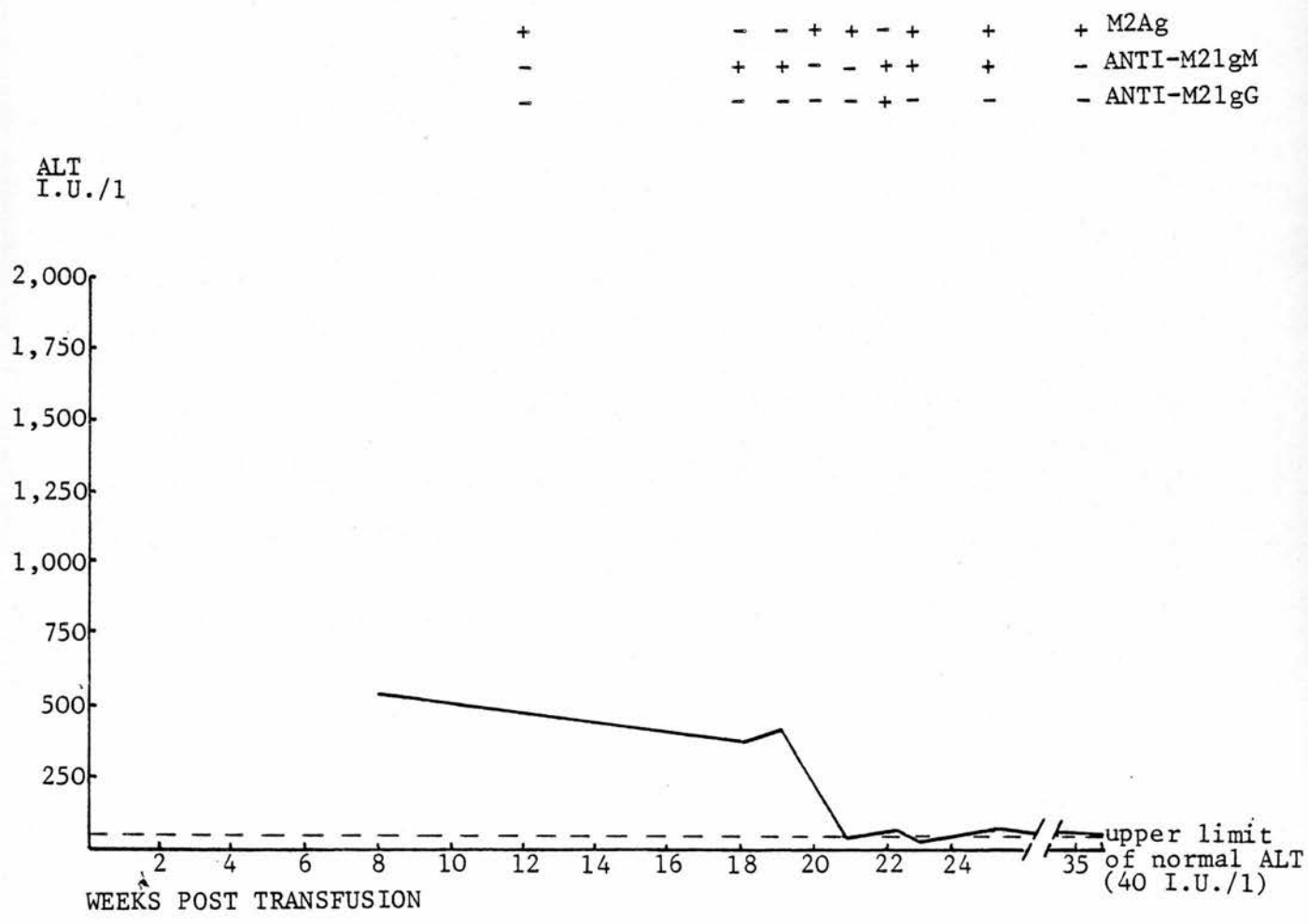


FIGURE 4.3xii

M2 MARKERS AND SERUM ALT VALUES FOR PATIENT D.M.

WEEKS POST TRANSFUSION	8	12	18	19	20	21	22	23	25	35	36
ALT I.U./1	590	NT	380	415	220	37	45	28	54	27	59
M2Ag	NT	+	-	-	+	+	-	+	+	+	-
ANTI-M21gM	NT	-	+	+	-	-	+	+	+	-	-
ANTI-M21gG	NT	-	-	-	-	-	+	-	-	-	-

N.T. - NOT TESTED



Serum was not available for examination before I2 weeks after treatment, leaving the possibility of M2Ag being present before week I2. Although anti-M2 IgG appeared 22 weeks after treatment, usually indicative of a 'first-time' exposure, it is also possible that this episode was a reactivation of a latent infection in view of the patient receiving regular Factor VIII therapy.

Patients JA, TT and JW.

Patient TT exhibited an acute attack of non-A, non-B hepatitis with a characteristic biphasic serum ALT (Figure 4.3xiii). Although patient PW also exhibited multiphasic serum ALT levels, the peaks were not as pronounced and occurred at longer intervals. Serum ALT in patient JA was only slightly elevated and remained so; however, sera was not available for testing between weeks 7 and 25. There was no evidence of M2 markers in this patient. TT was positive for anti-M2 IgM at week 5, M2Ag at week 7 and anti-M2 IgG at week 27, indicative of a 'first-time' exposure. Patient PW exhibited M2Ag throughout with fluctuations between anti-M2 IgM prior to the next ALT elevation. It is probable that PW is a chronic carrier of the antigen.

FIGURE 4.3xiii

M2 MARKERS AND SERUM ALT VALUES FOR PATIENTS J.A., T.T. AND P.W.

PATIENT J.A.

WEEKS POST TRANSFUSION	5	6	7	25
ALT I.U./l	200	150	130	120
M2Ag	–	–	–	NT
ANTI-M21gM	–	–	–	NT
ANTI-M21gG	NT	NT	NT	–

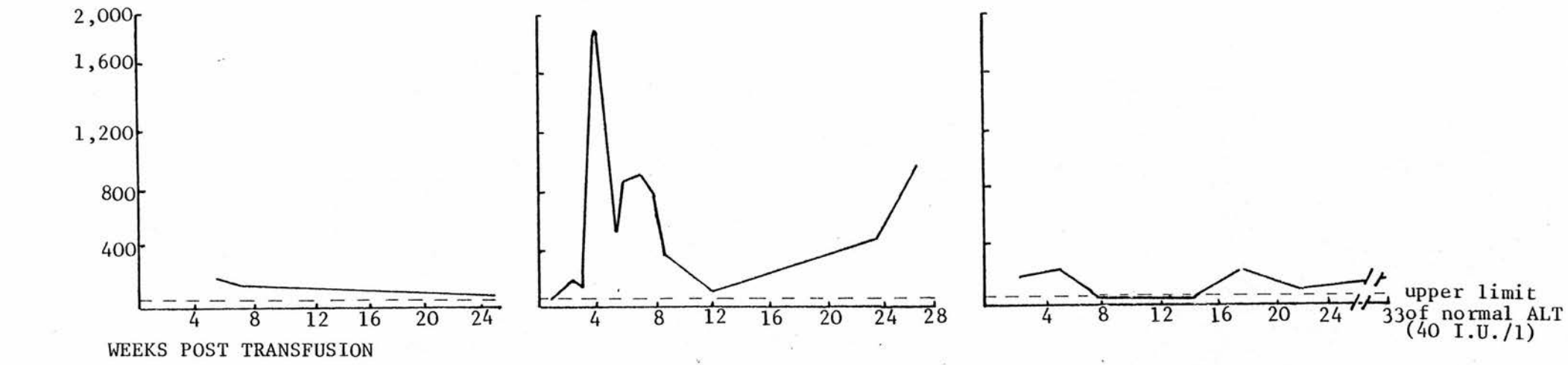
PATIENT T.T.

