STUDY OF THE SURFACE GLYCOPROTEINS OF RIFT VALLEY FEVER VIRUS USING MONOCLONAL ANTIBODIES

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DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

The necessary approval for all animal studies was obtained from the Animal Ethics Committee for the University of the Witwatersrand. Clearance certificates are included in Appendix D.

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5th day of October, 1992

ABSTRACT

The structural, functional and antigenic properties of the envelope glycoproteins of Rift Valley fever virus (RVFV) were analyzed using a panel of monoclonal antibodies (MAbs). In order to gain a better understanding of the role of the RVFV surface proteins in infection and pathogenesis, the mechanisms of antibody-mediated neutralization of the virus were examined, as well as the function of the glycoproteins in viral attachment and penetration.

Of the twenty three MAbs which were generated, fourteen were directed against the G1 and nine against the G2 protein of RVFV. The topological relationship of the antigenic determinants to each other on the viral glycoproteins was achieved using competitive binding assays with enzyme-labelled MAbs. For the RVFV G1 protein, four antigenic domains which may be interlinked were identified. The domains G1 I, II and IV were involved in virus neutralization and haemagglutination, while G1 III was associated with low level C′-dependent neutralization.

With regard to the G2 protein, four antigenic domains which appear to be spatially distinct were identified. Domain G2 I exhibited significant neutralizing and haemagglutination activity, while G2 II was involved in haemagglutination and weak C′-dependent neutralization. The remaining G2 regions neutralized to a low level only in the presence of C′. The majority of the epitopes on both viral glycoproteins were highly conformational, indicating that the native protein structure is necessary for the recognition and expression of the functional activities of these particular antibodies.

Protective determinants were shown to occur on both G1 and G2, demonstrating that both RVFV envelope proteins are important in viral pathogenesis. The neutralization studies, in turn, revealed that the inhibition of virus attachment is not the principal means of antibody-mediated neutralization of RVFV. Instead, such neutralization appears to be the result of several different processes, including synergistic neutralization by combinations of different antibodies, prevention of virus binding, virus internalization and the blocking of the viral life cycle at an intracellular stage. Further insight into RVFV infectivity was obtained by showing that both glycoproteins are involved in virus entry into the host cell. Finally, the present findings strongly support an endosomal route of entry and penetration for RVFV, associated with concomitant allosteric changes in the G1 protein.

PREFACE

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1. GENERAL INTRODUCTION

1.1 General aspects of RVFV

1.1.1 Epidemiology and transmission

Rift Valley fever is an important arthropod-borne disease of both livestock and humans in much of sub-Saharan Africa (Shope *et al*, 1982). Infection causes acute febrile illness, abortions and death in cattle and sheep, and a dengue like illness in man (Easterday, 1965; McIntosh *et al*, 1973; Peters and Meegan, 1984).

The etiologic agent, Rift Valley fever virus (RVFV), was first isolated in 1930 from the blood of a newborn lamb during an epizootic of fatal hepatic necrosis and abortion in sheep in Kenya (Daubney *et al*, 1931). It has subsequently become evident that this zoonosis had existed in this area since at least 1912 (Davies, 1975) and that it is widespread in Africa (Hoogstraal *et al*, 1979; Madkour, 1978; Meegan, 1981) with extensive epizootics described in countries such as South Africa (Van Velden *et al*, 1977). Zimbabwe (Swanepoel *et al*, 1979), Sudan (Davies, 1990) and Mauritania (Jouan *et al*, 1989).

In South Africa, RVF infection was first identified in 1951 (Gear *et al*, 1951). In this outbreak it is estimated that 20 000 people were infected and at least 100 000 sheep and cattle died. The disease reappeared in South Africa in epizootic form in 1953 (Gear *et al*, 1955) and again in 1955-1956 (Weiss, 1957). In 1974/1975 a severe epizootic of RVF swept through the sheep and cattle-farming districts of the Orange Free State and the Cape Province, also affecting livestock in Namibia (Barnard and Botha, 1977). Many humans were infected and for the first time in South Africa human deaths due to RVF were recognized (Van Velden *et al*, 1977).

Until 1977, the disease was restricted to man and domestic animals in sub-Saharan Africa (Shope *et al*, 1982). However, an explosive epidemic of RVF occurred

in Egypt in 1977 and 1978, demonstrating the potential for this disease to escape its normal endemic area, as well as to spread to livestock populations worldwide (Hoogstraal *et al*, 1979; Meegan *et al*, 1979). The Egyptian RVF epizootic resulted in widespread involvement of human populations, with 598 fatal human cases reported (Meegan, 1979). More recently, a large epizootic occurred in the Senegal river basin in 1987 and again there was extensive severe human disease and mortality (Jouan *et al*, 1989).

In contrast to the epizootic situation, RVF infection in other parts of sub-Saharan Africa is enzootic/endemic as indicated by the isolation of the virus from mosquitoes, sporadic cases in animals and man, and serological surveys. These include Mali, Nigeria, Gabon, Uganda, the Congo, Chad (Easterday, 1965), Zambia (Davies and Highton, 1980; Davies *et al*, 1992), Angola (Kokernot *et al*, 1965), Botswana (Tessier *et al*, 1987) and Mozambique (Valadaão, 1969). As well as being present in Africa, RVFV has also been isolated in Madagascar (Mathiot *et al*, 1984) and a RVFV outbreak has recently been reported there (Morvan *et al*, 1991a; b).

Epizootic transmission of RVFV is primarily by mosquito (Shope, 1985) and epidemics are very often linked with unusually high rainfall or warm seasons which favour mosquito density (Davies *et al*, 1985; McIntosh *et al*, 1980a). Other modes of transmission are nevertheless not uncommon and may be subsequently important when virus loads are high (Hoogstraal *et al*, 1979).

During epidemics in sub-Saharan Africa, RVFV has been isolated frequently from several culicine mosquitoes (Gear *et al*, 1955; McIntosh, 1972; McIntosh *et al*, 1980b). In South Africa the main epizootic vector among sheep and cattle on the inland plateau is *Culex theileri* where it probably causes some human infection (McIntosh *et al*, 1980b). Although the vector responsible for the 1977-1978

Egyptian epizootic was not proved, epidemiologic and laboratory evidence have implicated *Culex pipiens* (Meegan *et al*, 1980).

The identity of the possible arthropod vector as well as the vertebrate reservoir which serve to maintain the virus in the absence of epizootics is still uncertain (Peters and Le Duc, 1991). Attempts to implicate rodents, primates, birds, game animals and reptiles as the predominant maintenance reservoir host have been largely unsuccessful (Davies, 1975; Davies and Addy, 1979; McIntosh and Jupp, 1981; Swanepoel, 1981; Swanepoel *et al*, 1978).

An alternative to vertebrate reservoir hosts could be the maintenance of the virus by transovarial transmission in insect vectors (Alexander, 1957; Meegan *et al*, 1980). This would explain the dormancy of the virus over several years when rainfall is low and the sudden outbreaks in livestock during years of excess rainfall and large mosquito populations. In Kenya, transovarial transmission of RVFV has been reported for *Aedes mcintoshi* (Linthicum *et al*, 1985), but despite extensive studies undertaken in South Africa, the results have been inconclusive (Gargan *et al*, 1988; Jupp, pers comm).

1.1.2 RVFV pathogenesis, diagnosis and control

RVFV is pathogenic for sheep, cattle, goats, mice, rats and hamsters. The natural disease in domestic ruminants causes abortions and many deaths in pregnant and newborn animals (Davies, 1975; Scott *et al*, 1956). RVF infection may also affect buffalo, camels and antelopes (Easterday, 1965; Gear *et al*, 1955). In susceptible animals, the liver is the primary site of viral replication and the characteristic lesion is focal liver necrosis (Easterday *et al*, 1962; Erasmus and Coetzer, 1981).

The virus multiplies readily in both suckling and weaned mice following intracranial (i.c.), intraperitoneal (i.p.), or subcutaneous injection, causing death

within four days (Peters and Meegan, 1981). While the virus is a predominantly hepatotropic pathogen, it has a latent neurotropic attribute (Kitchen, 1950). Unlike virulent RVFV isolates, neurotropic strains of RVFV obtained by numerous serial intracerebral passages in mice do not kill mice after i.p. inoculations, but will do so if inoculated i.c. (Smithburn, 1949; Weinbren *et al*, 1957).

Man is susceptible to RVF virus infection via the bite of infected mosquitoes and exposure to aerosols or infected tissues. Human infection is usually a self-limiting, acute febrile disease, but in some cases severe complications such as haemorrhagic fever, encephalitis and retinal lesions ensue (Laughlin *et al*, 1979; Meegan *et al*, 1981; Van Velden *et al*, 1977).

The virus can be readily isolated in suckling and adult mice, hamsters (Anderson *et al*, 1989; Weiss, 1957) and Vero or *Aedes pseudoscutellaris* cells (Digoutte *et al*, 1989). An alternative approach to virus isolation is the detection of circulating viral antigen in serum by enzyme-linked immunoassay (ELISA) (Meegan *et al*, 1989) or immunoperoxidase demonstration of antigen in fixed liver tissue (Arborio and Hall, 1989).

Demonstration of RVFV specific IgM antibodies in acute phase sera can be performed using an IgM capture ELISA (Soliman *et al*, 1988), indirect fluorescent antibody (IFA) assay (Swanepoel *et al*, 1976) or a solid-phase immunosorbent technique (SPIT) (Soliman *et al*, 1988). If paired sera are available, serologic diagnosis by haemagglutination-inhibition (HI), complement-fixation (CF) or neutralization is also possible (Swanepoel *et al*, 1976).

Control of RVF infection is necessary to limit spread of the disease and prevent the possible dissemination of the virus outside Africa (Shope *et al*, 1982). One approach is that of mosquito control by ultra low volume spraying of chemical

pesticides to reduce populations of adult mosquitoes (Meegan, 1979). Nevertheless, such measures are of little practical use in widespread epizootics (Shope, 1985).

A more effective approach to preventing spread of RVF is that of immunization of domestic animals (Assaad *et al*, 1983; Rossi and Turell, 1988; Shope *et al*, 1982). Several vaccines against RVF for veterinary use are currently available. A live attenuated vaccine for sheep and cattle was originally developed by Smithburn by neuroadaptation to mice (Smithburn, 1949). Some years later, a live veterinary vaccine derived from the Smithburn neurotropic strain was prepared in South Africa at the Onderstepoort Veterinary Research Laboratory (Randall *et al*, 1962). These vaccine strains are efficacious and inexpensive, and have played a major role in limiting epizootic disease in countries such as South Africa and Kenya (Shope *et al*, 1982). However, since these vaccines may cause abortions and have shown changes in mouse virulence under certain circumstances (Coetzer and Barnard, 1977; Shimshany and Barzilai, 1983), they are not recommended for use in unaffected enzootic areas (Shope *et al*, 1982).

In addition to the live attenuated vaccines, inactivated tissue culture vaccines (Barnard, 1979) are produced in South Africa and Kenya. These are safe and induce antibodies in sheep and cattle in a high percentage of recipients, but require frequent boosting (Barnard, 1979).

Several formalin-inactivated monkey cell culture vaccines are also available for humans at high-risk (Meadors *et al*, 1986; Niklasson, 1982; Randall *et al*, 1962; 1964). While these vaccines appear to offer good protection, annual booster injections are needed to maintain detectable antibody levels (Eddy *et al*, 1981; Niklasson, 1982). Unfortunately, these vaccines are expensive to produce and available in limited supply, so that widespread use in Africa is not feasible. An experimental RVF vaccine candidate has since been developed by passaging a

human isolate in tissue culture under the influence of the mutagen 5-fluorouracil (Caplan *et al*, 1985). This mutant virus variant (MV P12) is currently being evaluated for use as a live attenuated vaccine (Hubbard *et al*, 1991; Morril *et al*, 1991; Saluzzo and Smith, 1990; Turell and Rossi, 1991).

1.2 Properties of RVFV

1.2.1 Classification, morphology and physicochemical properties

RVFV is a member of the genus *Phlebovirus* of the family *Bunyaviridae* (Bishop *et al*, 1980). The 45 members of the phlebotomus fever serogroup (Peters and Le Duc, 1991) have similar morphological, physical and biochemical properties (Bishop *et al*, 1980; Elliot, 1990; Murphy *et al*, 1973; Rice *et al*, 1980).

Morphologically, the viruses are lipid-enveloped spherical structures typically 90-120 nm in diameter (Ellis *et al*, 1988). Associated with the RVFV envelope are two structural glycoproteins, G1 and G2 (Collett *et al*, 1985), which are present in equivalent numbers on the virus surface (Robeson *et al*, 1979). By electron microscopy, these appear as a fringe of regularly arranged spikes measuring 10-18 nm in length (Ellis *et al*, 1988; Martin *et al*, 1985). In addition to the two external glycoproteins G1 and G2, there are two internal proteins, the N (nucleocapsid) and L (transcriptase component) proteins (Bishop and Shope, 1979). The virus has a single-stranded RNA genome consisting of three segments (Bishop *et al*, 1980; Collett *et al*, 1985). Each virus particle contains three nucleocapsids corresponding to the three RNA segments, and containing the RNA, the N protein, and the L protein in a supercoiled fashion (Obijeski *et al*, 1976). The central nucleocapsid structure in turn incorporates the internal ends of the estimated 350-375 glycoprotein spikes, as well as ribosomes (Ellis *et al*, 1988).

The physical and chemical properties of RVFV are similar to the other members of the phlebovirus genus (Bishop *et al*, 1980; Rice *et al*, 1980). A sedimentation coefficient of 450 S, as well as an additional slower sedimenting 156 S infectious

particle has been reported for RVFV (Polson and Levitt, 1963). The buoyant density of RVFV is 1,21 g/ml in caesium chloride gradients (Rice *et al*, 1980). The virus, like other phleboviruses, is destroyed easily by heating at 56°C, is inactivated by acidic pH and is also sensitive to lipid solvents and detergents (cited in Calisher, 1991).

1.2.2 Molecular and antigenic characteristics

The three RNA segments comprising the RVFV genome are designated L (2,7 x 10^6d), M (1,38 x 10^6d), and S (0,6 x 10^6d) (Collett *et al*, 1985; Rice *et al*, 1980). The corresponding sediment coefficients of the RNA are 27-31 S for the L segment, 22-26 S for the M and 16-20 S for the S (Bishop *et al*, 1980).

The viral envelope glycoproteins G1 and G2 are coded for by the M segment, the expression of which has been studied in detail (Collett *et al*, 1985; Kakach *et al*, 1988; Suzich and Collett, 1988). It consists of 3884 nucleotides and has a single, major open reading frame in the viral complementary-sense RNA (Collett *et al*, 1985; Takehara *et al*, 1989). The regions coding for the start of the mature G2 and G1 polypeptides are located at the codons corresponding to positions 481 and 2092 respectively (Collett *et al*, 1985). Thus the alignment of the protein-coding information with respect to the genomic M RNA is (3′)-G2-G1-(5′) (Collett *et al*, 1985; Ihara *et al*, 1985). The expression strategy employed by the RVFV M segment is complex, not only involving multiple translational initiation events, but distinct biosynthetic pathways for the two major viral glycoproteins G1 and G2 (Suzich *et al*, 1990).

In addition to the structural glycoproteins G1 and G2, two other proteins are encoded by the M gene : a glycosylated 78K and a non-glycosylated 14K NSm protein (Kakach *et al*, 1988). All four proteins can be detected in RVF virus-infected cells (Kakach *et al*, 1988; Wasmoen *et al*, 1988). The 78K protein probably represents an unprocessed polyprotein of NSm and G2 sequences, while

the 14K protein contains pre-G2 sequences (Kakach *et al*, 1988; Suzich and Collett, 1988). The significance of these nonstructural proteins is not known, but they are apparently not required for synthesis or processing of mature G2 or G1 (Kakach *et al*, 1988; Suzich and Collett, 1988; Takehara *et al*, 1990; Wasmoen *et al*, 1988). All the M segment gene products have a high cysteine content, suggesting that extensive disulphide bridge formation may occur (Collett *et al*, 1985; Ihara *et al*, 1985).

The S segment RNA encodes the viral nucleocapsid protein (N, 26-28 K) in the viral complementary sense RNA, and a nonstructural protein, NSs (29-31 K) in the virion-sense RNA (Parker *et al*, 1984). The latter is a phosphorylated protein which is synthesized early in infection (Smith *et al*, 1990). While the function of the NSs protein in virus replication is not yet known, studies have suggested that this protein carries protective determinants and that an altered NSs can result in significant virus attenuation (Dreier *et al*, 1990).

Little information is available regarding the L segment of RVFV. It is, however, believed to contain information coding for the viral transcriptase or L protein and to employ a negative-sense coding strategy (Suzich *et al*, 1990) as has been substantiated experimentally for some bunyaviruses (Endres *et al*, 1989).

As has been shown for other members of the *Bunyaviridae* family (Bishop and Shope, 1979), the segmented nature of the RVFV genome allows the rapid generation of virus reassortments when multiple strains of RVFV infect the same cell *in vitro* or *in vivo* (Saluzzo and Smith, 1990; Turell *et al*, 1990). These reassortant viruses presumably have the potential to be transmitted in nature, thereby possibly providing a mechanism for increased heterogeneity (Turell *et al*, 1990).

1.2.3 Morphogenesis

The early events in the infection process of RVFV and other *Bunyaviridae* members are not well defined (Schmaljohn and Patterson, 1990). Several viruses in the *Bunyaviridae* family appear to fuse infected cells at low pH values (Arikawa *et al*, 1985; Gonzalez-Scarano, 1985; Gonzalez-Scarano *et al*, 1984) suggesting an entry pathway involving endocytosis into acidic intracellular vesicles (Marsh, 1984; Marsh and Helenius, 1980; 1989). While direct evidence for this process with RVFV has not yet been obtained (Schmaljohn and Patterson, 1990), by electron microscopy RVF viral particles appear to enter cells in phagocytic vacuoles (Ellis *et al*, 1988), which is consistent with the endocytic mode of entry (Marsh and Helenius, 1980). The mildly acidic pH in the endosomal compartment (Tycko and Maxfield, 1982) then triggers virus-cell membrane fusion, resulting in the release of the nucleocapsid into the cytoplasm and the initiation of the replication process (Lenard and Miller, 1982; Lentz, 1990; Marsh, 1984; Marsh and Helenius, 1989; Matlin *et al*, 1981; White *et al*, 1983).

The first sign of replication of RVFV within an infected host cell is the appearance in the nucleus of a fibrillar rod containing viral antigen (Ellis *et al*, 1988; Swanepoel and Blackburn, 1977). Viral components aggregate next in the cytoplasm to form cores which bud into vacuoles to assemble mature particles (Ellis *et al*, 1988). The site of virus maturation and intracellular transport of RVFV membrane proteins varies as a function of both the cell type and strain of virus (Anderson and Smith, 1987). RVFV replication in hepatocytes, for example, is associated with maturation at cellular surface membranes in addition to the smooth internal membranes of the Golgi and endoplasmic reticulum observed in other cell types (Anderson and Smith, 1987).

Virus release is believed to be both by rupture of cells and fusion of particle-containing vacuoles with the plasma membrane and subsequent egestion (Murphy

et al, 1973). A single cycle of RVFV replication in Vero cell cultures is complete in about 13 hours (Ellis *et al*, 1988).

1.3 Envelope glycoproteins G1 and G2

1.3.1 Functional role and antigenic properties

Knowledge of the structural, functional and antigenic properties of the viral envelope proteins is an essential requirement for the understanding of virus infection at both the molecular level and the host immune response to infection (Keegan and Collett, 1986; Parsonson and McPhee, 1985).

Studies on the function of the G1 and G2 structural proteins of several members of the *Bunyaviridae* family have shown that they play a critical role in viral infection and pathogenesis and elicit neutralizing and haemagglutination-inhibition antibodies (Arikawa *et al*, 1989; Dantas *et al*, 1986; Kingsford and Hill, 1981; 1983; Pifat *et al*, 1988). One or both surface glycoprotein spikes appear to be involved in virus attachment and possibly penetration and subsequent virus replication in a susceptible host cell (Gonzalez-Scarano *et al*, 1982; Ludwig *et al*, 1989; 1991).

1.3.2 Analysis of viral epitopes by monoclonal antibodies

The antigenic reactivity of viruses represents their capacity to undergo specific binding with antibodies and cellular receptors, and resides in restricted parts of the viral surface (Crumpton, 1974; Milton and Van Regenmortel, 1979; Van Regenmortel and Neurath, 1985; Westhof *et al*, 1984) These antigenic determinants or epitopes possess a three-dimensional structure complementary to that of the binding site of the antibody molecule. The binding site in turn is composed of a series of hypervariable loops forming a cleft at the distal end of the Fab regions of the immunoglobulin molecule (reviewed in Capra and Kehoe, 1975; Hasemann and Capra, 1989). Recent X-ray crystallographic studies have

revealed that the antibody binding site is able to accommodate protein epitopes consisting of about 15-22 amino acid residues (reviewed in Laver *et al*, 1990).

Antigenic sites have been broadly divided into two categories ie "sequential" or "conformational" depending on whether their specificities rely on the amino acid sequence only, or on the conformation of the whole protein (Atassi, 1980; Crumpton, 1974; Sela, 1969). A more recently held concept is that all determinants recognized by antibodies are conformational, in that antibodies will bind with measurable affinity only to those molecules presenting in the right conformation (reviewed in Laver *et al*, 1990). Conformational determinants may be continuous or discontinuous (Atassi and Smith, 1978). In the former, all the residues in contact with the antibody are contained within a single segment of the amino acid sequence of the antigen. Discontinuous sites, on the other hand, are composed of distantly located amino acid sequences which are brought together by folding of the polypeptide chain (Atassi and Smith, 1978). Both non-covalent interactions as well covalent bonds, such as disulphide bridges, play important roles in maintaining protein conformation (cited in Berzofsky and Berkower, 1989; Crumpton, 1974).

Since the accessibility of any individual antigenic determinant on the antigenic molecule is necessary for its interaction with either antibodies or specific cell receptors (Arnon, 1985), many epitopes can be localized to those parts of the protein that are on the outer surface of the folded molecule (Atassi, 1978; Nowak and Wengler, 1987; Westhof *et al*, 1984). The antigenic epitopes of viral envelope proteins, for instance, are often associated with hydrophilic protein domains, particularly regions of the folded polypeptide chain that jut out, such as loops, carboxy termini, and turns between helices (Atassi, 1978; Eisenberg *et al*, 1985; Nowak and Wengler, 1987; Pellet *et al*, 1985; Westhof *et al*, 1984).

The localization of epitopes in the sequence of the viral proteins has been accomplished experimentally by using a number of methods (reviewed in Berzofsky and Berkower, 1989; Carter and ter Meulen, 1984; Heinz, 1986; Horsfall *et al*, 1991). Continuous antigenic sites have been identified and mapped by binding of antibodies to cleavage fragments and short synthetic peptides from the protein sequence (Ball *et al*, 1992; Eisenberg *et al*, 1985). To map discontinuous topographic sites, more complex approaches have been necessary (Burnens *et al*, 1987; Geysen *et al*, 1987a; Horsfall *et al*, 1991; Jemmerson and Paterson, 1986). These include the mapping of epitopes by the selection of virus variants (Carter and ter Meulen, 1984; Pellet *et al*, 1985), by chemical modification of free and antibody-bound protein antigen (Burnens *et al*, 1987) and by proteolysis of antigen-antibody complexes (Jemmerson and Paterson, 1986). An alternative has been to use the 'mimotope' strategy (Geysen *et al*, 1987a).

In addition to the experimental approaches, continuous antigenic determinants have also been predicted by computer analysis using programmes that provide information on the hydrophilicity, surface probability, flexibility and antigenicity index of the amino-acid sequence in the viral polypeptide chain (Becker, 1990; 1992). The putative antigenic domains have then been compared with the experimentally reported domains for the envelope glycoproteins of several viruses (Becker, 1990; 1992). Crystallographic studies of the three- dimensional structure of proteins have permitted the complete structure of epitopes to be defined (reviewed by Laver *et al*, 1990).

Fine analysis of the antigenic structures of viral proteins (Heinz, 1986; Roehrig *et al*, 1982) has been made possible by the development of hybridoma technology by Köhler and Milstein (1975; 19.6). These somatic hybridization techniques allow the production of cell lines that continuously synthesize monoclonal antibodies (MAbs) of uniform specifity. The use of such chemically homogeneous antibodies which react with constant avidity to single antigenic

determinants thus permits exquisite analysis of epitope/antibody interactions (Carter and ter Meulen, 1984).

By means of MAbs, specific viral epitopes involved in biological functions such as neutralization and haemagglutination can be identified, and the topological relationship of the epitopes to each other on the surface of the molecule can be examined (Heinz, 1986; Stone and Nowinski, 1980; Yewdell and Gerhard, 1981). Analysis of the spatial arrangement of the antigenic sites can be achieved by a competitive binding assay (CBA) using an ELISA or radioimmunoassay (Heinz, 1986; Stone and Nowinski, 1980), although selection of virus variants can also be used (Carter and ter Meulen, 1984; Pellet *et al*, 1985).

Since MAbs are capable of detecting differences of one amino acid, it is possible to identify structural alteration in the target molecule (Carter and ter Meulen, 1984) and perform a precise analysis of intervirus relationships at both the structural and biological levels (Massey and Schochetman, 1985; Roehrig *et al*, 1983). Defined MAbs also have the potential for the assessment and production control of modern vaccine manufacturing (Heinz, 1986).

MAbs have been used to identify physical and functional antigenic domains within the envelope glycoproteins of several members of the *Bunyaviridae* family (Arikawa *et al*, 1989; Dantas *et al*, 1986; Gonzalez-Scarano *et al*, 1982; Kingsford *et al*, 1983; Najjar *et al*, 1985) including the phlebovirus Punta Toro (Pifat *et al* 1988).

The structural topography of the surface glycoproteins of several of these viruses has been examined using MAbs in competitive binding assays. Epitope mapping studies on La Crosse virus (LACV) have for example revealed the presence of eight antigenic regions on the G1 glycoprotein (Kingsford *et al*, 1983). In the study by Arikawa *et al* (1989), nine distinct, partially-overlapping antigenic sites,

two on G1 and seven on G2, were demonstrated for Hantaan virus. Monoclonal antibodies reactive with the Punta Toro G1 protein were shown to bind to epitopes in two distinct topological sites (Pifat *et al*, 1988).

Functional antigenic domains involved in virus neutralization and haemagglutination have furthermore been identified on the envelope glycoproteins of these viruses. MAbs have revealed that virus neutralization sites were located on the G1 protein of La Crosse virus (Gonzalez-Scarano *et al*, 1982; Grady *et al*, 1983; Kingsford *et al*, 1983), G1 and G2 proteins of Hantaan virus (Arikawa *et al*, 1989; Dantas *et al*, 1986) and Punta Toro virus (Pifat *et al*, 1988). With the exception of Hantaan virus (Arikawa *et al*, 1989), most of the glycoprotein epitopes with neutralizing activity were also found to be involved in viral haemagglutination. Some of these antibodies were moreover shown to be capable of passive protection from lethal virus infection (Pifat *et al*, 1988; Schmaljohn *et al*, 1990).

1.3.3 RVFV monoclonal antibodies

RVFV MAbs prepared against the Egyptian ZH501 RVF isolate (cited in Battles and Dalrymple, 1988) and the Entebbe strain (Meegan *et al*, 1983) have been useful in defining a limited number of the glycoprotein antigenic determinants (Keegan and Collett, 1986) and identifying several epitopes involved in virus neutralization (Collett *et al*, 1987; Schmaljohn *et al*, 1989). In the study by Keegan and Collett (1986), three neutralizing sites on G2 of the ZH501 strain were identified and the corresponding gene coding assignments were defined. Antibodies directed against the G2 protein have furthermore been shown to be capable of passive protection in mouse studies (cited in Battles and Dalrymple, 1988).

RVFV MAbs produced against the glycoproteins and the nucleocapsid protein have also been used to examine possible antigenic variation amongst strains

isolated from Egypt, Central Africa and Madagascar (Meegan *et al*, 1983; Morvan, pers comm; Saluzzo *et al*, 1989 a; b). These have shown that Zinga virus is a strain of RVFV (Meegan *et al*, 1983) and are able to distinguish between virus strains collected in Egypt and sub-Saharan Africa (Saluzzo *et al*, 1989a; b). MAbs have also been generated against the RVFV NSs protein in order to assess the function and antigenic structure of this nonstructural protein (Smith *et al*, 1990). The findings have revealed that the NSs protein appears to be highly conserved antigenically among RVFV strains isolated from various regions in Africa.

MAbs have furthermore provided the opportunity to examine the potential of segment reassortment to serve as a mechanism for generating genetic diversity among RVF viruses. In a recent study the origin of the S and M RNA segments of reassortment viruses was determined using MAbs capable of differentiating the nucleocapsid protein (S segment marker) or the G1 (M marker) of the parental RVFV strains (Turell *et al*, 1990). The antigenic analysis of reassortment viruses generated from the attenuated RVFV MV P12 candidate vaccine strain and a wild strain isolated in Senegal has also been performed using these MAbs (Saluzzo and Smith, 1990). Several G1 and G2-specific MAbs have also been used to study the candidate MV P12 vaccine strain by examining the properties of baculovirus-expressed RVFV glycoproteins synthesized in insect cells (Takehara *et al*, 1990).

1.4 Scope of this study

The RVFV envelope glycoproteins G1 and G2 play a critical role in viral infection and pathogenesis. The structural, functional and antigenic properties of the surface glycoproteins, however, have not been fully characterized. There is little published data, for example, on the actual antigenic determinants responsible for eliciting RVFV neutralizing, haemagglutination-inhibition and protective antibodies, particularly for the G1 protein.

In order to gain a better understanding of the role of the RVF viral surface proteins in infection and neutralization, the objectives of the present study will be to analyse the antigenic structure and function of these proteins using monoclonal antibodies.