	2	5	7	25	27
	200	1080	895	485	870
	–	–	+	–	–
	–	+	–	–	–
	NT	NT	NT	–	+

PATIENT P.W.

2	5	8	15	18	22	33	
150	250	20	30	250	75	150	
+	+	+	+	+	+	+	+ M2Ag
+	–	–	+	–	+	–	– ANTI-M21gM
–	–	–	–	–	–	–	– ANTI-M21gG

–	–	–		NT	–	–	+	–	–	+	+	+	+
–	–	–		NT	–	+	–	–	–		+	–	–
NT	NT	NT		–	NT	NT	NT	–	+	–	–	–	–



NT — NOT TESTED

4.4 DISCUSSION

Donors from South-East London exhibit a higher prevalence of M2 markers compared to the donor population in South-East Scotland. The anti-HAV prevalence in the donor population in the two areas is approximately equal (80 percent): HBsAg prevalence is greater in the South-East London donor population that the South East Scotland donor population the prevalence being 0.2 percent and 0.02 percent respectively. This is probably the result of a greater influx of immigrants to London than Scotland.

In countries where the HBV carrier rate is high, the prevalence of M2 increased, the low level of anti-M2 IgM in Greece and Taiwan may be indicative of a carrier state for the antigen in these populations. (Although the prevalence of the blood group B also increased in these areas, there was no increased association between type B blood group and M2 markers).

The prevalence data obtained from the donors in Taiwan may be influenced by the exclusion of HBsAg positive sera. Approximately 20 percent of the donor population carry HBsAg (Beasley et al I983). It is probable that some HBsAg carriers will also show serological evidence of M2 markers, and these will have been excluded from testing.

Of interest is the finding that the prevalence of M2

markers increased in HBsAg positive donors in South-East Scotland. This may imply that the transmission of M2 is similar to those of HBsAg as it does appear that the prevalence of M2 markers in other donor populations (Greece, Tanzania, Taiwan) parallels that of HBV.

Donors with a history of jaundice in South-East Scotland exhibited a significantly higher prevalence of anti-M2 IgM than the local donor population. This could indicate that it is related to the cause of jaundice. However, it must be remembered that a history of jaundice does correlate well with a past HAV infection. Also donors were only accepted if the episode of jaundice occurred at least one year before. Evidence from the retrospective investigation of post transfusion non-A, non-B hepatitis suggests that persistance of anti-M2 IgM could indicate continued virus replication.

ALT elevations alone are often assumed to be indicative of a non-A, non-B hepatitis event. However, ALT elevations amongst Edinburgh plasmapheresis donors were not associated with any significant increase in the prevalence of M2 markers. HAV and HBV status also remained constant throughout the period of investigation. This is in keeping with the finding of Mijović et al (I983) who studied a group of 42 North London plasmapheresis donors. In the North London study, I6 of these donors were overweight, I2 had high alcohol intake and 4 were exposed to toxic chemicals. Similar observations were

not available for the Edinburgh patients. Of particular interest is the increase of anti-M2 IgG (although the numbers tested were small) in plasmapheresis donors with normal serum ALT levels. It is possible that regular plasmapheresis may regulate or enhance an underlying immune response since anti-HBs plasma donors usually retain high levels of anti-HBs despite frequent plasmapheresis (Hopkins, personal communication I983).

Findings from the outbreak of hepatitis on a North Sea oilrig are of interest since they allow serological study in an isolated self-contained community. Although there is a two-fold increase in the occurrence of M2 markers in the patient versus control group of oilrig workers, the I7 percent prevalence among the latter group is over three times that of the Scottish blood donor population. Subdivision of the control group based upon the level of serum ALT, however, indicates that 5 of 6 controls (83 percent) with raised ALT values possessed serological evidence of current or recent M2 involvement, while only 2 of the remaining 35 controls (5.7 percent) with normal ALT levels were positive for evidence of exposure to M2Ag, a figure comparable to that (4.5 percent) for blood donors in the South-East of Scotland. It is tempting to speculate that one or more of the M2 reactive asymptomatic controls (particularly those with raised ALT values) may represent carriers of M2 infectivity and a possible source of infection since 5 were involved with the catering service aboard the oilrig. A more obvious

parenteral spread of infection through the sharing of needles for intravenous drug abuse can be excluded, as rigorous preventative procedures are enforced among off-shore oilrig workers.

The finding that prisoners exhibited a significantly ($p > 0.001$) higher prevalence of M2Ag or anti-M2 IgM than blood donors andother patient groups studied (with the exception of renal patients) is in keeping with the previously reported studies of comparative HBsAg in prisoners (Chiaramonte et al I982). The absence of detectable anti-M2 IgG in these prisoners is hard to explain. As most donations tested originated from young adult short term prisoners it is possible that infection had occurred near the time of or during detention and that a detectable immune response had not developed. (Anti-M2 IgG is usually detected 4-6 months after primary exposure). A proportion of the young offenders could have committed drug-related offences. It is also possible that the prevalence of specific IgG may increase with age, as is the case with anti-HBs.

The relative frequent finding of M2Ag among intravenous drug addicts is not altogether surprising in view of the well recognised opportunities for the spread of blood borne infections among this group. Sixty percent of addicts tested in this area have been exposed to HBV. The finding that this was the group in which M2Ag occurred most frequently agrees with a recent report by Francis

et al I984 who defined drug abuse as the greatest risk factor associated with occurrence of non-A, non-B hepatitis in the U.S.A.

Prior to HBsAg screening, HBV infection was not uncommon in dialysis units. Development of a carrier state was almost certainly influenced by natural or treatment related underlying immune deficiency, a phenomenon which may similarly influence the pattern of M2 related infection and account for the significantly ($p > 0.00I$) higher prevalence of M2Ag in this group compared with local blood donors. There appears to be a trend following increased risk of exposure to M2Ag from:

(a) exposure to blood;

(b) transplantation;

(c) hospital dialysis.

This trend ($p > 0.I$) may become significant as more patients are studied. Of the 28 renal patients with M2 markers, 53.4 percent had received kidney transplants while 3I.4 percent of M2 negative renal failure patients had been transplanted (Table 4.4a and 4.4b). It is possible that transplant associated immunosuppressive therapy may account for the finding that all transplant patients exhibiting serological evidence of M2 markers possessed M2Ag. Furthermore 54.3 percent of M2 negative patients had received predominantly home dialysis treatment compared with 35.7 percent of M2 marker positive patients. Although not statistically significant, these

TABLE 4.4a

RELATIONSHIP BETWEEN M2Ag AND EXPOSURE TO BLOOD IN PATIENTS WITH CHRONIC RENAL FAILURE.