The research proposals are thus to:

1) Generate a panel of MAbs to the RVFV G1 and G2 glycoproteins and use them to identify functional epitopes on the viral proteins.

2) Analyse the structure of the glycoproteins by mapping the antigenic sites and examining the conformation of the epitopes.

3) Correlate the epitope specificities with functional activities.

4) Analyse the mechanisms of antibody-mediated neutralization of the virus.

5) Examine the role of the glycoprotein epitopes in virus attachment and penetration into the host cell.

2. MONOCLONAL ANTIBODY PRODUCTION AND CHARACTERIZATION

2.1 Introduction

Fine analysis of the antigenic determinants of viral proteins (Roehrig *et al*, 1982) is possible using hybridoma technology (Köhler and Milstein, 1975; 1976) which allows the production of cell lines that continuously synthesize antibodies of uniform specificity. By means of these somatic cell hybridization techniques, immunocompetent lymphocytes are rendered 'immortal' by fusion with myeloma cells. The latter are plasmacytoma cell lines which have for example been selected to lack the enzyme hypoxanthine guanosine [illegible] syl transferase. Such mutants cannot grow in medium containing [illegible] supplemented with hypoxanthine and thymidine (HAT medium) (Littlefield, 1964) because they are unable to utilize the salvage pathway. Hybrids between the myeloma cells and spleen cells can be selected from the parental components as the only cells that actively multiply in HAT selective medium. The antibodies produced by the resulting cell hybrid (hybridoma) clones are by definition monoclonal antibodies (MAbs) and are, in effect, homogeneous immunological reagents of defined specificity, avidity, high specific activity and selected isotype (Nowinski *et al*, 1983). These MAbs react with constant avidity to single antigenic determinants, thereby permitting exquisite analysis of viral epitope/antibody interactions and antigenic structure in the target molecule (Carter and ter Meulen, 1984; Heinz, 1986).

In recent years RVFV MAbs have been prepared against the Entebbe and the Egyptian ZH501 strains (Meegan *et al*, 1983; Smith, cited in Battles and Dalrymple, 1988). These have been useful in defining a limited number of the envelope glycoprotein antigenic determinants (Collett *et al*, 1987; Keegan and Collett, 1986; Schmaljohn *et al*, 1989). However, unlike Punta Toro virus, a

related phlebovirus, a comprehensive study using MAbs to analyse the antigenic determinants of the RVFV surface glycoproteins has not been undertaken to date.

Accordingly, it is intended to study the RVFV envelope glycoproteins by generating a panel of MAbs against the South African 1830 strain of RVFV and identifying functional epitopes. The latter will be determined on the basis of reactivity of the MAbs in haemagglutination-inhibition, neutralization and complement fixation tests. The possibility that certain of the RVFV epitopes are more conserved than others will be examined by determining the cross-reactivity of the MAbs with another member of the phlebovirus genus. The antibodies will also be tested for their ability to protect otherwise lethally infected mice.

2.2 Materials and Methods

2.2.1 Virus

2.2.1.1 Preparation of stock virus

Stocks of RVFV for use in the various serological assays and for the preparation of purified virus were grown in Vero cells. Monolayers of the cells in 150 cm^3 flasks (Cel-Cult, Sterilin Ltd., U.K.) were inoculated with 2 ml of the South African prototype RVF strain AN 1830 (tissue culture passage 2, mouse passage 6) and incubated at 37°C for 1 h to allow adsorption to take place. The inoculum was then removed, and replaced with Earle's minimum essential medium (EMEM) containing 2% foetal calf serum (FCS) and 0,3% gentamicin. The flasks were reincubated at 37°C and the supernatant harvested when the cells showed advanced cytopathic effect (CPE). The medium was clarified by centrifugation at 3000 rpm for 8 min in a Beckman J2-21 centrifuge at 4°C. The resulting supernatant was amped out and stored at -70°C.

The titre of each batch of stock virus was subsequently determined by titration in flat-bottomed 96 well microtitre plates (A/S Nunc, Roskilde, Denmark). Serial ten-fold dilutions of the virus were prepared in Leibovitz-15 medium

supplemented with 5% FCS and 0,3% gentamicin. Volumes of 0,1 ml virus were added to the wells of the microtitre plates in quadruplicate. An additional 0,1 ml Leibovitz medium was added to each well, followed by 3 x 10^4 Vero cells per well in 0,025 ml medium. The plates were incubated in a humidified container at 37°C and on the sixth day examined for CPE. The viral titre was calculated using the Kärber method as described by Hawkes (1979).

2.2.1.2 Purification of RVFV and glycoprotein isolation

RVFV was propagated in Vero cell monolayers as described above and harvested 2-3 days post-infection at maximum CPE. The supernatant fluids were clarified at 7000 g for 30 min at 4°C in a Beckman J2-21 centrifuge. The clarified supernatant was precipitated with polyethylene glycol 6000 (PEG) by the addition of 7,5% (w/v) PEG and 2,5% (w/v) NaCl and held at 4°C overnight. Following centrifugation at 10 000 g for 1 h at 4°C the resulting pellets were resuspended in TNE buffer (0,01M Tris; 0,1M NaCl; 0,001M EDTA pH 7,2). These were clarified at 5000 rpm for 15 min at 4°C and the cleared supernatants layered over a discontinuous CsCl gradient consisting of 2 ml of 35% (w/w) CsCl and 3 ml of 20% (w/w) CsCl in TNE buffer. The tubes were spun at 15°C for 16 h at 35 000 rpm (155 000 g) in a Beckman L8-60M ultracentrifuge using a SW40 Ti rotor. The visible viral band was harvested and dialysed extensively against TNE buffer at 4°C. The protein concentration of the purified virus was determined spectrophotometrically at 280 nm.

The method used to extract the RVFV glycoproteins was based on that of Pifat *et al* (1988). Firstly, RVFV was grown and PEG-precipitated as above. Purified glycoprotein preparations were obtained by disruption of the virus supernatants with an equal volume of 2% (v/v) Nonidet P40 in TNE for 20 min at room temperature (rt). The lysate was layered over a discontinuous CsCl gradient and centrifuged as described above. The supernatant fractions containing G1 and G2 were harvested and dialysed against TNE at 4°C. The extracted glycoprotein

preparation was then concentrated by ultrafiltration using immersible CX-10 ultrafiltration units (Millipore Corp., Bedford, U.S.A.) and the protein concentration determined spectrophotometrically.

2.2.2 Production of monoclonal antibodies

2.2.2.1 Cells

a) Myeloma cells

The FO-clone of SP2/0 (Fazekas de St. Groth and Scheidegger, 1980) was obtained from ATCC (U.S.A.). The cells were grown in standard medium consisting of RPMI-1640 with 15% foetal calf serum, 0,001M sodium pyruvate, 0,0375% sodium bicarbonate, 0,05% of 0,1M 2-mercaptoethanol, 0,01% amphotericin B and antibiotics. For the selective medium, 50 x hypoxanthine aminopterin thymidine (HAT) and hypoxanthine thymidine (HT) concentrates (Flow Laboratories, Irvine, U.K.) were added to give a final concentration of 1%. Care was taken to maintain the myeloma cultures in an exponential growth phase so as to optimize the amount of fusion competent cells. As the FO myeloma line has a doubling rate of 8,7 h (Fazekas de St. Groth and Scheidegger, 1980), this entailed subculturing the cells almost daily.

b) Immune spleen cells

Balb/c mice were immunized by intraperitoneal (i.p.) inoculation of 0,5 ml of a 10^{-1} suspension of beta-propriolactone inactivated RVF 1830 mouse brain antigen. Three days later the inoculation was repeated using inactivated virus, and on days 13 and 18 the mice were boosted i.p. with live virus. After a further ten days, the mice were given 0,05 ml of purified glycoprotein preparation (17 mg/ml) plus 0,2 ml i.p. for two successive days. Spleens were removed three days later for fusion.

c) Peritoneal macrophages

Adult mice were killed and the abdominal skin removed aseptically. Five ml of a 11,6% sterile sucrose solution was injected peritoneally, the abdomen gently massaged and the fluid withdrawn. The cells were collected in 15 ml polypropylene tubes (Falcon, Becton Dickinson and Co., Oxnard, U.S.A.), spun down at 1000 rpm in a Beckman TJ-6 benchtop centrifuge and resuspended in HAT media to yield 6 x 10^4/ml macrophages.

2.2.2.2 Cell fusion

The method for production and maintenance of monoclonal antibodies was based on that of Fazekas de St. Groth and Scheidegger (1980).

A. On the day before fusion:

i) Myeloma cells

Myeloma cultures were subcultured to ensure that the cells were in a logarithmic growth phase for fusion the following day.

ii) Preparation of microcultures

0,15 ml freshly prepared HAT-medium was added to the wells of six microtitre tissue culture plates (A/S Nunc, Roskilde, Denmark). Macrophages were prepared as described above and 0,05 ml drops were distributed in the microtitre wells. The plates were incubated in a humidified container at 37°C.

B. On the day of fusion

i) Preparation of parental cells for fusion

a) Myeloma cells

Myeloma cells were harvested and pelleted in 50 ml centrifuge tubes at 1000 rpm for 7 min. The resulting pellets were pooled, topped up to 50 ml with saline (Dulbecco buffer with 0,28% glucose pH 7,2) and an aliquot mixed with Trypan

blue and counted to determine that the viability was greater than 90%. The remaining cells were spun down again and resuspended in saline at a concentration of 10^7 cells/ml.

b) Spleen cells

The spleens from two hyperimmune mice were removed under sterile conditions and carefully washed in saline. Using forceps, the spleen cells were gently teased into 10 ml saline and transferred to a 15 ml centrifuge tube. The cells were dispersed by pipetting and the remaining clumps and pieces of connective tissue allowed to sediment for 10 min. The supernatant was transferred to a 50 ml centrifuge tube, topped up with saline and spun for 7 min at 1000 rpm. The cell pellet was taken up in saline, counted in Turck's solution and adjusted to a concentration of 10^8 cells/ml.

ii) **Fusion**

Five ml myeloma cell suspension was combined with 3 ml spleen cell suspension in a 50 ml centrifuge tube and saline added to a final volume of 50 ml. The suspension was centrifuged at 1000 rpm for 7 min, the supernatant carefully tipped off and the pellet loosened by flicking the bottom of the tube. One ml polyethylene glycol fusing solution (PEG-4000; Merck Chemicals, Hohenbrunn, Germany) was added over 1 min under constant agitation of the tube. The tube was then immersed for 1 min in a 37°C water bath, keeping the contents swirling all the time. The fusion was stopped by slowly adding 20 ml saline, 1 ml over the first 30 sec, 3 ml over the next 30 sec and the rest over the second minute. The mixture was topped up with saline, allowed to stand 5 min and then spun as before. The saline supernatant was replaced with standard medium and the solution centrifuged again. The cell pellet was recovered and resuspended in 30 ml HAT-medium by gentle pipetting. 0,05 ml drops were distributed in the wells of the microtitre plates prepared the previous day, and the plates returned to the humidified container at 37°C.

On the sixth day after fusion, approximately 50% of the supernatant fluid from each well was removed and replaced with fresh HAT-medium. Feeding of the cultures was subsequently carried out every second day. The plates were inspected daily from the sixth day and growing colonies were transferred to new 96 well plates containing freshly prepared macrophages before they were screened for the presence of RVFV antibodies. This was done to exclude detecting any antibodies in the supernatants which may have been excreted by the spleen lymphocytes prior to their death shortly after plating out. The hybridoma colonies were scored as follows: >50 cells = 1+; 50 to 500 cells = 2+; 500 to 1000 = 3+; >1000 = 4+. An aliquot of the supernatant fluid of wells containing 3+ and 4+ sized colonies was removed for screening for antibody secretion by ELISA and indirect immunofluorescent antibody assay.

2.2.2.3 Detection of positive clones

a) ELISA

In order to screen for hybridomas excreting antibodies specifically directed against the RVFV glycoproteins, an ELISA using extracted glycoprotein (2.2.1.2) as the capture antigen was developed. The optimal concentration of viral glycoprotein for use as the solid phase antigen was first established. This was achieved by preparing a starting dilution of 1:50 of the antigen in carbonate-bicarbonate buffer pH 9,6 (Appendix A). Serial two-fold dilutions of the viral antigen were then prepared in polystyrene microtitre ELISA plates (Nunc - Immuno II A/S, Nunc, Roskilde, Denmark). Following overnight incubation at 4°C, the plates were washed three times with phosphate buffered saline (PBS) containing 0,01% Tween-20 using an automated microplate washer (Bio-Tek Instruments, Flow Laboratories, Irvine, U.K.) The wells were then blocked with 0,2 ml of 4% bovine serum albumin (BSA) in PBS for 30 min at room temperature. After washing as before, 0,1 ml volumes of RVFV hyperimmune ascitic fluid diluted 1:1000 in ELISA diluent (PBS with 5% foetal calf serum) were added to the wells in duplicate. Controls included RVFV hyperimmune ascitic fluid in wells without

antigen, antigen coated wells without antibody, and antigen coated wells with the hybridoma supernatant from a MAb prepared against West Nile virus (Besselaar and Blackburn, 1988). Following incubation for 1 h at 37°C, the plates were washed again and incubated with a 1:1000 dilution of peroxidase conjugated anti-mouse immunoglobulins (Zymed Laboratories Inc., San Francisco, C.A., U.S.A.) in ELISA diluent for a further hour at 37°C. ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, U.S.A.) was added after washing and optical densities (O.D.) were measured at 405 nm with a Titertek multiscan apparatus (Flow Laboratories, Irvine, U.K.).

The supernatants of the hybridoma cultures were screened using plates which had been coated with the predetermined optimal dilution of glycoprotein extract (8,5 μg/well) and blocked with BSA as above. The hybridoma supernatants were added to the wells in duplicate (0,1 ml/well) and incubated for 1 h at 37°C. The plates were washed three times, and incubated with peroxidase conjugated anti-mouse immunoglobulins for 1 h, followed by ABTS substrate as described above.

b) Indirect immunofluorescence antibody assay (IFA)

Colonies positive by ELISA for excretion of antibodies directed against the RVFV glycoproteins were then tested by IFA as a confirmatory assay using 8-well multitest slides (Flow Laboratories) with RVFV infected cells. The slides were prepared earlier as follows: Vero cells were infected with RVF 1830 tissue culture passage two or three (TC2 or 3) at an input multiplicity of 0,1 - 1 and harvested when early signs of cytopathic effect (CPE) vident. The cells were adjusted to 1,5 x 10^6/ml and mixed in a 1:3 ratio with uninfected cells. The cell suspensions were spotted onto the slides, air dried, then fixed with acetone and stored at -70°C until use. Immunofluorescence tests were carried out by incubating slides with neat hybridoma culture fluids for 2 h at 37°C. The slides were then washed in PBS and treated with affinity purified anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (Zymed Laboratories).

After a further 30 min incubation at 37°C, the slides were washed, mounted with glycerol and viewed with a Nikon HFX-II Optiphot ultra-violet microscope.