	Units of blood transfused			
	0	1-10	11-20	20+
Number of patients	10	22	17	18
Number M2Ag positive	2	8	9	9
+ve:-ve ratio	1:4	1:1.7	1:0.9	1:1

TABLE 4.4b

INFLUENCE OF (i) TRANSPLANTATION AND (ii) PLACE OF DIALYSIS ON THE PREVALENCE OF M2 MARKERS IN 63 PATIENTS.

	M2 markers present	M2 markers absent
Number of patients	28	35
Transplanted	15 (53.4%)	11 (31.4%)
Home dialysis	10 (35.7%)	17 (54.3%)

Ten patients had not received a transplant and were not on home dialysis.

findings favour an increased potential for spread of M2 within a hospital dialysis environment, particularly in association with transplantation.

In contrast, mentally retarded patients and those attending a clinic for sexually transmitted diseases possessed a significantly ($p > 0.001$) higher prevalence of anti-M2 IgG inferring a high risk of exposure to M2Ag; similar to exposure to HBV as reflected by antibody prevalence.

Among the same group of haemophiliacs there was a predominance of anti-M2 IgG upon initial screening. Ninety percent had experienced exposure to HBV as indicated by the presence of anti-HBs. It is unlikely that the latter antibody was passively acquired as the Scottish Factor VIII and Factor IX concentrates tested to date have been negative for anti-HBs (Hopkins and Peutherer; unpublished data). Subsequent sampling of several serial serum donations from a number of haemophiliacs suggests a cycling of M2Ag (reinfection or reactivation) following exposure to clotting factor concentrates. It is possible that passive transfer of M2Ag may lead to detectable serum levels in some haemophiliacs in the absence of appropriate donor screening.

Testing sera from mentally retarded patients and from patients attending a sexually transmitted disease clinic for anti-HBs revealed a degree of positivity in both

groups 45 times that of the local donor population (Peutherer personal communication I982). It is possible that M2Ag is sexually transmitted. Sexual transmission of non-A, non-B hepatitis has been suggested by Farrow et al I98I.

As there are currently no serological assays available for the detection of the agents of non-A, non-B hepatitis, haemophiliacs are continually at risk of exposure to these agents by virtue of therapy with blood components and products. Although M2Ag was not detected in all products tested this may reflect the relative sensitivity of the assays current state of development. The apparent absence of M2Ag in commercial Factor VIII produced in I983 is in contrast to those manufactured before I98I. The finding that 7/9 batches were reactive for anti-M2 IgG in I983 is difficult to explain purely on the basis of a change in the local donor population. It inevitably leads to the speculation that an amount of immune globulin may have been added to each batch in the hope of neutralising non-A, non-B hepatitis agents present. As there is no apparent decline in the incidence of non-A, non-B hepatitis this may be further indirect evidence of the non-protective but possibly attenuating effect of this antibody resulting from infusion of infectious immune complexes.

Distribution of T:N ratios for M2Ag showed a skew towards the higher ratio in Factor VIII preparations not observed

in Factor IX preparations. The nature of the manufacturing process favours the majority of heavy particulate matter being concentrated in Factor VIII preparations which supports the theory that M2Ag is a determinant on a particulate structure. Positivity in Factor IX may reflect determinants that have been sloughed off the coat of the particle during the manufacturing process.

Testing of normal immune serum globulin showed fluctuations in the distribution of anti-M2 IgG with an approximate five year variation mimiced by a lower titre of anti-M2 IgG in hepatitis B immune globulin possibly reflecting the similarity in transmission of these agents.

Investigation of a single serum sample from each haemophiliac drawn in I98I revealed 75 percent positivity for M2 markers, 90 percent had anti-HBs. Of particular interest is the finding that most patients were reactive for anti-M2 IgG. This is contrast to the belief that many haemophiliacs experience chronic liver dysfunction which by hepatitis B standards one would expect to associate with antigen rather than antibody. Regular donations from 7 haemophiliacs in Edinburgh have revealed fluctuating serum ALT levels mostly unrelated to specific treatment. This is in contrast to the report by Myers et al (I980) that fluctuations in serum ALT levels were the result of an antigenic overload precipitated by infusion of Factor VIII.

Six of 7 haemophiliacs studied over a period of months

possessed M2 markers. M2Ag was detected at the peak of ALT elevation, followed by an anti-M2 IgM response. Anti-M2 IgG was transient in nature, disappearing with the reappearance of M2Ag. Apart from inferring the non-protective nature of this antibody, it also supports the findings of other authors that haemophiliacs tend to have a high level of chronic liver disease and, once infected with the agents of non-A, non-B hepatitis, a chronic carrier state develops - a situation observed in studies with chimpanzees (Bradley et al I983a, Prince et al I984).

Study of 'isolated' cases of non-A, non-B hepatitis in haemophiliacs presents a confusing picture. Assuming that the patient had previously been exposed to M2 markers via Factor VIII therapy, the question has to be posed: is the current hepatitis due to a recrudescence of the disease, brought about by the apparent lack of immunity due to a non-protective or transient antibody? Noteworthy features include the rapid appearance of anti-M2 IgG in one patient six weeks after infusion of Factor VIII, reminiscent of an anamastic response. This is in contrast to the response seen in post-transfusion cases where anti-M2 IgG appears after 4-6 months. Other patients tended to carry the antigen with a transient appearance of anti-M2 IgM, inferring they had become a carrier of M2Ag as a result of Factor VIII infusion. The picture is further confused by the possibility of passive transfer of M2Ag, via blood products.

Of particular interest is the development of an acute HBV infection in two patients undergoing non-A, non-B hepatitis. Both events occurred in the presence of M2Ag. It is possible that HBV caused further disruption of liver cells, resulting in further release of M2Ag into the serum. Dual infection leading to possible interference or attenuation of the disease has been reported, both with HBV and non-A, non-B hepatitis and HAV and non-A, non-B hepatitis (Bradley et al I983c, Brotman et al I983b, Tsiquaye et al I983).

The findings of the epidemiological studies significantly enhances the possibility that M2Ag and anti-M2 IgM may prove useful serological markers for the diagnosis and prevention of at least one form of transfusion transmitted non-A, non-B hepatitis.

PART FIVE

FINAL DISCUSSION AND SUGGESTIONS FOR THE DIRECTION OF FUTURE RESEARCH.

5.I FINAL DISCUSSION

The aim of this research was to characterise a non-A, non-B hepatitis associated antigen, M2Ag, and to clarify its association with a form of parenterally transmitted non-A, non-B viral hepatitis.