2.2.2.4 Cloning and expansion

Hybridoma colonies excreting antibodies specific for the RVFV glycoproteins were cloned by limiting dilution by preparing 10^{-1}, 10^{-2}, and 10^{-3} dilutions of the cells in HAT-selective medium. Drops (0,05 ml) of the resulting cell suspensions were then distributed in a number of wells in a 24 well tissue culture plate (Costar, Cambridge, MA, U.S.A.) each containing 1 ml medium and 2 x 10^4 macrophages. The medium was replaced every third day until the secondary colonies were large enough to be retested by ELISA. Those colonies producing RVF antibodies were transferred to six well tissue culture plates and later to 15 ml tissue culture flasks (25 cm^3, Costar). The medium was changed to HT-selective medium at this stage as the aminopterin was no longer necessary since the only surviving cells were the myeloma-lymphocyte hybridomas. The cells were expanded by passaging them firstly into 75 cm^3 and later into 150 cm^3 flasks, and used to prepare both frozen stocks of hybridoma clones and monoclonal antibody-rich ascitic fluid.

Frozen stocks were prepared as follows: the hybridoma cells were harvested, spun down and adjusted to a concentration of 10^7/ml in standard media containing 10% dimethylsulphoxide. The cell suspensions were aliquoted in 1 ml volumes and frozen down slowly by cooling first at 4°C for 1 h, then storing at -70°C overnight before transferring them to liquid nitrogen (N_2).

2.2.2.5 Production of ascitic fluid

Balb/c mice were injected i.p. with 0,5 ml Pristane (2,6,10,14-tetramethylpentadecane; Sigma Chemicals, St. Louis, Mo, U.S.A.) as this is essential for ascites formation (Brodeur *et al*, 1984). Two weeks after priming, the mice were injected i.p. with 10^5 hybridoma cells which corresponded to the

optimum number of hybrid cells for ascitic fluid production as determined previously (Besselaar and Blackburn, 1988). Ten to fourteen days later, the ascitic fluid was aseptically collected and clarified at 3000 rpm for 15 min. The fluid was stored at 4°C overnight, then spun at 7000 g for 30 min in a Beckman J2-21 centrifuge and the supernatant aliquoted and frozen at -70°C.

The initial batch of fluid was produced using cloned hybrid cells which had not yet been frozen down. For subsequent batches, hybridoma stocks stored in liquid N_2 were thawed quickly, resuspended in standard RPMI medium supplemented with 5% macrophage extract and grown in 25 cm^3 flasks. The macrophage extract was produced by culturing peritoneal macrophages in standard media with 5% FCS in a 150 cm^3 flask and harvesting the supernatant fluid after seven days. This was then passed through sterile 22 μm Millex GS filter unit (Millipore Corp., Bedford, U.S.A.) and stored at -70°C as a stock of growth enhancing medium. The hybridoma cells were passaged once or twice until sufficient viable cells for inoculation into mice were reached.

2.2.3 Characterization of MAbs

2.2.3.1 Isotyping of monoclonal antibodies

The monoAb-ID EIA kit (Zymed) was used for isotype determination of the monoclonal antibodies. ELISA plates (Nunc Immuno II) were coated with goat anti-mouse antibody (10μg/ml) in coating buffer (pH 9,6) at 4°C overnight. The wells were washed with PBS-Tween and blocked with 0,2 ml/well of PBS containing 0,5% bovine serum albumin at 37°C for 1 h. After washing the plates, 0,05 ml of hybridoma culture supernatants were added to each of 6 wells and incubated at 37°C for 1 h. The wells were again washed and 0,05 ml of subclass-specific rabbit anti-mouse immunoglobulins or normal rabbit serum were added to each of the 6 wells. After incubation at 37°C for 1 h, the plates were washed thoroughly and 0,05 ml/well of alkaline phosphatase-labelled anti-rabbit immunoglobulins was added. The plates were incubated at 37°C for 1 h, washed

as before and 0,1 ml of p-nitrophenyl phosphate substrate solution dispensed in the wells. The colour reaction was monitored for 2 h and positive results were read quantitively at 405 nm.

2.2.3.2 Radioimmune precipitation assay (RIPA)

a) Preparation of radiolabelled intracellular virus induced proteins

Specificity of the monoclonal antibodies was determined by RIPA using detergent extracts of infected cells. Vero cells in 75 cm^3 tissue culture flasks were infected at a multiplicity of infection of 1-10 and incubated in serum-free Eagle's minimum essential medium with Earle's salts (EMEM). At 3 h post-infection the medium in each flask was replaced with 8 ml methionine-deficient EMEM (Flow Laboratories) for 1 h, followed by the addition of L-[^{35}S] methionine (20μCi/ml, sp.act. 1000 Ci. mmol; Amersham Int., U.K.) for 1-20 h post-infection at 37°C. Mock infected cultures were labelled in the same way. After discarding the supernatant, the cells were scraped off using a disposable cell scraper (Costar, MA, U.S.A.), resuspended in cold PBS and transferred to centrifuge tubes. The cells were washed three times by spinning at 1000 rpm for 8 min and the final cell pellet used for immunoprecipitation.

b) Immunoprecipitation

The labelled cell pellets were resuspended in NET/BSA buffer (0,15M NaCl, 0,005M EDTA, 0,05M Tris-HCl pH 7,4; 5% Nonidet P40; 1 mg/ml BSA) at a concentration of 1 x 10^5 cells/ml. Following incubation on ice for 30 min, nuclei were removed by centrifugation at 3000 rpm for 15 min. The supernatants were made 0,1% (w/v) with respect to SDS, disrupted with a probe sonicator and clarified by centrifugation at 15000 rpm (18000 g) for 30 min. Immunoprecipitation was performed by adding 50 μl of the required monoclonal immune ascitic fluid to a 1 ml aliquot of the clarified radiolabelled supernatants. The virus-antibody mixtures were incubated for 4-6 h rt.

Protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) were resuspended in NET-BSA buffer and added to each mixture at 10 mg/ml for 2 h at rt, followed by 4°C overnight. The immune complex was collected by centrifugation and washed five times with NET-BSA buffer. The bead pellet was solubilized by treatment with dissociation buffer (Appendix B) and heated at 100°C for 5 min. The beads were pelleted at 1000 rpm for 10 min, the sample buffer recovered and stored at -70°C until analysis by PAGE.

c) Polyacrylamide gel electrophoresis (PAGE)

The immunoprecipitated viral proteins were analysed on linear 12% polyacrylamide gels essentially by the method of Smith and Brown (1977). PAGE was performed in 1,5 mm thick slab gels using a 4% stacking gel and a 12% resolving gel in the Tris-glycine buffer system of Laemmli (1970) (Appendix B). In both the stacking and resolving gels, diallyltartardiamide (DATD, Sigma Chemicals, St. Louis, Mo, U.S.A.) was used as a cross-linker instead of bisacrylamide to achieve better resolution of the G1 and G2 proteins. Protein molecular weight determinations were made by running ^{14}C-methylated protein molecular weight standards (Amersham, U.K.) on the same gel. The marker proteins were: myosin, 200 kilodaltons (K); phosphorylase b, 100 and 92,5 K; BSA, 69 K; ovalbumin, 46 K; carbonic anhydrase, 30 K and lysozyme, 14,3 K. The gels were electrophoresed at 16 mA for 16 h at 4°C treated with Amplify (Amersham, U.K.) for 30 min and the separated proteins visualized by autoradiography (Appendix B).

2.2.3.3 Serological tests

a) ELISA titres

The titres of the MAbs in the ELISA were determined by preparing serial $\log_{10}$ dilutions of the ascitic fluids and testing these for reactivity against purified glycoprotein antigen as described in 2.2.2.3 (a). The reactivity of the MAbs

against purified whole virus was also examined using purified virus (2.2.1.2) at a predetermined optimal dilution as the solid phase antigen.

b) Indirect immunofluorescence antibody (IFA) titres

Indirect fluorescent antibody titres were assessed by titrating the MAb ascitic fluids in serial two-fold steps on multitest slides against RVFV infected Vero cells as described in section 2.2.2.3 (b).

c) Haemagglutination-inhibition test

The haemagglutination-inhibition (HI) test used was a micro-adaption (Swanepoel *et al*, 1986) of the technique of Clarke and Casals (1958). For the haemagglutination titration, sucrose-acetone extracted RVFV antigen was reconstituted at a 1:10 dilution in borate buffered saline (BBS), pH 9,0. Serial two-fold dilutions were made in 0,025 ml volumes of BBS containing 0,4% bovine albumin (BABS diluent) in microtitre plates (Linbro Division, Flow Laboratories Inc., Hamden, Conn., U.S.A.). Goose erythrocyte suspensions containing 10% cells were diluted 1:30 in phosphate adjusting diluents to yield the predetermined optimal pH for the antigen and 0,05 ml volumes were added to each well. The plates were read after 30 min to determine the antigen dilution containing 4 units of haemagglutinin (HA) per 0,025 ml volume.

For the HI test, serial two-fold dilutions of extracted monoclonal immune ascitic fluids were prepared in 0,025 ml volumes of BABS diluent from 1:10. Equal volumes of antigen containing four HA units were added to each well. After overnight incubation at 4°C, 0,05 ml volumes of goose erythrocyte suspensions were added to all wells to yield the final optimal pH for the antigen. In addition to cell and antigen controls, ascitic fluid controls were included to ensure that the test specimens were free of nonspecific agglutinins. Haemagglutination-inhibition titres were recorded as the highest dilutions of monoclonal ascitic fluids which caused complete inhibition of haemagglutination.

d) Complement fixation test

The complement fixation (CF) method followed was adapted from Bradstreet and Taylor (1962), using 0,025 ml volumes in microtitre plates (Linbro Division, Flow Laboratories). Rabbit haemolytic serum (Wellcome Reagents Ltd., Beckenham, U.K.) and guinea pig complement were standardized for use in the test by checkerboard titration. The optimal dilution of RVFV antigen was determined by chessboard titration against RVFV hyperimmune ascitic fluid (HAF).

The hybridoma-induced ascitic fluids were diluted 1:4 in veronal buffered saline (VBS) diluent, pH 7,2 and inactivated at 56°C for 30 min. Doubling dilutions of the monoclonal immune ascitic fluids were prepared in 0,025 ml volumes of VBS in round-bottomed microtitre plates. Equal volumes of complement and antigen were added and the tests left overnight at 4°C. The following day the plates were incubated at 37°C for 30 min, then one volume of 2% sensitised sheep erythrocytes was added to each well. The plates were incubated at 37°C for 30 min, shaking after 15 min. The complement fixation titre of each monoclonal antibody was recorded as the highest dilution which produced complete fixation of complement. Controls for all reagents and test specimens were included in each test.

e) Cytopathic effect neutralization test (CPENT)

The CPENT tests were based on the technique of Swanepoel *et al* (1986) using flat-bottomed tissue culture microtitre plates (Sterilin). Two-fold dilutions of the hybridoma-induced ascitic fluids were prepared in quadruplicate in the plates with Leibovitz-15 medium supplemented with 5% FCS. Equal volumes of virus containing 100 tissue culture infective doses$_{50}$ (100 $TCID_{50}$) as determined by previous titration were added to each well and the plates incubated for 1 h at 37°C before seeding with 3 x 10^4 Vero cells per well. A concurrent titration of the virus was performed to establish that the test dose actually contained the

calculated infective dose. The plates were incubated in a humidified container at 37°C and examined after 4 days for ability of the monoclonal antibodies to inhibit cytopathic effect of the virus. Final readings were made on the sixth day and end points recorded as the mean reciprocal of the highest dilutions which prevented cytopathic effect. Complement-enhanced neutralization was determined by the addition of 5% guinea pig serum as a source of complement to the virus-antibody preparations prior to the 1 h incubation step.

f) Plaque reduction neutralization test (PRNT)

Assays were performed in duplicate on Vero cell monolayers in 24 well tissue culture plates (Costar) essentially as described by Swanepoel *et al* (1986). The wells were seeded with 2 x 10^5 Vero cells in Leibovitz medium with 5% FCS, incubated in a humidified container at 37°C and the monolayers used 24 h later. Serial four-fold dilutions of heat-inactivated hybridoma ascitic fluids were prepared in sterile tubes in the Leibovitz medium. An equal volume of virus containing a calculated 80 plaque forming units (PFU) per 0,05 ml, was added to each tube and the MAb-virus mixtures incubated for 2 h at 37°C. Medium was removed from the monolayers and 0,1 ml of each mixture added to each well and allowed to adsorb for 1 h at rt. The cells were then overlaid with Hanks'-based minimum essential amino acid medium (HMEM) containing agarose at a final concentration of 0,5% and 2% FCS. The cultures were incubated at 37°C for two days, then stained with agarose overlay medium containing neutral red at a 1:5000 dilution. Plaques were counted after a further 24 h incubation and MAb neutralizing titres were recorded as the reciprocals of the highest dilutions which produced an 80% reduction in the number of plaques relative to the virus controls. The effect of complement (C') on the MAb neutralization titres was investigated by adding guinea-pig serum at a 1:40 final dilution to the MAb-virus mixtures prior to incubating and staining as above.

2.2.3.4 MAb cross-reactivity

To assess whether the MAbs were cross-reactive with another member of the phlebovirus genus, the hybridoma ascitic fluids were also tested by the indirect immunofluorescence and HI assays against Gordil virus. For the immunofluorescence tests, Vero cells infected with Gordil virus at mouse passage 11 and tissue culture passage 2 were used to prepare multitest slides as described in 2.2.2.3b. The IFA assays were carried out with the RVF hybridoma ascitic fluids at a 1:100 dilution in PBS. The HI tests were performed with sucrose-acetone extracted Gordil virus antigen using the method outlined in section 2.2.3.3c.

2.2.3.5 Passive protection studies

The in vivo protective ability of the MAbs against RVFV was assayed in four week old mice. The virus used in these experiments was stock tissue culture virus (2.2.1.1) with a titre of 10^9/ml. In order to determine the LD_{50} (50% mortality endpoint) for mice, serial ten-fold dilutions of the stock virus were prepared in serum-free EMEM. Groups of four mice were inoculated intraperitoneally (i.p.) with 0,2 ml volumes of each virus dilution ranging from 10^{-3} to 10^{-6}. The mice were monitored for three weeks and euthanased when moribund. The LD_{50} was calculated using the Kärber method as described by Hawkes (1979). The plaque forming units (PFU) were subsequently determined for the virus dilution which corresponded to $10LD_{50}$ by plaque assay as described earlier (2.2.3.3 f).

For the protection studies, monoclonal antibody ascitic fluids were diluted 1:2 in EMEM and 0,2 ml volumes were inoculated i.p. into groups of five mice. Twenty-four hours later, mice were challenged i.p. with $10LD_{50}$ (360 PFU) of the stock RVF virus diluted in EMEM while controls received virus only. The mice were observed for three weeks as outlined above. Statistical significance of the difference in the time of death between the test and control groups was assessed

by the Student's t-test. The level of minimal significance was calculated using both $p < 0,05$ and $p < 0,01$.