Preliminary biochemical and biophysical characterisation of M2Ag have shown distinct differences with other reported serological markers for non-A, non-B hepatitis, and with other known human hepatitis viruses (HAV, HBV). Whereas M2Ag exhibits many of the physical and epidemiological characteristics associated with a viral product, other reported markers detected by techniques such as immuno-diffusion, counterimmune electrophoresis, RIA or EIA have the characteristics reminiscent of rheumatoid factor like molecules (Hellings et al I983). The sedimentation coefficient, polypeptide composition and molecular weight of M2Ag show similarities with the 22nm spherical HBsAg particle. Electron microscope studies have shown the presence of 20-40nm particles which appear to be aggregated by anti-M2. These particles are unlike any known intact virus but bear some resemblance to the 22nm HBsAg bearing structures representing excess protein coat released into the serum during HBV infection. The lack of nucleic acid in two gradient purified M2Ag preparations studied could be due to a very low concentration of intact infectious virus particles (as has been demonstrated by infectivity studies in chimpanzees) in the serum. Alternatively,

the infectious agents of non-A, non-B hepatitis may band at a different position of the gradient than the bulk of M2Ag bearing structures (similar to the difference in buoyant density between Dane particles and 22nm spherical HBsAg particles). There appears to be no difference in the sedimentation coefficient (within experimental error) of protease treated and untreated material, the antigen is ether resistant but chloroform sensitive. Chloroform alters the structure of proteins (possibly rendering the antigenic site unrecognisable) as well as being a lipid solvent. Evidence accumulated so far is indicative of M2Ag as an antigenic determinant on a protein structure of possible viral origin.

Chimpanzee infectivity studies indicate that agents of non-A, non-B hepatitis are inactivated, and their antigenicity destroyed, by formalin treatment (Tabor and Gerety I980). Three colony born chimpanzees were inoculated with formalin treated (I:4,000 at 37 C for 96 hours) infectious serum; none developed recognisable non-A, non-B hepatitis during 7 months observation, and the chimpanzees were subsequently found to be susceptible to non-A, non-B hepatitis when challenged with 0.Iml of the same untreated infectious serum 3I weeks after initial inoculation. It is of interest to note that antigenicity of M2Ag was destroyed when positive sera was treated with formalin under similar conditions to those of Tabor and Gerety (I980).

M2Ag illicits an immune response in both animals and man.

Immunisation of rabbits, mice and guinea pigs leads to the production of anti-M2-IgM detectable after absorption of anti-human activity. In man, anti-M2-IgM is the first class of anti-M2 to be detected after a (presumed) primary exposure resulting in a transfusion-related hepatitis episode involving M2Ag, followed after several months by anti-M2-IgG. This is very similar to the late appearance of anti-HBs IgG after primary exposure to HBV.

Serial follow-up of haemophiliacs has highlighted an interesting and possibly atypical feature of the immune response to repeated M2 exposure. Study of M2 markers and serum ALT levels showed a fluctuation between antigen and antibody with associated fluctuations in serum ALT levels. The 'cycling' phenomenon may indicate that the initial antibody produced is neutralising but not necessarily protective. If anti-M2 is protective it may be a quantitative phenomenon since apparently susceptible patients are often shown to possess anti-M2-IgG I-3 years prior to M2Ag exposure but are M2 marker negative in the months immediately prior to exposure. It is probable that a partially protective antibody could facilitate the development of a chronic carrier state of the virus. This has been observed in HBsAg carriers where there appears to be a strong association between the major histocompatability locus and immune response and the development of a HBsAg carrier state. (Thomas I984). A memory or anamastic immune response has

been observed in certain haemophiliacs who have been reactive for one of the M2 markers, upon re-exposure to strongly M2Ag reactive clotting concentrates there is a relatively rapid appearance of a high titre anti-M2 IgG within 4-7 weeks (as opposed to 6-7 months following presumed first-time exposure). Again this antibody appears not to be fully protective since M2Ag and anti-M2 IgM have been shown to appear weeks after the production of anti-M2 IgG. This feature introduces a certain amount of confusion concerning the interpretation of the cross challenge experiments performed in chimpanzees. Without a specific serological marker to distinguish different agents of non-A, non-B hepatitis it is not certain whether the second episode of non-A, non-B hepatitis is due to a reactivation of the first agent or in response to infection with an antigenically distinct agent. Recrudescence of a non-A, non-B episode has been shown to occur spontaneously in chimpanzees, three years after the initial episode (Bradley et al I983a). This adds further indirect evidence to the possibility of the non-protective and transient nature of the antibody and may help to explain some of the difficulties encountered when trying to establish a specific immunoassay.

One of the aims of this thesis was to produce a rapid screening test for M2Ag, anti-M2 IgM and anti-M2 IgG for potential use within the Blood Transfusion Service if the presence of M2 markers in donor blood proved to be predictive in the transmission of non-A, non-B hepatitis. M2Ag was first detected using an immuno-

diffusion system involving precipitation of antigen-antibody complexed in agarose. A solid phase sandwich EIA was subsequently developed for the detection of M2Ag. Utilisation of the anti-μ capture principle allowed greater flexibility in that anti-M2 of either the IgM or IgG class could be separately identified. Furthermore, EIA is more suited to large scale screening and the reagents are more economical and have a longer shelf life than RIA reagents. It also conforms to the general trend within the UK of a move away from RIA for reasons of safety and economy.

Although the sensitivity specificity and reproducibility of the EIA for (mainly) M2Ag has improved over the past three years as various aspects of the test system have evolved, it is still relatively insensitive largely due to the lack of a high affinity anti-M2. Whereas hyper-immune sera from a rabbit can readily be used on the solid phase, it is not so efficient as a label. However, recent studies have shown that guinea pig anti-M2 has proved a most efficient label when conjugated with alkaline phosphatase. The antibody used throughout the thesis was obtained from a haemophiliac and contained both anti-M2 IgM and anti-M2 IgG. This tends to suggest that an IgM antibody should be used for a more sensitive conjugate as has been found using monoclonal anti-HBs IgM.

For practical purposes, screening of blood donations needs to be a rapid test so results can be released within

one working day. The problem regarding the two overnight incubation steps for M2Ag might be overcome if a simultaneous monoclonal assay could be developed and employed or if the assay sensitivity could be improved (possibly) by the use of bioluminescence or fluorescence which may be offset by allowing shorter incubation times to make donor screening a realistic proposition.

All three assays appear to be specific for detecting their appropriate markers. There appears to be little interference by rheumatoid factor for the individual assays. This is supported by data obtained from testing M2Ag positive sera and rheumatoid factor positive sera before and after absorption of rheumatoid factor. Exchange of coded panels from Dr. Duermeyer and Dr. Thomas indicate a lack of relationship between M2Ag and DSAg, a rheumatoid factor like molecule. The relationship between M2Ag in anti-M2 immune complexes and rheumatoid factor is less clear. M2Ag appears not to be associated with any normal liver components.

The prevalence of anti-M2 IgG is almost certainly an underestimate. The anti-human IgG commercial conjugate (Miles-Yeda) is purified on a Protein A column which selectively binds IgG subclasses but has a poor affinity for IgG subclass 3. In those viral infections studied the major IgG subclasses produced are I and 3 and it is possible that the conjugate will not detect a proportion of the IgG_3 present.

The development of enzyme immunoassays for the detection of M2-related markers has allowed epidemiological studies to be performed. Although each epidemiological group has been discussed in some detail, one of the most striking features is the apparent similarity in implied route of transmission of M2Ag with HBV, a parenterally transmitted human hepatitis virus. This is shown by the very high prevalence of M2 markers in groups known to be at high risk of exposure to HBV and in countries where the prevalence of HBV in the donor population is higher than that in the UK. In groups that are immunosuppressed e.g. those undergoing renal dialysis M2Ag is predominant with very little antibody. Bradley et al (I984) have shown that infection of immunosuppressed chimpanzees with the 'tubule forming' agent leads to a subclinical hepatitis, but on biopsy there is evidence of extensive liver damage characteristic of the non-A, non-B hepatitis agent, implying a form of carrier state in such circumstances. Liver extract from such chimpanzees is infectious. Like HBV, it may be that the immune response to the agent is the cause of clinical illness, not replication of the agent per se. In institutionalised mentally retarded patients it appears that infection with M2Ag has 'burnt-out' as evidenced by the high level of anti-M2 IgG, a situation analogous to HBV. It would be interesting to examine serial samples from the same patients at the institution and compare the duration of anti-M2 IgG with that found in haemophiliacs. In the latter group this antibody is short-lived and

may not be fully protective.

The most convincing evidence for the value of M2 markers in predicting non-A, non-B hepatitis transmission has come from retrospective investigation of post-transfusion non-A, non-B viral hepatitis. There appears to be a definite association with a 'short' 3-7 week incubation period, where transfusion with either M2Ag or anti-M2 IgM (in the absence of co-transfused anti-M2 IgG) results in a hepatitis event, associated with the appearance of M2 markers in the recipient. Of interest is the fact that transfusion of anti-M2 IgM results in a hepatitis episode, inferring M2Ag is complexed with, and masked by, anti-M2 IgM. This is analogous to HAV, where anti-HAV IgM is often the only serological marker of recent infection, but transfusion of positive units can result in a post-transfusion HAV infection.

None of the patients in the retrospective study showed evidence of being transfused with anti-M2 IgG. This was somewhat unexpected as the prevalence of all three markers of M2 is approximately equal in the Edinburgh donor population. Since all patients except S.H. were jaundiced it is possible that co-transfusion of anti-M2 IgG with M2Ag or anti-M2 IgM has a protective or attenuating effect resulting in no infection or a subclinical infection.

Screening of blood donations for raised serum ALT levels would reduce, but not eliminate, the incidence of post

transfusion non-B hepatitis (Aach et al I98I). However, the non-specificity of ALT elevations and the lack of knowledge concerning the prevalence of elevated ALT levels among otherwise healthy adults as well as the likelihood of elevated and normal ALT levels in sequential samples from a single individual have discouraged the use of ALT screening to eliminate post-transfusion non-A, non-B hepatitis. Likewise, although there are reports of an increased association between non-A, non-B hepatitis and the transfusion of anti-HBc and/or anti-HBs positive blood (reflecting the likely exposure to non-A, non-B hepatitis agents of persons exposed to HBV) screening donations for these HBV markers would not eliminate all post-transfusion non-A, non-B hepatitis episodes. Effective prevention of parenterally transmitted non-A, non-B hepatitis would only be feasible if a specific serological assay could be employed to screen blood donations for a marker able to specifically predict the capability of transmitting non-A, non-B hepatitis.

Several putative serological markers and 'virus-like' particles have been reported although none have yet proved reproducible in other laboratories or been shown to exhibit characteristics usually associated with a virus or viral component. Research seems to be hampered by the lack of a good antibody and the probability (from chimpanzee studies) that the infectious agent(s) are present at a much lower titre than in transmitters of hepatitis B, rendering them undetectable by

conventional immunoassays such as RIA or EIA, or by electron microscope studies. Many false positive reactions appear to be caused by a 56 'S' IgM, while some reported serological markers have been found to be host related antigens. (Neurath et al I98I, Hellings et al I983).

There have been reports suggesting that at least a proportion of non-A, non-B hepatitis is due to HBV infection in which serological markers are not expressed. Trepo et al I98I have reported a virus like particle very similar in morphology and antigenicity to HBV but found in HBsAg negative cases. However, it was subsequently reported that 4 percent of post-transfusion non-A, non-B patients showed a weak HBsAg activity in pelleted serum at the onset of illness, with I3 percent of donors with raised ALT showing the same phenomenon. (Trépo I984).

Use of monoclonal IgM anti-HBs as a solid phased antibody in HBsAg screening to identify potential transmitters of HBV led to the detection of HBsAg in serum of patients negative for HBsAg by a conventional polyclonal radio-immunoassay (Wands et al I98I). Examination of the monoclonal immunoreactive material showed that it was not identical with HBV but shared some common properties. It was suggested that this could explain for some of the previously diagnosed 'non-A, non-B' hepatitis cases. The immunoreactive material also contained nucleic acid

sequences complementary to HBV-DNA by molecular hybridisation analysis.

Other reports have failed to find HBV-DNA in sera obtained from chimpanzees infected with a Factor VIII derived non-A, non-B agent (Fields et al I983). HBV-DNA : non-A, non-B hybridisation studies may be prone to false positives arising from contamination by bacterial DNA in stored serum which hybridises with HBV-DNA prepared by cloning HBV in E. coli. At the present time it seems likely that the agents of non-A, non-B hepatitis and HBV are not closely related antigenically.

Despite intensive worldwide research when it became clear that human hepatitis viruses other than HAV or HBV existed (Ratazan et al I97I; Prince et al I974) the causative agents of non-A, non-B hepatitis, both parenteral and epidemic forms, have yet to be isolated and characterised. The need for a means of identifying these agents has been stressed by Dr. Alter at the International Symposium on Viral Hepatitis held in San Francisco during March I984. He stated that although the initial illness following a transfusion episode was mild, the majority (66 percent) of patients developed chronic hepatitis with 3 percent progressing to cirrhosis. Upon repeat biopsy several months later, 43 percent had progressed to a more severe chronic active hepatitis with 35 percent developing cirrhosis. The long term consequence of post transfusion non-A, non-B hepatitis is therefore severe and it may

account for at least some previously unexplained cases of cirrhosis (Alter et al I984).

The data presented in this thesis provides sufficient encouragement for the association of M2Ag with one form of parenterally transmitted non-A, non-B hepatitis. However, further intensive research involving at some stage a prospective study of blood donors and recipients is required before the true relationship of M2Ag to non-A, non-B viral hepatitis is clarified.

5.2 FUTURE RESEARCH

Although the EIA's for M2 markers have improved over the course of this research, they are still relatively insensitive due to the reagents currently used. Recent studies using a guinea pig anti-M2 alkaline phosphatase conjugate have proved encouraging and these reagents need to be investigated further. An alternative is provided by the production of monoclonal antibodies: however caution is required as there have been numerous problems in developing EIA's for HBsAg using monoclonal antibodies as both solid phase and tracer antibodies. Another approach to increase the sensitivity of the assays is to employ a more sensitive technique such as bioluminescence or fluorescence.

Availability of monoclonal antibodies to subclasses of IgG will allow investigation of the subclasses produced in response to M2Ag. Of interest would be a comparison between the subclasses produced in haemophiliacs and those of other epidemiological groups as in the former the immune response does not appear to be fully protective.