2.3 Results

2.3.1 Production of MAbs

Screening of the hybridomas was performed by ELISA using the viral glycoprotein at a dilution of 1:200 for the capture antigen. This corresponded to the highest dilution which gave maximum absorbance (Table 1) and represented a protein concentration of 8,5 μg/well. By the screening ELISA, 23/273 (8,4%) hybridoma clones were found to be excreting antibodies specifically against the glycoprotein antigens. Six of these exhibited weak reactivity, but were confirmed by means of the IFA test.

While all 23 hybridomas were successfully cloned and frozen stocks prepared, the viability of the hybridomas varied considerably on resuscitation from liquid nitrogen. Growth of the cells was greatly promoted by the addition of freshly prepared macrophages or 5% macrophage extract. All 23 clones appeared to be stable as they remained positive for RVFV antibody excretion.

Table 1. Titration of RVFV glycoprotein for use as capture antigen in ELISA

Antigen dilution[1]	Optical density (405 nm)
50	2.000
100	2.000
200	2.000
400	1.757
800	1.254
1600	0.795
No Ag	0.054

[1] Dilutions expressed as reciprocals

2.3.2 Characterization of MAbs

2.3.2.1 Determination of immunoglobulin isotype

The immunoglobulin subclasses of the 23 MAbs are shown in Table 2. The majority of the antibodies were of the IgG1 subclass.

Table 2. Immunoglobulin subclass of MAbs

MAb	Subclass
3E5, 5E1, 3D2, 3H1, 4B3, 5A1, 9E4, 5A6, 9C4, 7F2, 8C2, 7F1, 4D10, 8E6, 6E10, 5C12	IgG1
5E11, 8A3, 5E9, 8G2	IgG2a
5F2	IgG2b
1E4, 8G10	IgM

2.3.2.2 Determination of MAb specificity

The optimum period for radiolabelling the infected cells for the detection of the virus-specific proteins by polyacrylamide gel electrophoresis (PAGE) was investigated by labelling at various times post-infection (p.i.). Labelling for the period 4-20 h p.i. was found to be the most suitable and was used in subsequent immunoprecipitation experiments.

By SDS-PAGE, polypeptides of the following molecular weights were detected from infected cell lysates which had been immunoprecipitated with RVFV hyperimmune ascitic fluid : 65K, 56K, and 26K (Fig. 1 lane 2). The apparent molecular weights of these bands correspond to the known weights for RVFV G1, G2 and NC proteins respectively. The fact that similar polypeptides were not

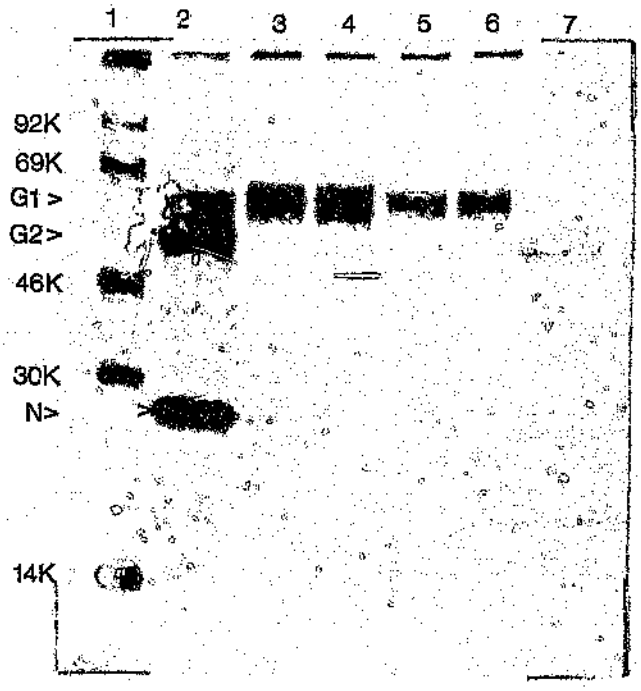


Fig.1. Immunoprecipitation of [^{35}S] methionine-labelled RVFV-specific proteins by selected MAbs. Proteins were electrophoresed on a 12% SDS-polyacrylamide gel. Lane 1, ^{14}C molecular weight markers (Amersham); lane 2, RVFV-infected cell lysate immunoprecipitated with RVF polyclonal hyperimmune ascitic fluid; lane 3, MAb 4B3; lane 4, MAb 3H1; lane 5, MAb 5E1; lane 6, MAb 3E5; lane 7, MAb 8G2.

detected in the uninfected cell controls (data not shown) indicates that these proteins were indeed virus-specific.

The specificity of the MAbs is shown in Table 3. Fourteen of the MAbs precipitated the G1 protein while the remaining nine were specific for the G2 protein. Examples are shown in Fig. 1 (lanes 3-7). While the G2 polypeptide was easily visible when immunoprecipitated by the polyclonal hyperimmune ascitic fluid, the intensity of the G2 band when precipitated by the MAbs was very weak. By increasing the concentration of the ^{35}S label from 10 to 50 μCi/ml and increasing the exposure time, the G2 band was detectable but still weak.

Table 3. Specificity of the RVFV MAbs as determined by the radioimmune precipitation assay

MAb	Specificity
3E5, 5E1, 3D2, 3H1, 4B3, 5A1, 9E4, 5E11, 8A3, 5E9, 5F2, 5A6, 1E4, 8G10	G1
9C4, 8G2, 7F2, 8C2, 7F1, 4D10, 8E6, 6E10, 5C12	G2

2.3.2.3 Serological reactivities

a) Enzyme-linked immunosorbent assay (ELISA) titres

Table 4 shows the results of the reactivity of the MAbs in the ELISA using either purified whole RVFV or the extracted glycoprotein preparation as capture antigen. The titre of most of the MAbs against the extracted glycoprotein antigen was one to two logs higher than against the whole virus.

b) Indirect immunofluorescence antibody titres

IFA titres of hybridoma ascitic fluids ranged from 1:1024 to 1:128 000 (Table 4). For each individual MAb, the titres of different batches of ascitic fluids remained

Table 4. Reactivity of the RVFV MAbs by indirect immunofluorescence and ELISA

MAb	Specificity	IFA titre[1]	ELISA titre[2]	
			Purified virus	Glycoprotein
3E5	G1	64 000	10^5	10^6
5E1	G1	16 000	10^4	10^5
3D2	G1	64 000	10^5	10^7
3H1	G1	32 000	10^7	10^8
4B3	G1	128 000	10^5	10^6
5A1	G1	64 000	10^6	10^7
9E4	G1	8 000	10^5	10^6
5E11	G1	16 000	10^5	10^6
8A?	G1	32 000	10^5	10^7
5E9	G1	64 000	10^4	10^6
5F2	G1	4 096	10^3	10^5
5A6	G1	1 024	10^3	10^4
1E4	G1	16 000	10^3	10^3
8G10	G1	64 000	10^3	10^5
9C4	G2	128 000	10^5	10^6
8G2	G2	64 000	10^5	10^6
7F2	G2	32 000	10^5	10^5
8C2	G2	32 000	10^4	10^5
7F1	G2	64 000	10^5	10^6
4D10	G2	64 000	10^4	10^6
8E6	G2	32 000	10^4	10^6
6E10	G2	4 096	10^3	10^3
5C12	G2	64 000	10^3	10^3

[1] Titres expressed as dilution reciprocals

[2] Titres expressed as - $\log_{10}$ dilution reciprocals

relatively constant. Differences in the intensity of the fluorescence induced by the various MAbs were evident i.e. while the majority exhibited strong fluorescence, several displayed a weaker intensity of fluorescence (5C12 and 5A6).

c) Haemagglutination-inhibition and complement-fixation activities

The biological activities of the MAbs in various serological tests are shown in Table 5. Nine of the G1-specific MAbs and four of the G2-reactive MAbs displayed haemagglutination-inhibition (HI) activity, with titres ranging from weak (1:20) to very strong (1:81920).

In the complement-fixation test, only four G1- and G2-specific MAbs were reactive. MAb 8A3 exhibited the highest activity, with a titre of 1:1024.

d) Neutralizing activities

Two MAbs (3E5 and 9C4) were able to neutralize RVFV in the CPENT (Table 5). Many of the others, however, appeared to delay the appearance of CPE and were therefore tested by the more sensitive PRNT (Table 6). These assays revealed that nine of the anti-G1 MAbs neutralized the virus *in vitro* in the absence of C′, with MAb 3E5 exhibiting the highest neutralization titre. When C′ was added, the majority of these antibodies neutralized to higher titres; furthermore the remaining five non-neutralizing MAbs showed low level neutralizing activity.

With regard to the G2-specific MAbs, three were able to neutralize the virus *in vitro* without C′. Of these, MAb 9C4 was strongly neutralizing whereas MAb 7F2 and 8G2 were much weaker. In the presence of C′ the remaining anti-G2 MAbs, with the exception of MAb 5C12, were able to neutralize at a low level.

Table 5. Haemagglutination-inhibition, neutralizing and complement fixation activities of the RVFV MAbs

MAb	Specificity	HI	CPENT	CF
3E5	G1	2 560[1]	4 096	-
5E1	G1	-	-	-
3D2	G1	10 240	-	-
3H1	G1	81 920	-	-
4B3	G1	40 960	-	-
5A1	G1	81 920	-	-
9E4	G1	10 240	-	-
5E11	G1	-	-	-
8A3	G1	-	-	1 024
5E9	G1	5 120	-	32
5F2	G1	-	-	-
5A6	G1	-	-	-
1E4	G1	5 120	-	512
8G10	G1	10 240	-	-
9C4	G2	40 960	1 024	-
8G2	G2	160	-	8
7F2	G2	40	-	-
8C2	G2	-	-	-
7F1	G2	20	-	-
4D10	G2	-	-	-
8E6	G2	-	-	-
6E10	G2	-	-	-
5C12	G2	-	-	-

[1] Titres expressed as dilution reciprocals

Table 6. Neutralizing activity of the RVFV MAbs by the plaque reduction neutralization test (PRNT)

MAb	Specificity	PRNT	PRNT + C'
3E5	G1	32 768[1]	262 144
5E1	G1	-	512
3D2	G1	32	256
3H1	G1	1 024	16 384
4B3	G1	4 096	8 192
5A1	G1	1 024	4 096
9E4	G1	128	2 048
5E11	G1	-	128
8A3	G1	-	256
5E9	G1	1 024	2 048
5F2	G1	-	512
5A6	G1	-	512
1E4	G1	4 096	524 288
8G10	G1	4 096	16 384
9C4	G2	8 192	65 536
8G2	G2	32	1 024
7F2	G2	256	256
8C2	G2	-	128
7F1	G2	-	128
4D10	G2	-	32
8E6	G2	-	128
6E10	G2	-	32
5C12	G2	-	-

[1] Titres expressed as dilution reciprocals

2.3.2.4 MAb cross-reactivity

The results of the reactivity of the MAbs with Gordil virus by indirect immunofluorescence are shown in Table 7. Three of the MAbs directed against the G1 protein ie 4B3, 5A1 and 9E4 reacted strongly, while the remaining antibodies displayed weak or no reactivity.

With regard to the HI test using Gordil virus antigen, only the three G1-specific MAbs which fluoresced strongly against Gordil virus by IFA exhibited haemagglutination-inhibition activity (Table 8). For each of these antibodies, the HI titres against Gordil virus were lower than those for RVFV.

2.3.2.5 Passive protection

From the initial virus titration in mice it was established that a dilution of $10^{-4,5}$ of this particular batch of RVFV yielded the 50% mortality dose (LD_{50}). The $10LD_{50}$ ($10^{-3,5}$) used in the subsequent protection studies in turn corresponded to 360 PFU.

Due to constraints imposed by the University's Animal Ethics Committee, it was not possible to test the in-vivo protective ability of all the MAbs. Instead, those MAbs which exhibited the highest in-vitro neutralizing activity were examined, as well as several non-neutralizing antibodies.

The G1-reactive MAb 3E5, which exhibited the highest in-vitro neutralization activity, provided complete protection from lethal RVF infection (Table 9). Another G1-specific MAb which possessed a relatively high in-vitro neutralizing titre, (MAb 3H1), protected 60% of the animals.

In the case of the MAbs specific for the G2 protein, MAb 9C4, a strong in-vitro neutralizing antibody, provided complete protection from infection while MAb 7F1 protected 60% of the mice.

Two other MAbs, 8A3 and 8G2, specific for G1 and G2 respectively, significantly prolonged the mean survival time of the infected animals at the 99% confidence level. The remaining antibodies tested were not able to significantly delay death even at the 95% confidence level.

Table 7. Reactivity of MAbs with Gordil virus by indirect immunofluorescence

MAb	RVFV AN 1830	Gordil virus
3E5	+	-
5E1	+	-
3D2	+	±[1]
3H1	+	±
4B3	+	+
5A1	+	+
9E4	+	+
5E11	+	-
8A3	+	±
5E9	+	-
5F2	+	-
5A6	±	±
1E4	+	-
8G10	+	-
9C4	+	±
8G2	+	-
7F2	+	±
8C2	+	±
7F1	+	±
4D10	+	±
8E6	+	±
6E10	+	-
5C12	±	±

[1] ± = weak immunofluorescence

Table 8. Haemagglutination-inhibition activity of the RVFV MAbs with Gordil virus

MAb	Specificity	RVFV 1830	Gordil virus
3E5	G1	2 560	-
3D2	G1	10 240	-
3H1	G1	81 920	-
4B3	G1	40 960	1 280
5A1	G1	81 920	5 120
9E4	G1	10 240	1 280
1E4	G1	5 120	-
8G10	G1	10 240	-
9C4	G2	40 960	-
8G2	G2	160	-
7F2	G2	40	-
7F1	G2	20	-

Table 9. Protection of mice from infection with RVFV[1] by passive administration of MAbs

MAb	Specificity	No.of survivors/ No. tested	Mean day of death[2]	p[3]
Expt 1				
No MAb		0/5	3.2	
3E5	G1	5/5	-[4]	< 0.01
5E1	G1	0/5	2.8	> 0.05
9C4	G2	5/5	-	< 0.01
7F[illegible]	G2	0/5	4.4	> 0.05
Expt 2				
No MAb		0/5	2.8	
1E4	G1	1/5	4.6	> 0.05
3H1	G1	3/5	8.8	< 0.01
7F1	G2	3/5	8.4	< 0.01
Expt 3				
No MAb		0/5	2.02	
4B3	G1	0/5	4.2	> 0.05
8A3	G1	2/5	7.4	< 0.01
8G2	G2	2/5	7.5	< 0.01

[1] Virus $10LD_{50}$ (360 PFU)

[2] Mice euthanased when moribund

[3] p values for the difference in death time between each test group and the control group assessed by Students t-test

[4] All animals survived > 21 days

2.4 Discussion

The successful generation of hybridomas excreting specific monoclonal antibodies depends on many factors, each of which plays a critical role at the various stages of production (Fazekas de St. Groth and Scheidegger, 1980; Galfré and Milstein, 1981; Lane, 1985; Oxford, 1982; Reading, 1982; Sinkovics and Dreesman, 1983; Westerwoudt, 1985). The main factors influencing the production of the RVFV MAbs will firstly be discussed, and thereafter the characterization of the antibodies with respect to their antigen specificity and biological activities.