M2Ag needs to be characterised more fully and the development of a more sensitive M2Ag EIA may lead to the detection of intact virus particles opening the way for intensive nucleic acid studies. One approach to ensure a continuous production of M2Ag would be to

propagate the antigen in a tissue culture system, assuming a suitable cell line can be identified.

By these means, a sensitive assay system could be applied to the diagnosis of individual cases and most importantly to prospective studies of the correlation between positive donors and the outcome of transfusion in the recipient. This would require the inclusion of a large number of donors and considerable effort to follow up recipients. Such a study would be justified on the basis of the evidence presented in this thesis.

PART SIX

APPENDIX

APPENDIX I. BUFFERS USED THROUGHOUT RESEARCH.

One mg/ml sodium azide was added to all buffers to prevent microbial growth.

I. Phosphate Buffered Saline (P.B.S.).

Stock solutions.

(a) Di-sodium hydrogen orthophosphate, anhydrous ($Na_2 HPO_4$; BDH 'AnalaR' grade m.w. I4I.96).
A 0.I5M stock solution was prepared with 42.588g Na_2HPO_4 and made up to 2 litres with distilled water.

(b) Potassium dihydrogen orthophosphate (KH_2PO_4; BDH 'AnalaR' grade, m.w. I36.09).
A 0.I5 stock solution was prepared using 40.827g $KH_2 PO_4$ and made up to 2 litres with distilled water.

(c) Sodium chloride (NaCl, BDH 'AnalaR' grade m.w. 58.44).
A 0.I5M stock soultion was prepared using I7.532 NaCl and made up to 2 litres with distilled water.

Stock solutions were stored at room temperature.
P.B.S. pH 7.2 was prepared by mixing the following volumes of stock solutions:-

Na_2HPO_4 7I5ml.

KH_2PO_4 285ml.

NaCl I000ml.

Total volume, 2000ml. stored at room temperature.

2. Carbonate-Bicarbonate Buffer pH 9.6.

A 0.05 solution of carbonate-bicarbonate buffer was prepared using the following reagents:-

(a) Sodium carbonate (Na_2CO_3 anhydrous BDH ('AnalaR' grade, m.w. I05.99).

(b) Sodium Hydrogen carbonate ($NaHCO_3$ BDH 'AnalaR' grade, m.w. 84.0I).

3. Substrate buffer for p-nitrophenyl phosphate pH 9.8.

(a) Sodium carbonate, anhydrous (Na_2CO_3 BDH ('AnalaR' grade, m.w. 203.30).

2.675g Na_2CO_3 and 0.Ig $MgCl_26H_20$ were made up to 500ml. with distilled water. pH was adjusted using concentrated Hydrochloric Acid (HCl; BDH 'AnalaR').

4. Tris/HCl - $MgCl_2$ Buffer.

A 0.05M Tris: 0.000IM $MgCl_2$ buffer was prepared using the following reagents:-

(a) Tris (hydroxymethyl) methylamine ($NH_2C(CH_3OH)_3$ BDH 'AnalaR' grade, m.w. I2I.I4.

(b) Magnesium chloride, hexahydrate ($MgCl_2$, $6H_20$) BDH 'AnalaR' grade, m.w. 203.30.

I2.IIg Tris and 0.4Ig $MgCl.6H_2O$ were made up to 2 litres with distilled water. The pH was adjusted using concentrated hydrochloric acid (HCl, BDH 'AnalaR').

5. Tris/HCl - EDTA Buffer pH 8.0.

A 0.05 Tris, 0.005M EDTA buffer was prepared using the following reagents:-

(a) Tris (hydroxymethyl) methylamine $(NH_2.C(CH_2OH)_3$ BDH 'AnalaR' grade, m.w. I2I.I4.

(b) Ethylenediaminetetra-acetic acid disodium salt ($CH2_2.N(CH_2COOH)CH_2COONa)\ 2H_2O$. BDH 'AnalaR' grade, m.w. 372.24) (E.D.T.A.).

I2.IIg Tris and 3.72g EDTA were made up to 2 litres with distilled water. The pH was adjusted using concentrated hydrochloric acid (HCl, BDH 'AnalaR').

APPENDIX 2. PREPARATION OF SUCROSE GRADIENT.

Three ml of a 64 percent sucrose solution was added to a 25ml. polypropylene centrifuge tube. Sixteen ml of a 30 percent sucrose solution was added gently to the tube. The sample was layered on to this gradient and the volume made up to 25ml. with distilled water when necessary.

APPENDIX 3. PREPARATION OF CAESIUM CHLORIDE GRADIENT.

Four ml. each of a I.5g/ml., I.4g/ml., I.3g/ml., I.2g/ml. and a I.Ig/ml. solution of caesium chloride (CsCl) was added in that order to 2.5ml. polypropylene centrifuge tubes. One ml. of the sample was layered on to the gradient and the volume made up to 25ml. with distilled water.

Solutions of CsCl:-

I.Ig/ml	2.8g CsCl in 20ml. distilled water.
I.2g/ml.	5.6g CsCl in 20ml. distilled water.
I.3g/ml.	8.0g CsCl in 20ml. distilled water.
I.4g/ml.	II.0g CsCl in 20ml. distilled water.
I.5g/ml.	I4.0g CsCl in 20ml. distilled water.

APPENDIX 4.

Density in caesium chloride and refractive index data. (From Griffith, O.W. 'Techniques of Preparative, Zonal and Continuous Flow Ultracentrifugation, p.II).

Density g/ml.	Refractive index
I.I059	I.3432
I.II5I	I.344I
I.I245	I.3450
I.I340	I.3459
I.I437	I.3468
I.I536	I.3478
I.I637	I.3488
I.I739	I.3498
I.I843	I.3508
I.I948	I.35I8
I.2055	I.3529
I.2I64	I.3539
I.2275	I.3550
I.2387	I.356I
I.2502	I.3572
I.26I9	I.3584
I.2738	I.3596
I.2858	I.3607
I.298	I.36I9
I.3II	I.363I
I.324	I.3644
I.336	I.3657
I.3496	I.3670

APPENDIX 5. ONE STEP GLUTARALDEHYDE METHOD OF AVRAMEAS AND TERNYNCK (1969).

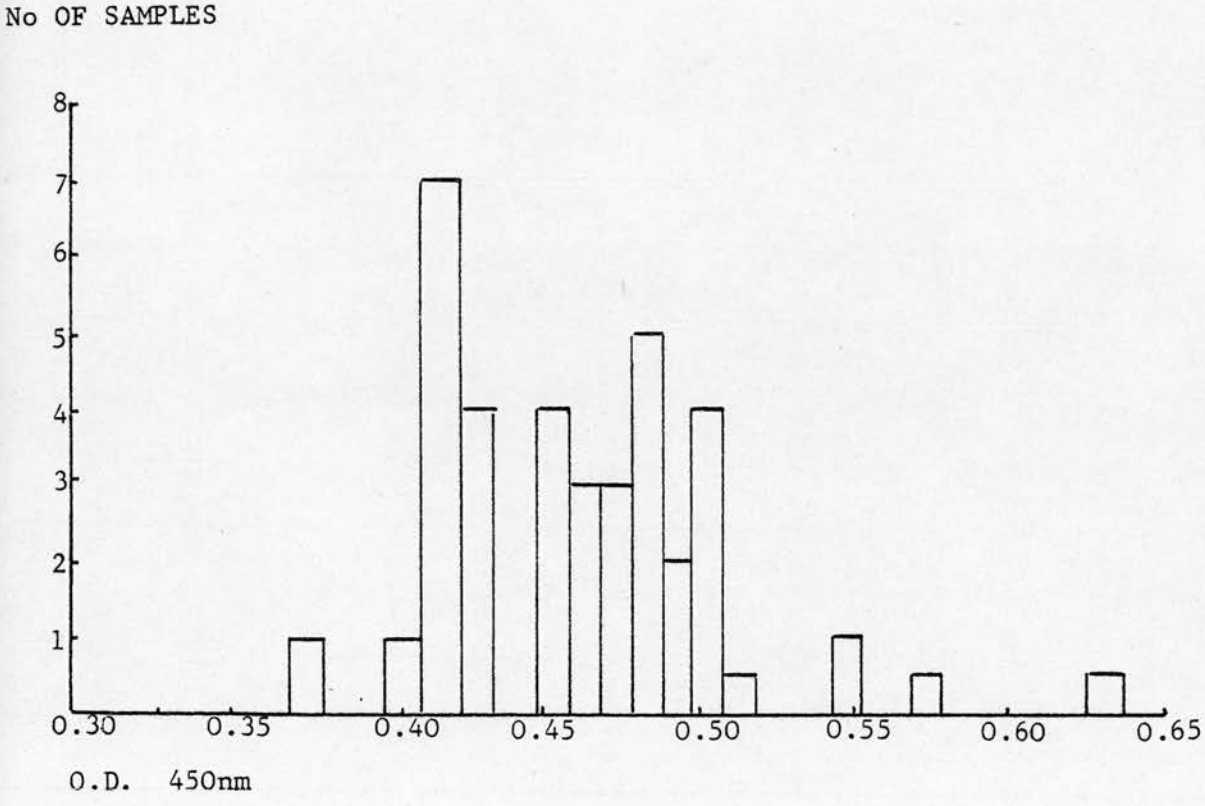
The immunoglobulin fraction (obtained by ammonium sulphate precipitation) was resuspended in PBS at a concentration of Img/ml., added to 5,000 units of alkaline phosphatase (Sigma: Type VIIS), and dialysed against PBS pH 7.2 overnight at 4°C. Twenty five percent glutaraldehyde was added to give a final concentration of 0.2 percent, and mixed at room temperature for I.5 hours. The solution was then dialysed against 0.05M PBS pH 7.2 overnight at 4°C, followed by a further overnight dialysis against 0.05M Tris/HCl containing 0.00IM $MgCl_2$ pH 8.0 at 4°C. The solution was recovered and stabilised with I percent BSA. Conjugated antibody was separated by precipitation with an equal volume of saturated sodium sulphate.

APPENDIX 6. PREPARATION OF M2Ag AFFINITY COLUMN.

4.73mg gradient purified M2Ag was dialysed against 0.I5M NaCl (without azide) and added to Igm. of gluteraldehyde activated affinity adsorbant (BCL) and mixed at room temperature for 4 hours. The mixture was poured into a column (PDI0, Pharmacia) and then washed with saline. 0.3M ethanolamine/HCl pH 7.5 was added, left for 50 minutes at room temperature. The column was then subsequently washed with 0.9% saline, followed by 0.5M propionic acid and then a final wash with 0.9% saline. The M2Ag affinity column was stored at $4^{\circ}C$ in 0.9% saline.

APPENDIX 7

HISTOGRAM SHOWING O.D. VALUES OBTAINED BY TESTING 40 REPLICATES OF A HBsAg POSITIVE CONTROL USING A COMMERCIAL HBsAg E.I.A.



APPENDIX 8. IDENTIFYING BANDS BY SEDIMENTATION COEFFICIENTS: PRINCIPLE AND WORKED EXAMPLES.

It is possible to estimate sedimentation coefficients of sample zones from a knowledge of run time, rotor speed and band position in a known gradient. The method of McEwen (1967) for estimating sedimentation coefficients is summarised below:-

Z_0 for the rotor and gradient is obtained from

$$Z_o = \frac{Z_I r_2 - Z_2 r_I}{r_2 - r_I}$$

where Z_o = solute concentration corresponding to extrapolation of a linear gradient distribution to zero radius.

Z_I = minimum percent w/v of sucrose gradient.

Z_2 = maximum percent w/v of sucrose gradient.

r_I = meniscus of gradient i.e. minimum radial distance from centrifugal axis (cm)*.

r_2 = maximum radial distance from centrifugal axis (cm)*.

* obtained from rotor handbook.

Tables from McEwen (1967) were used to obtain time integral (I) values for sucrose at the meniscus of the gradient and at the separated zone for the particle.

$s_{20,w}$ is calculated from the formula

$$s = \frac{\Delta I}{w^2 t}$$

where w^2 = $(0.10472 \text{ rpm})^2$

t = time (sec)

Calculation of 's' values for HBsAg and M2Ag.

Experimental conditions:

5-30 percent w/v linear sucrose gradient in 0.05M PBS.

t = 3600 sec.

w^2 = $(0.10472 \quad 57{,}000)^2$ = $3.56 \quad 10^7$.

Temperature = 5°C.

$$\text{Pre-run } Z_0 = \frac{5 \times 10.431 - 30 \times 4.264}{10.431 - 4.264} = -12.285$$

$$\text{Final } Z_0 = \frac{4 \times 10.321 - 24 \times 4.264}{10.431 - 4.264} = -9.828$$

Z_0 was taken as -10.

From Table and Figure 's' values were calculated for fractions 9, (HBsAg) 10 (HBsAg and M2Ag) and 11 (M2Ag).

Fraction 9 corresponds to 18.5 percent w/v sucrose.

From tables taking particle density of 1.30; Z_0 - 10; temperatures 5°C.