The first step for efficient production of MAb-secreting hybridomas generally requires that the donor animal be well immunized against the antigen of interest (Lane, 1985). Both virus-infected mouse brain and purified virus as antigen have been used to elicit hybridomas specific for members of the *Bunyaviridae* family (Arikawa *et al*, 1989; Gonzalez-Scarano *et al*, 1982; Pifat *et al*, 1988). Since in the present study it was necessary to obtain a large number of MAbs directed against the glycoproteins relative to the highly immunogenic nucleocapsid protein, a purified glycoprotein preparation was the immunogen of choice. Extraction of the glycoproteins from the RVF virion was performed using mild nonionic detergent lysis since antigens prepared this way should not expose the epitopes buried within the secondary or tertiary structures of the protein (Roehrig *et al*, 1982).

One reason for the success of the actual immunization protocol followed for generating the RVFV hybridomas probably lay in scheduling two final booster doses four and three days before explantation of the spleens, thus improving on the single booster which reportedly increases the amount of hybridomas that secrete antibody against the immunizing virus (Prabhakar *et al*, 1984; Sander and Dietzgen, 1984). Another factor of paramount importance is choice of the parent myeloma cell line for hybridization with the immune B lymphocytes since the stability of the resulting hybrids is in part determined by the myeloma cell type

(Reading, 1982). The Fo myeloma cells, a fast-growing mutant clone of the SP 2/0 line (Fazekas de St. Groth and Scheidegger, 1980) were chosen for the production of the RVFV hybridomas as they have been shown to generate hybrids which are more stable than those from the X63 Ag.8.653 line (Besselaar and Blackburn, 1988).

One of the factors which is extremely crucial at the stage of cell fusion is the exposure period of the cells to the fusing agent polyethylene glycol (Fazekas de St. Groth and Scheidegger, 1980; Lane *et al*, 1986). A shorter fusion period than the 90 seconds advocated by Fazekas de St. Groth and Scheidegger (1980) was used here since exposure times longer than one minute appeared to have a detrimental effect upon the fusion efficiency, possibly due to an increased sensitivity of the newly formed hybrids to the cytotoxic effects of this chemical (Lane, 1985).

Another critical step in the procedure for generating MAbs is the screening of hybridoma cultures for those hybrids secreting the desired antibodies. Since screening represents the stage of hybridoma production which is extremely labour intensive, an assay that is both rapid and sensitive is required so that an immediate decision can be made as to whether or not to clone the hybridoma (Goding, 1980). The choice of assay can moreover greatly affect the selection of antibodies with different specificities (Prabhakar *et al*, 1984). Screening of the RVFV antibody secreting clones by ELISA utilizing extracted glycoprotein as antigen not only permitted the detection of MAbs specific for the envelope proteins, but was rapid to perform. The confirmatory screening test by indirect immunofluorescence, on the other hand, was particularly valuable for those MAbs which reacted weakly in the ELISA.

In order to avoid contamination of the antibody-secreting hybridomas with clones producing no globulin, it is advisable not to aim for a high multiplicity of hybrids

in the fusion cups (Fazekas de St. Groth and Scheidegger, 1980). Since the precursor parental cells forming hybrids are independently distributed throughout the wells, the number of hybrids per well follow the Poisson distribution, thereby allowing the lowest number of parental cells required to form hybrids to be calculated (Cianfriglia *et al*, 1986). As the number of hybridomas formed depends on the number of spleen cells and not on the number of myeloma cells, the multiplicity of spleen cells used for fusion is critical (Westerwoudt, 1985; 1986). It is thus sound strategy to follow a method such as that of Fazekas de St.Groth and Scheidegger (1980) where the spleen cell input (2,5 x 10^5/well) has been calculated to ensure that positive cultures arise predominantly from single hybrids. This eliminates the need for stringent cloning. In most instances, cloning once by limiting dilution was found to be adequate for the RVFV hybridomas, thereby having the added advantage of saving time, material and the risk of contamination associated with increased cell manipulation.

During the stage of expansion of the RVF hybrid clones, one of the problems experienced was that of fungal and yeast contamination of the cultures. Although contamination of this kind is difficult to confine due to the rapid spread of spores, the inclusion of 0,8 mcg/ml Amphotericin B (Fungizone, Squibb, Princeton, NJ, U.S.A.) in the culture medium helped to contain it without exerting any toxic effects on the cells.

Another difficulty encountered at this stage was that of poor cell growth of some hybrid clones. As has been reported by others (Fazekas de St. Groth and Scheidegger, 1980; Galfré and Milstein, 1981; Prabhakar *et al*, 1984; Sanders and Dietzgen, 1984; Sugasawara *et al*, 1985) the presence of either a peritoneal macrophage feeder layer or macrophage conditioned medium greatly promoted hybridoma cell growth. This was particularly evident for a few clones where, in the absence of macrophages, a very low percentage of the original antibody-secreting cells were recovered upon resuscitation from liquid nitrogen. This

enhancing effect of the macrophages has been suggested to be due in part to the production of monokines which are growth supporting for the hybridomas (Reading, 1982; Westerwoudt, 1986).

With regard to the characterization of the MAbs, knowledge of antibody isotype is important for the interpretation of various immunological assays such as binding to protein A or complement, and for antibody competition (Ey *et al*, 1978; Kronvall and Williams, 1969; Yewdell and Gerhard, 1981). Since, for example, immunoglobulins of IgG1 subclass bind poorly to protein A (Ey *et al*, 1978; Kronvall and Williams, 1969) it was necessary to "arm" the protein A with anti-mouse immunoglobulin in the RIPA to ensure that it would react with the RVFV MAbs of this isotype.

A major problem experienced during determination of the specificity of the MAbs by the RIPA was that of poor labelling of the RVFV G2 protein. This phenomenon has been reported by others for various *Bunyaviridae* (Bishop and Shope, 1979; Gonzalez-Scarano *et al*, 1982). The weak reaction with G2 may have been due to low affinity of the antibodies (Yolken, 1982), although it is unlikely that all nine anti-G2 MAbs would possess similar poor antigen-binding activity. A more probable explanation is that the poor reactivity could be due to lack of stable antigen binding under the conditions of the assay (Fernie *et al*, 1982). MAbs are often sensitive to minor conformation changes (Carter and ter Meulen, 1984; Lussenhop *et al*, 1988; Niesters *et al*, 1987) and consequently may be adversely affected in the RIPA where the detergents necessary to prevent non-specific contamination of the precipitate have some destabilizing effect on protein structure (Yewdell and Gerhard, 1981).

The possibility that certain of the epitopes defined by these MAbs were more conserved than others was examined by determining the cross-reactivity of the antibodies with Gordil virus. The latter was chosen as it represents an African

phlebovirus (Tesh *et al*, 1976) which exhibits an antigenic relationship with RVFV as established by various serological tests with RVF convalescent sera and reference antisera (Shope *et al*, 1980; 1981; Swanepoel *et al*, 1986; Tesh *et al*, 1982). The strong reactivity of three of the G1-specific MAbs with Gordil virus indicates that the antigenic determinants defined by these antibodies are not specific for RVFV, but may be highly conserved in some other members of the phlebovirus genus.

Examination of the serological reactivities of the RVFV MAbs revealed that some MAbs directed against both G1 and G2 were capable of neutralizing virus in the absence of C′. This suggests that their epitope specificities are localized to sites which contribute to the role of the glycoproteins in virus infectivity. Of interest was the fact that all the neutralizing sites identified by these antibodies also demonstrated haemagglutination activity (HA). While several neutralizing epitopes have been identified in other studies on RVFV (Collett *et al*, 1987; Schmaljohn *et al*, 1989) no data regarding the HA of these antigenic sites has been published. The findings here are similar to those reported to the phlebovirus Punta Toro, where all except one of the neutralizing MAbs also had haemagglutination-inhibition activity (Pifat *et al*, 1988). The close correlation of neutralizing and HA responses strongly suggests that the RVFV G1 and G2 domains which bind to erythrocytes also bind to receptors on virus-susceptible cells in culture.

In addition to C′-enhanced virus neutralization exhibited by these *in vitro* neutralizing antibodies, the majority of the non-neutralizing RVFV MAbs were also able to neutralize virus in the presence of C′. C′-enhanced and C′-dependent virus neutralization by MAbs is a well-documented phenomenon (Holland *et al*, 1983; Kimuro-Kuroda and Yashui, 1983; McCullough, 1986; Russell *et al*, 1983). Both RVFV IgG and IgM subclass MAbs exhibited increased neutralization when C′ was added. In the case of IgG antibodies, C′ may function together with

immune IgG to lyze (Oldstone, 1974; Oroszlan and Gilden, 1970), aggregate (Oldstone *et al*, 1974) or coat viruses (Linscott and Levinson, 1969), resulting in destruction or inactivation. IgM also binds C′ under certain conditions (Feinstein *et al*, 1986). The addition of C′ plus anti-viral IgM to the nairovirus Dugbe, for example, increased neutralization 16-fold (Armstrong *et al*, 1990). It is possible that those MAbs which are dependent on C′ for neutralization cannot alone induce sufficient conformational changes in the virus to effect neutralization (McCullough, 1986).

The passive immunization studies clearly demonstrate that antibodies to individual epitopes on both the RVFV G1 and G2 proteins can provide protection to otherwise lethally infected mice, indicating that both envelope proteins play an important role in virus infectivity and pathogenesis. This finding is of particular interest since previously only anti-G2 or a mixture of anti-G1 and G2 antibodies had been shown to be protective *in vivo* (Battles and Dalrymple, 1988).

Of the four MAbs capable of protecting mice from lethal RVFV infection to varying degrees, three were also able to neutralize *in vitro*. The remaining antibody (7F1) was, however, non-neutralizing *in vitro*. A similar finding has been shown for several other viruses (Boere *et al*, 1985; Schmaljohn *et al*, 1982). A number of possible explanations for in-vivo protection by non-neutralizing MAbs are apparent, including complement-dependent lysis or antibody-dependent, cell-mediated lysis of virus infected cells (Boere *et al*, 1985; Lefrançois, 1984; Schmaljohn *et al*, 1982). Since MAb 7F1 was of the IgG1 subclass, the underlying mechanism for this phenomenon is unlikely to be C′-dependent cytolysis (Spiegelberg, 1974). Similarly, enhanced uptake of virus in non-permissive macrophages is not probable, as this is most likely to be mediated by IgG2a subclass antibodies (Boere *et al*, 1985). The 7F1 antibody may instead protect by inhibition of virus maturation (Boere *et al*, 1985) or antibody-dependent cell mediated cytotoxicity (Stanley *et al*, 1986).

In addition to the four MAbs that were able to protect at least 60% of the infected animals, two other antibodies significantly prolonged the mean survival time of the mice. The G1-specific MAb 8A3 was non-neutralizing *in vitro* while the anti-G2 MAb 8G2 exhibited very low level neutralizing activity in the absence of C′. Since the *in vitro* neutralizing activity of both antibodies was potentiated by C′, the underlying mechanism for the prolonged survival time could be C′-dependent cytolysis mediated by the non-neutralizing antibodies as suggested by Schmaljohn *et al* (1982). Alternatively, as both antibodies are of the IgG2a isotype, the partial protection might be due to the clearance of virus-antibody complexes in nonpermissive macrophages as suggested for Semliki Forest virus (Boere *et al*, 1985).

The results of the characterization of the RVFV MAbs with respect to their antigenic specificity and their biological activities thus indicate that they are involved in various biological functions. This in turn suggests that they are directed against different antigenic determinants on the G1 and G2 proteins. In order to assess this, and to assign the protective, neutralizing and haemagglutination functions to antigenic regions on the glycoproteins, it is necessary to examine the topological relationship of the epitopes to each other.

3. EPITOPE MAPPING OF RVFV G1 AND G2

3.1 Introduction

The topological relationship of antigenic determinants to each other on the surface of the virus protein molecule can be conveniently examined by utilizing MAbs in a competitive binding assay (CBA) (Heinz, 1986; Stone and Nowinski, 1980; Yewdell and Gerhard, 1981). Competitive binding is frequently performed by ELISA or radioimmunoassay, where one MAb is labelled with an appropriate marker, and unlabelled antibodies are assessed for their capacity to inhibit the competition of the labelled antibody with antigen (Carter and ter Meulen, 1984). The definition of epitopes by CBA is based on the assumption that a MAb binding to a specific site hinders the attachment of another antibody to the same or proximal site (Stone and Nowinski, 1980).

CBA's with MAbs have been used to map the antigenic determinants of the envelope glycoproteins of several members of the *Bunyaviridae* family (Arikawa *et al*, 1989; Dantas *et al*,1986; Gonzalez-Scarano *et al*, 1982; Kingsford *et al*, 1983; Najjar *et al*, 1985) including the phlebovirus Punta Toro (Pifat *et al*, 1988). Epitope mapping studies on La Crosse virus (LACV) have, for example, revealed the presence of eight antigenic regions on the G1 glycoprotein (Kingsford *et al*, 1983). In the study by Arikawa *et al* (1989), nine distinct, partially-overlapping antigenic sites, two on G1 and seven on G2, were demonstrated for Hantaan virus. Monoclonal antibodies reactive with the Punta Toro G1 protein were shown to bind to epitopes in two distinct topological sites (Pifat *et al*, 1988).

In contrast, a topographical analysis of the surface glycoproteins of RVFV has not been undertaken to date. This may partly be due to the difficulty in generating RVFV MAbs to the glycoproteins relative to the highly immunogenic nucleocapsid (Pifat *et al*, 1988).

The present objectives therefore are to use the RVFV MAbs prepared against the South African AN 1830 strain to map the antigenic sites on G1 and G2. The epitope mapping will be carried out using competitive binding ELISAs with MAbs conjugated with an enzyme label. The biological functions of the MAbs will then be assigned to the corresponding mapped domains.

3.2 Materials and Methods

3.2.1 Purification of virus for CBA

a) RVFV was PEG-precipitated and purified using CsC1 gradients as described in section 2.2.1.2.

b) Purification of RVFV using potassium tartrate-glycerol gradients (Robeson *et al*, 1979) was also performed in an attempt to obtain a higher yield of intact virus particles. The potassium tartrate-glycerol gradients were prepared by successively layering 1,5 ml volumes of solutions A to G (Appendix C) in Beckman Ultra-Clear centrifuge tubes. Two ml PEG-precipitated RVFV (2.2.1.2) was then loaded over the combination equilibrium-viscosity gradients of glycerol and potassium tartrate. The tubes were spun at 35 000 rpm (155 000 g) for 90 min at 4°C in a SW 40 Ti rotor using a Beckman L8-60M ultracentrifuge. The visible virus band in each tube was harvested by collecting 0,5 ml fractions using a 19 gauge needle. The fractions were tested for protein by measuring the absorbance at 280 nm.

Positive fractions were pooled and dialyzed overnight against TSE buffer (0,01 M tris-hydrochloride buffer, pH 7,6, containing 0,15 M NaC1 and 0,003 M EDTA, pH 7,4). The final protein concentration was determined spectrophotometrically as above.

3.2.2 Purification of MAbs

Prior to purification the monoclonal antibody rich ascitic fluids were treated with Lipoclean (Behring, Hoechst, Germany) to remove any lipid-containing material. The ascitic fluids were mixed with the Lipoclean reagent at a ratio of 1,5:1, vortexed and clarified by spinning at 2500 rpm for 15 min in a Beckman TJ-6 benchtop centrifuge. The top phase was carefully removed for antibody purification.

IgG MAbs were purified from the clarified ascitic fluid by protein A-linked agarose (Affi-gel MAPS; Bio-Rad Laboratories, Richmond, CA, USA) chromatography. Fractions of 1 ml were collected and tested for protein using the Bio-Rad Protein assay (Bio-Rad Laboratories). Positive fractions were pooled and concentrated by ultrafiltration using CX-10 ultrafiltration units (Millipore). The antibodies were then dialyzed against 0,1 M phosphate buffer, pH 7,4 with 0,075 M NaCl and the resulting protein concentrations determined spectrophotometrically at 280 nm.

MAbs of the IgM subclass were isolated by gel filtration on Ultragel AcA 34 (Spectrum Medical Industries, Los Angeles, CA, USA) using a 50 x 1,5 cm column. The gel was equilibrated with 0,5 M NaCl buffered with 0,02 M sodium phosphate buffer, pH 7,3. Clarified ascitic fluids (1 ml per run) were applied to the column and eluted with the same phosphate buffer as above. Fractions of 3 ml were collected and the protein contents checked using the Bio-Rad Protein assay kit. The fractions in the first peak were pooled, concentrated by ultrafiltration and the protein concentrations determined at 280 nm.

3.2.3 Determination of MAb avidities

The abilities of MAbs to bind to solid-phase viral antigen were evaluated by indirect ELISA using both purified virus and glycoprotein preparations. ELISA microtitre plates were coated overnight at 4°C with an optimal dilution of

gradient-purified RVF virions or glycoprotein extract determined as in Section 2.2.2.3a. Non-specific binding was blocked by addition of 0,2 ml/well of 4% BSA for 1 h at 37°C. Log_{10} dilutions of purified MAbs adjusted to a starting concentration of 0,1 mg/ml were prepared and 0,1 ml volumes added to the wells and incubated for 2 h at 37°C. The bound antibody was determined with anti-mouse peroxidase conjugate as described for the screening ELISA (2.2.2.3a). To provide an approximate comparison of MAb-binding properties, relative binding avidity was defined as the amount of antibody (μg MAb protein) required to yield an absorbance of 0,5 at 405 nm (Boere *et al*, 1984).

3.2.4 Peroxidase conjugation of MAbs

Antibodies were initially coupled to horseradish peroxidase (HRPO) (Sigma Chemicals, St Louis, Mo, USA) by the method of Wilson and Nakane (1978). Immunoglobulin fractions were isolated from ascitic fluids by ammonium sulphate precipitation and dialyzed overnight against 0,01 M sodium carbonate buffer (pH 9,5). HRPO (10 mg) was dissolved in 2,5 ml distilled water and activated with 0,5 ml freshly prepared 0,1 M sodium periodate for 20 min at rt. The solution was then dialyzed against 0,01 M sodium acetate buffer (pH 4,4). The pH of the solution was then raised to 9,5 with 0,2 M sodium carbonate buffer (pH 9,5) and 4 mg immunoglobulin was incubated with the activated HRPO for 2 h at rt. After the addition of 0,25 ml freshly prepared sodium borohydride solution (4 mg/ml), the mixture was left for 2 h at 4°C, then dialyzed against PBS overnight at 4°C.

A modified peroxidase conjugation method in which ascorbic acid is used instead of sodium borohydride as the reducing agent (Henning and Nielson, 1987) was also assessed. The labelling procedure was identical to that above, with the exception that 0,25 ml of ascorbic acid (4 mg/ml) was added to the HRPO-antibody mixture instead of the sodium borohydride. The mixture was left at 4°C for 24 h to stabilize and then sterilized by filtration through a 0,22 μm filter (Millipore Corp, Bedford, USA).

To remove uncoupled IgG and free enzyme, peroxidase conjugates were purified by gel filtration by the method of Nakane and Kawaoi (1974). Half the amount of the HRPO-conjugates (2 mg protein) was chromatographed on a 85 x 1,5 cm column of Sephadex G-100 equilibrated in PBS. Fractions of 20 drops were collected and their absorbances at 280 and 403 nm determined. The enzymatic activity of the fractions were tested by ELISA (3.2.7) to detect the presence of active conjugates. Positive fractions were pooled and concentrated by ultrafiltration. The purified conjugates were then compared to the other half of the non-purified conjugate for antigenic activity by ELISA (3.2.7).

3.2.5 Biotin labelling of MAbs

Labelling of the MAbs with biotin was assessed using two methods:

a) Amersham biotinylation kit (Amersham Int., U.K.): MAb immunoglobulin fractions (precipitated by ammonium sulphate) were dialyzed overnight against 0,05 M borate buffer (pH 8,5). The protein concentration of each sample was adjusted to 2 mg/ml and 40 µl biotin was added for 1 h at rt with shaking. The labelled preparation was then fractionated through the Sephadex G25 column supplied and fractions of 0,5 ml were collected.

b) Biotinylation using the succinimide ester: Labelling of the MAbs with a biotin ester (biotin-XX-NHS) which has an extra spacer arm interposed between the reactive ester and the biotin molecule was also examined. Purified as well as ammonium sulphate precipitated RVFV MAb immunoglobulins were adjusted to 2 mg/ml in 0,05 M borate buffer (pH 8,5). The biotin-XX-NHS (Calbiochem, Behring, Hoechst, Germany) was prepared by dissolving 1 mg in 1 ml of dimethylsulphoxide (DMSO). Fifty µg and 250 µg quantities of the biotin ester were then added to duplicate 2 mg MAb immunoglobulin samples. These were reacted for 4 h with shaking at rt followed by the addition of 20 µl of 1,0 M

ammonium chloride per 250 μg biotin to block unreacted biotin ester. The labelled preparations were then fractionated through Sephadex G25 as described above.

3.2.6 Conjugation of MAbs via their carbohydrate moietics

Conjugation of antibodies via their carbohydrate moieties using the hydrazide derivatives of peroxidase and biotin was evaluated using RVFV hyperimmune ascitic fluid (HAF) and several representative MAbs. The RVFV antibodies were oxidized by mixing 2 mg of ammonium sulphate precipitated immunoglobulin diluted in 0,5 ml of 0,1 M sodium acetate (pH 5,5) with 0,5 ml 0,01 M sodium periodate for 20 min at rt. The mixtures were then dialyzed against 0,1 M sodium acetate (pH 5,5).

a) Peroxidase-hydrazide

Crystalline horseradish peroxidase (5 mg) (HRPO, Sigma) was dissolved in 1 ml of 0,15 M sodium chloride. To this was added 0,1 ml of a 0,5 M aqueous solution of adipic acid dihydrazide (AADH, Sigma) adjusted to pH 5,0 with 1 N HC1, and 0,5 ml of a 0,1 M aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma). The reaction was allowed to proceed for 6 h at rt at which time the solution was dialyzed against 0,15 M sodium chloride and then against 0,1 M sodium acetate, pH 5,5. After dialysis, the HRPO-hydrazide was mixed with oxidized RVFV immunoglobulin at a protein ratio of 1:1 and allowed to react overnight at rt.

b) Biotin-hydrazide

Solid biotin-hydrazide (Sigma) was added to 2 mg/ml of oxidized RVFV immunoglobulin to yield a final concentration of 0,01 M. This was reacted for 2 h at rt with gentle shaking, followed by extensive dialysis against PBS.

3.2.7 Determination of the reactivity of the conjugated MAbs

a) To determine the efficiency of the labelling method, the enzymatic activity of the conjugated MAbs was tested by ELISA. The peroxidase conjugated preparations were diluted 1:50 in ELISA coating buffer (pH 9,6) and serial two-fold dilutions pr pared in ELISA plates. Following overnight incubation at 4°C, the wells were washed and blocked with 4% BSA for 30 min at 37°C. After washing again, ABTS substrate was added and the absorbance read at 405 nm. The reactivity of the biotin conjugated MAbs was determined in the same manner, except that peroxidase streptavidin (Amersham) at a dilution of 1:1000 was added after the blocking step. Following an incubation period of 1 h at 37°C, the wells were washed and the enzymatic activity detected with ABTS substrate.

b) The antigenic reactivity of the labelled MAbs was examined by adding serial two-fold dilutions of the conjugated antibodies to ELISA plates coated with extracted glycoprotein and whole virus antigen (3.2.3). After 1 h incubation at 37°C, the wells were washed and ABTS substrate added to the peroxidase conjugated MAbs. For the biotin labelled MAbs, peroxidase streptavidin was added instead and the ELISA carried out as described above.

3.2.8 Competitive binding assays

Competitive binding ELISAs were performed using ELISA plates which had been coated with an optimal concentration of extracted RVFV glycoprotein (80 μg/ml) and blocked with BSA as described in Section 2.2.2.3. Prior to carrying out the actual competitive assays, the amount of each labelled MAb needed to give an optical density (O.D.) of 0,5 to 1,0 in the absence of a second unlabelled antibody (Lussenhop *et al*, 1988) was determined by titrating the conjugated antibodies. A starting dilution of 1:25 was prepared in ELISA diluent for each enzyme-labelled antibody, and 0,1 ml volumes added in duplicate to the coated and

blocked ELISA plate wells. Serial two-fold dilutions were prepared in the plates and incubated at 37°C for 1 h. The plates were washed, ABTS substrate added and the absorbance values at 405 nm were read.

For the competitive binding assays, unlabelled purified MAbs were adjusted to 0,1 mg/ml in ELISA diluent and 0,1 ml volumes added in duplicate to the coated plates. After incubation for 3 h at 37°C, the plates were washed and peroxidase-labelled MAbs were added in duplicate (0,1 ml/well) for a further hour at 37°C. After washing as before, the colour was developed by adding ABTS substrate and the O.D. at 405 nm determined. Controls included antigen-coated wells without any competing unlabelled MAbs, as well as homologous unlabelled antibody for each respective conjugated MAb.

Percent inhibition or enhancement due to the competing antibody was calculated from average corrected absorbance readings using the formula described by Lussenhop *et al* (1988):
[(% $O.D._{405}$ with competing antibody) - (% $O.D._{405}$ without competing antibody)] x 100/(% $O.D._{405}$ without competing antibody).

Competition was rated strong if it was more than 75% and partial or intermediate if it was more than 25% (Arikawa *et al*, 1989). An increase in binding to more than 20% was considered significant enhancement.

3.3 Results

3.3.1 MAb avidities

The relative binding avidities of the MAbs, which serves to provide an approximate comparison of MAb-binding properties, are shown in Table 10. The MAb avidities differed greatly for the purified virus and extracted glycoprotein antigens. In general, the amount of MAb protein which was required to bind to the intact virus in order to give an ELISA absorbance value of 0,5 was about

Table 10. MAb relative avidities

MAb	Relative Avidity[1] (μg/ml)	
	Purified virus	Glycoprotein
3E5	25	15×10^{-3}
9E4	1.6	1×10^{-3}
5A1	1.7	1×10^{-3}
4B3	3.8	1×10^{-3}
3H1	5	1×10^{-3}
3D2	22	2×10^{-3}
5E9	0.8	1×10^{-3}
8A3	14	2×10^{-3}
5E11	>50	0.1
5E1	7.6	27×10^{-3}
5F2	33	0.3
5A6	>50	0.4
8G10	34	7.2
1E4	20	7.4
9C4	7	5×10^{-3}
7F2	2.5	2×10^{-3}
7F1	2.9	6×10^{-3}
8G2	3.2	17×10^{-3}
8C2	0.4	25×10^{-3}
4D10	9.3	27×10^{-3}
8E6	23	2×10^{-3}
6E10	12	0.2
5C12	5.8	3.8

[1] Amount of antibody (μg/ml) required to yield an absorbance of 0,5 at 405 nm.

three logs greater than that for the glycoprotein antigen eg for MAb 3E5, 25 μg/ml was needed to bind to the viral antigen compared to only 15 ng/ml for the glycoprotein preparation. The individual MAbs furthermore varied in their ability to bind to solid phase antigen, with relative avidities ranging from 1 x 10^3 to 7.4 μg/ml in the case of the glycoprotein preparation.

3.3.2 MAb labelling

Several different conjugation procedures were initially evaluated in order to determine the optimum method for enzyme labelling of the RVFV MAbs. Preliminary studies on peroxidase conjugation of the MAbs using the periodate method of Wilson and Nakane (1978) and the modified procedure of Henning and Nielson (1987) yielded conjugates of similar reactivity. The latter method was subsequently used as it was less toxic and generated conjugates with greater stability on storage. Optimal coupling efficiencies were obtained using a ratio of 10 mg peroxidase conjugated with 4 mg of protein.

By this method, seventeen MAbs were successfully coupled to peroxidase. The remaining six antibodies exhibited enzymatic activity, indicating that labelling with peroxidase had occurred. However, the antigenic reactivity (ability to bind antigen) of these conjugates was extremely weak. Purification of the peroxidase labelled antibodies by gel filtration to remove uncoupled IgG did not improve the activity of the conjugates.

With regard to biotin labelling, coupling of the MAbs using the biotin N-hydroxysuccinimide ester (biotinlyation kit; Amersham) or the biotin-XX-NHS (Calbiochem, Behring, Hoechst) yielded conjugates of comparable reactivity. While the enzymatic activity of all the biotinylated antibodies was excellent, the antigenic reactivity of the individual MAbs varied considerably. Several of the biotinylated MAbs, eg 3E5 and 7F1, exhibited a markedly reduced ability to bind antigen compared to the same MAbs labelled with peroxidase. The six MAbs

which displayed poor antigenic reactivity on coupling to peroxidase similarly did not retain their binding capacity on biotinylation.

Conjugation of RVFV antibodies via their carbohydrate moieties using hydrazide derivatives of peroxidase and biotin produced active conjugates as verified by testing the enzymatic activity. The antigenic activity of the conjugates, however, was very low compared to the other methods where labelling was performed via the amino groups of the antibodies.

3.3.3 Competitive binding assays

The seventeen MAbs which retained their binding capacity after coupling to horseradish peroxidase were used in reciprocal competition assays (Tables 11, 12, 13, 14). With regard to the G1-specific antibodies, MAbs 3E5 and 5E1 exhibited complete, symmetrical inhibition, indicating that they recognized identical or overlapping sites (Table 13). As the antibodies displayed different serological behaviour, the epitopes were considered to be overlapping rather than specific for the same epitope.