I (19%) = 2.3828

I (4%) = 0.5516

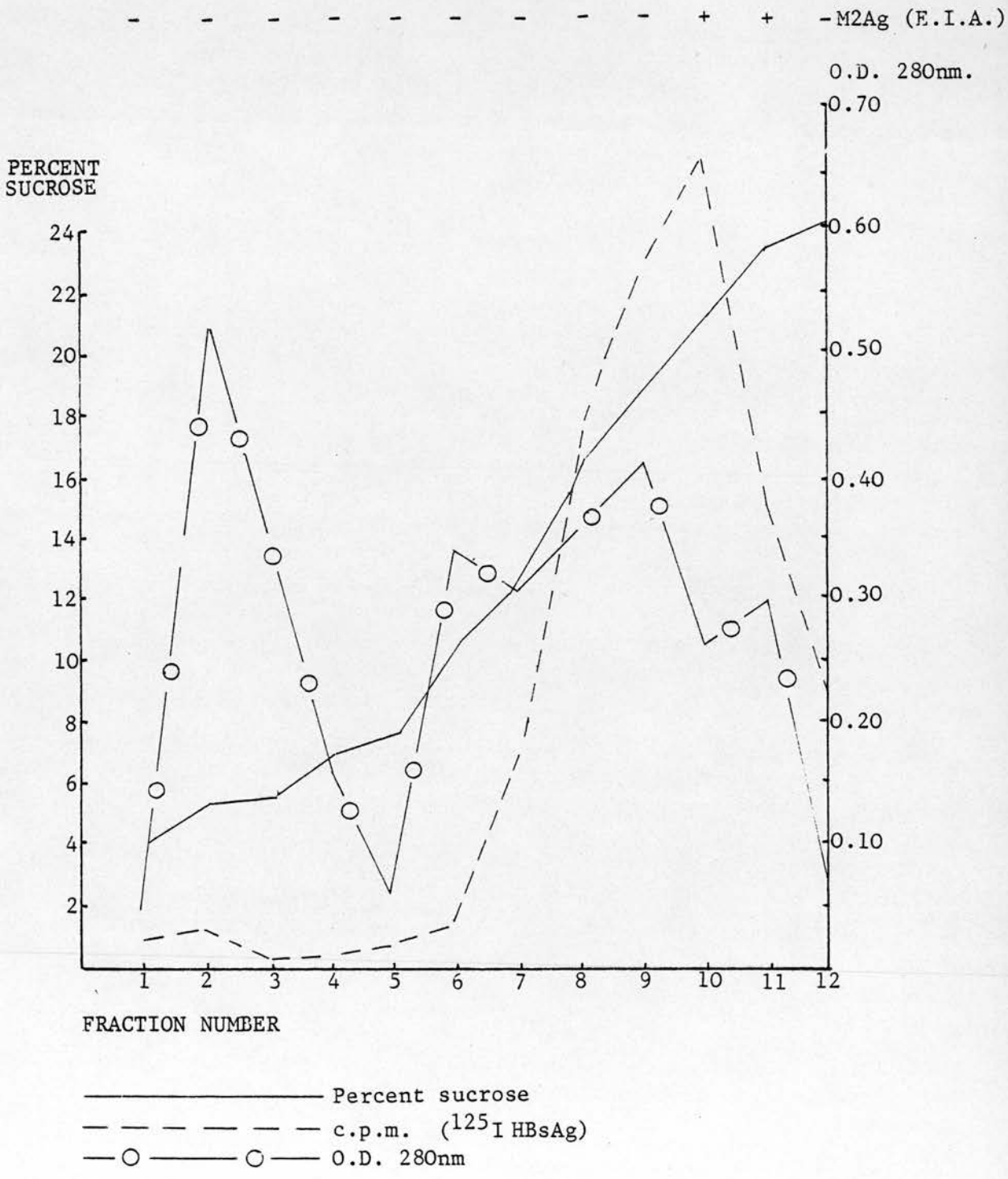
TABLE 6.8a

TABLE SHOWING SUCROSE CONCENTRATION, PROTEIN CONCENTRATION (O.D. 280nm) COUNTS PER MINUTE, HBsAg (RIA) AND M2Ag REACTIVITY (EIA).

Fraction number	Percent sucrose	O.D. 280nm	c.p.m.	HBsAg (RIA)	M2Ag (O.D. 410nm)
1	4.0	0.053	44.094	-	0.04
2	5.5	0.537	66.051	-	0.02
3	5.5	0.336	20.995	-	0.04
4	7.0	0.157	18.076	-	0.04
5	7.5	0.057	42.076	-	0.03
6	10.5	0.342	81.849	-	0.03
7	12.5	0.308	352,830	-	0.04
8	16.5	0.370	876.678	-	0.07
9	18.5	0.412	1147.389	+	0.09
10	21.5	0.257	1331.660	+	0.25
11	23.5	0.304	762.139	-	0.31
12	24.0	0.069	486.038	-	0.10

FIGURE 6.8i

GRAPH SHOWING PROTEIN CONCENTRATION (O.D. 280nm); HBsAg (R.I.A.) AND SUCROSE CONCENTRATION FOR THE DETERMINATION OF SEDIMENTATION COEFFICIENTS.



$$\Delta I = I.83I2$$

$$s_{20,w} = \frac{I.83I2}{I.9739 \quad I0^{II}}$$

$$= 92.77 \quad I0^{-I3}$$

$$s_{20,w} = 93$$

Fraction I0 corresponds to 2I.5 percent sucrose

$$I \ (22\%) = 2.8737$$

$$I \ (4\%) = 0.55I6$$

$$\Delta I = 2.322I$$

$$s_{20,w} = \frac{2.322I}{I.9739 \quad I0^{II}}$$

$$= II7.64 \quad I0^{-I3}$$

$$s_{20,w} = II8$$

Fraction II corresponds to 23.5 percent w/v surcrose

$$I \ (24\%) = 3.202I$$

$$I \ (4\%) = 0.55I6$$

$$\Delta I = 2.6506$$

$$s_{20,w} = \frac{2.6506}{I.9739 \quad I0^{II}}$$

$$= I34.28 \quad I0^{-I3}$$

$$s_{20,w} = I34$$

Values obtained for HBsAg ranged from 93S to II8S (published data II0S), for M2Ag the values ranged from II8S to I34S.

APPENDIX 9.

STATISTICAL FORMULA

χ^2 (chi - squared) test

$$(\Sigma\ x_{i'} - \bar{x}_I)^2 \Big/ \Sigma^2 + \Sigma x_{j2} - \bar{x}_2$$

Students t test

$$\frac{\bar{x}_I - \bar{x}_2}{\sqrt{\frac{I}{n_I} + \frac{I}{n_2}}} \Bigg/ \sqrt{\frac{I}{n_I} + n_2 - 2\ (\ (x_{iI} - \bar{x}_I)^2 + (x_{j2} - \bar{x}_2)^2)}$$

Standard deviation

$$S.D. = \sqrt{(x - \bar{x})^2/n}$$

APPENDIX IO. ADDRESSES OF COMPANIES.

Abbott Laboratories, Brighton Hill Parade, Basingstoke, Hants, RG22 4EH, England.

Agar Aids Ltd., 66[a] Cambridge Road, Stanstead, Essex, CM24 8DA, England.

Armour Pharmaceutical Company Limited, Eastbourne, England.

B.D.H. Limited, Laboratory Chemicals Division, Poole, England.

Boehringer and Mannheim, Bell Lane, Lewes, East Sussex, BN7 ILG, England.

Dynatech Laboratories Limited, Daux Road, Billingshurst, Sussex, RHI4 9SJ, England.

Miles Laboratories Limited, P.O. Box 37, Slough, SL2 4LY, England.

MSE Limited, Manor Royal, Crawley, Sussex, RHIO 2QQ, England.

Northumbria Biologicals Limited, South Nelson Industrial Estate, Cramlington, Northumberland, NE23 9HL, England.

Pharmacia (Great Britain) Limited, Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks, MK9 3HP, England.

Precision Instruments, London, England.

Seward Laboratories, Unit 5, Viking Industrial Estate, Norse Road, Bedford, MK4I, 09G, England.

Sigma (London) Chemical Company, Fancy Road, Poole, Dorset, BHI7 7NH, England.

Wellcome Diagnostics, Wellcome Research Laboratories, Beckenham BR3 3BS, Kent, England.

PART SEVEN

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