Partial symmetrical competition between various MAbs was also observed. Here differences in the degree of blocking efficiency between antibody pairs was seen despite having comparable avidities eg MAb 3H1 competed fully with MAb 3D2 and 3D2 only partially competed with 3H1. MAb 4B3 exhibited partial reciprocal binding with MAbs 3H1 and 3D2, as well as MAbs 5A1 and 9E4. As such partial competition may be due to a low degree of structural overlap of determinants, the epitopes identified by these five antibodies were consid[illegible] to be closely adjacent and were assigned to the same antigenic domain (G1 II).

A similar competition pattern was observed for MAbs 5E11 and 8A3 (G1 IIIa and IIIb), while MAb 5E9, which did not show any reciprocal competition with the other MAbs, was assigned to domain IV. Besides competition, enhancement was

also observed. MAb 9E4 (G1 IIe) induced enhanced binding of MAbs 3B5 (G1 Ia) and 5E11 (G1 IIIa) but not *vice versa* (Fig. 2).

The competition assays with the G2-specific MAbs showed that none of the MAbs displayed complete, symmetrical competition with any of the other antibodies and thus appeared to recognize different epitopes (Table 14). MAb 7F2 had the unique ability to compete fully or partially with all the other labelled G2 MAbs. In turn, 7F2 was partially blocked by only one MAb (8C2) which suggested that these two antibodies bound to closely adjacent epitopes in the same antigenic region (G2 Ic and Id).

Asymmetrical inhibition occurred between MAbs 9C4 and 8G2 ie 9C4 blocked the binding of 8G2 by 100% but 8G2 did not compete with 9C4 at all. A similar pattern of one-way inhibition was seen for MAbs 7F2 and 8G2 where 7F2 blocked 8G2 by 95% but 8G2 only blocked 7F2 by 10%. Since this type of competition could be explained by the lower avidity of 8G2 relative to that of 9C4 and 7F2, the epitope recognized by 8G2 was assigned to the same domain as the 9C4 and 7F2 determinants (G2 Ia, Ib, Ic). No two-way competition was observed for MAbs 7F1, 4D10 and 8E6 which were thus placed in different domains. In addition, reciprocal enhanced binding was seen for MAbs 7F1 and 8C2, 7F1 and 4D10, 7F1 and 8G2, 4D10 and 8C2, 4D10 and 8G2, and 8C2 and 8G2 (Fig. 3).

Data from CBAs performed with competitor G1-specific MAbs against labelled anti-G2 MAbs and *vice versa* revealed that none of the anti-G1 MAbs competed with the G2-specific antibodies for binding to the G2 protein. In the reverse situation, MAbs 8G2, 7F2 and 8C2 (mapping to G2 I) competed with the G1-specific MAbs for binding to domain G1 II (Table 15).

Table 11. Percentage inhibition of binding of labelled MAbs by unlabelled antibodies determined by competitive binding assay for RVFV G1

Antigenic Domain	Unlabelled MAb	HRPO Labelled MAb									
		3E5	5E1	3D2	3H1	4B3	5A1	9E4	5E11	8A3	5E9
G1 Ia	3E5	100	91	- 9	0	17	- 7	- 1	- 8	6	- 3
Ib	5E1	75	100	- 9	0	4	- 13	- 1	- 4	12	- 3
IIa	3D2	41	46	100	56	43	1	5	26	3	- 14
IIb	3H1	71	2	100	100	27	- 13	- 10	0	82	- 13
IIc	4B3	58	46	59	49	100	93	91	0	- 1	- 14
IId	5A1	65	23	2	- 2	36	100	100	- 2	11	- 17
IIe	9E4	- 94	26	- 15	- 8	25	54	100	- 56	60	- 14
IIIa	5E11	45	30	- 7	14	23	- 15	1	100	36	- 7
IIIb	8A3	39	17	- 7	3	14	- 6	- 4	57	100	- 6
IV	5E9	9	47	- 9	- 16	1	- 8	- 6	5	15	100

- = enhanced antibody binding

Table 12. Percentage inhibition of binding of labelled MAbs by unlabelled antibodies determined by competitive binding assay for RVFV G2

Antigenic domain		Unlabelled MAb	HRPO labelled MAb						
			9C4	8G2	7F2	8C2	7F1	4D10	8E6
G2	Ia	9C4	100	100	2	19	- 98	- 186	3
	b	8G2	20	100	15	- 13	- 74	- 81	- 1
	c	7F2	65	95	100	58	64	68	43
	d	8C2	- 5	- 104	26	100	- 59	- 82	8
	II	7F1	24	- 39	15	- 90	100	- 72	10
	III	4D10	- 90	- 256	- 3	- 146	- 62	100	14
	IV	8E6	3	20	1	- 14	18	4	100

- = enhanced antibody binding

Table 13. Competitive binding assay mapping of RVFV G1 glycoprotein epitopes based on patterns of antibody binding inhibition

Antigenic domain	Unlabelled MAb	HRPO labelled MAb[1]									
		3E5	5E1	3D2	3H1	4B3	5A1	9E4	5E11	8A3	5E9
G1 Ia	3E5	++	++								
Ib	5E1	++	++								
IIb	3D2	+	+	++	+	+			+		
IIb	3H1	+		++	++	+					
IIc	4B3	+	+	+	+	++	++	++			
IId	5A1	+				+	++	++			
IIe	9E4	0	+			+	+	++	0	+	
IIIa	5E11	+	+						++	+	
IIIb	8A3	+							+	++	
IV	5E9		+								++

[1] Binding inhibition of labelled MAb by unlabelled antibodies as defined by

++, >75% competition; +, 25 to 75% competition
0, one-way enhancement more than 20%

Table 14. Competitive binding assay mapping of RVFV G2 glycoprotein epitopes based on patterns of antibody binding inhibition

Antigenic domain	Unlabelled MAb	HRPO labelled MAb[1]						
		9C4	8G2	7F2	8C2	7F1	4D10	8E6
G2 Ia	9C4	++	++			0		
b	8G2		++			R	R	
c	7F2	+	++	++	+	+	+	+
d	8C2		0	+	++	R	R	
II	7F1		R		R	++	R	
III	4D10	0	R		R	R	++	
IV	8E6							++

[1] Binding inhibition of labelled MAb by unlabelled antibodies as defined by:

++, >75% competition; +, 25 to 75% competition
0, one-way enhancement more than 20%; R, reciprocal enhancement more than 20%

Table 15. Percentage inhibition of binding of labelled G1-specific MAbs by unlabelled G2-specific antibodies determined by competitive binding assay

Antigenic domain		Unlabelled MAb	HRPO MAb				
			3D2 (G1 IIa)	3H1 (G1 IIb)	4B3 (G1 IIc)	5A1 (G1 IId)	9E4 (G1 IIe)
G2	Ia	9C4	2	32	0	- 7	6
G2	Ib	8G2	37	52	82	83	100
G2	Ic	7F2	70	77	35	26	40
G2	Id	8C2	67	50	98	85	100

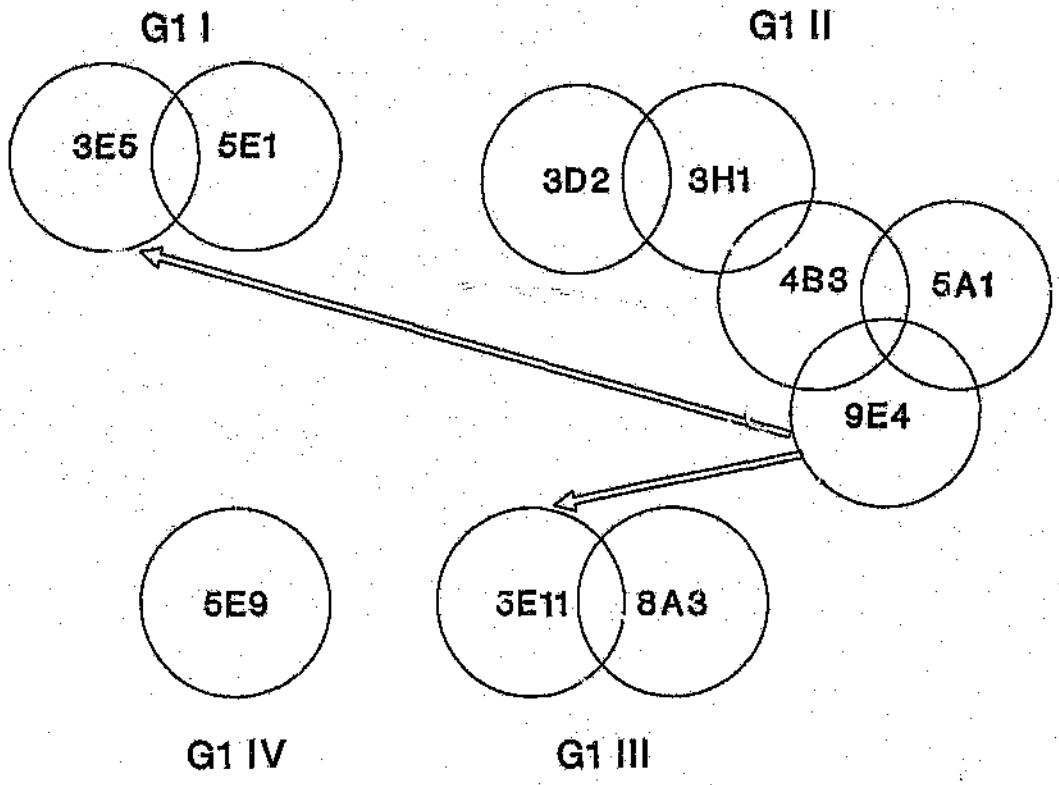


Fig. 2. Diagrammatic representation of the antibody interactions between the epitopes on the RVFV G1 glycoprotein. Circles represent epitopes recognized by each MAb. Overlapping circles indicate competition between MAbs. The unfilled arrows show unidirectional enhanced antibody binding.

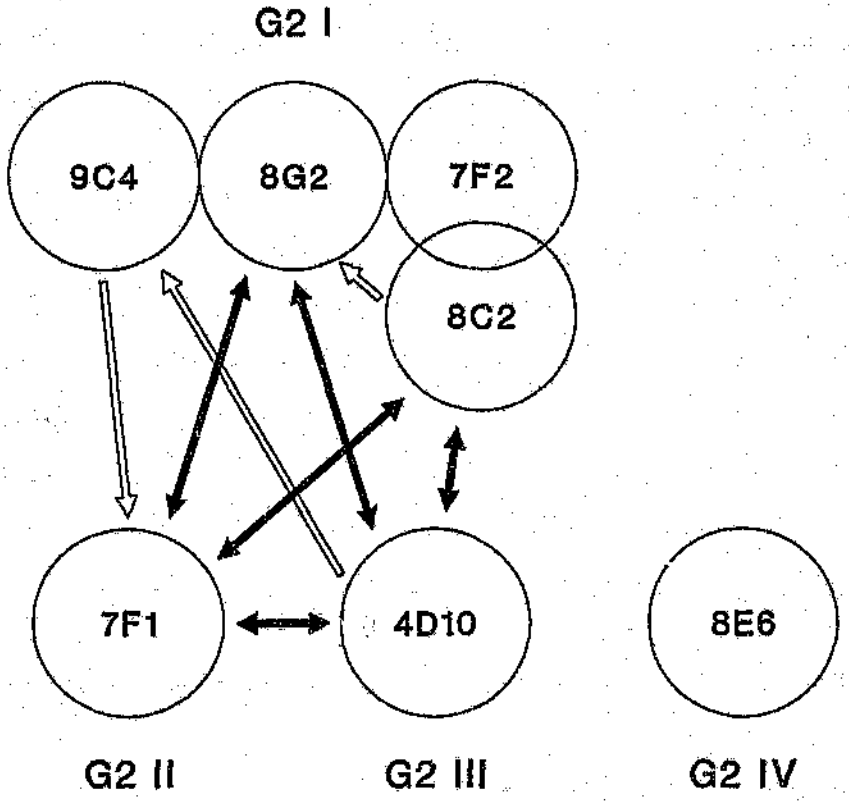


Fig. 3. Diagrammatic representation of the antibody interactions between the epitopes on the RVFV G2 glycoprotein. Circles represent epitopes recognized by each MAb. Overlapping circles indicate competition between MAbs. The unfilled arrows show unidirectional enhanced antibody binding. The solid arrows indicate bidirectional augmented antibody binding.

3.4 Discussion

The CBA utilizing MAbs is a powerful technique for the topographical analysis of antigens (Heinz, 1986; Roehrig *et al*, 1982; Stone and Nowinski, 1980). While this type of study can be conveniently performed by ELISA using enzyme labelled MAbs (Carter and ter Meulen, 1984), it is necessary to establish optimal conditions for the assay. Firstly, a uniform preparation of purified antigen suitable for use in the CBA is essential (Engvall, 1980). It is also critically important to determine the best method for enzyme conjugation of the MAbs (Howard *et al*, 1985; O'Shannessy and Quarles, 1985; O'Sullivan and Marks, 1981). Additionally, the avidities or binding strengths of the antibodies for the viral antigen have to be taken into consideration so as not to misinterpret the results of the CBAs (Stone and Nowinski, 1980).

With regard to the preparation of antigen, purification of RVFV led to substantial losses of intact virus particles. The problem encountered here in maintaining the structural integrity of the virus particles has been reported for various members of the *Bunyaviridae* family (Obijeski *et al*, 1976; Robeson *et al*, 1979). Although this can reportedly be overcome using potassium tartrate-glycerol gradients (Robeson *et al*, 1979), low yields of intact RVFV were still obtained by this method. In contrast, high yields of a purified glycoprotein preparation were produced by extraction of the surface glycoproteins from the RVF virion using mild nonionic detergent lysis.

Preliminary studies using some of the higher avidity MAbs in parallel with purified virus and glycoprotein as the solid phase antigen gave virtually identical competition results. The latter was used in subsequent competitive binding assays since the MAb avidities for the purified viral antigen were in general much weaker than for the glycoprotein extract. This weak reactivity in the ELISA may

have been due to disintegration of the virus or "deformation" of antigenic determinants during the purification procedure (Boere *et al*, 1984; Robeson *et al*, 1979).

While many procedures have been described for enzyme-antibody conjugation, their efficiencies differ greatly (Jeanson *et al*, 1988; O'Sullivan and Marks, 1981). A method widely used for enzyme-labelling of MAbs is the periodate-oxidation technique developed by Nakane and Kawaoi (1974), whereby horseradish peroxidase is coupled to amino groups of the immunoglobulins. Nevertheless, since labelling with an enzyme such as peroxidase may cause loss of antigen-binding capacity due to the higher molecular weight of the conjugate (Boorsma and Kalsbeek, 1975), it may be valuable to use a low molecular weight marker such as biotin instead (Simkins *et al*, 1989). However, these types of amino-directed labelling procedures can also result in the destruction of antigen binding activity (Howard *et al*, 1985; O'Shannessy *et al*, 1984). This can reportedly be overcome using a method based on coupling the enzymes to the carbohydrate moieties of immunoglobulins (O'Shannessy and Quarles, 1985), as the latter are not involved in the antigen binding site.

Of the various enzyme coupling procedures investigated in the present study, labelling using the HRPO-periodate method yielded MAb conjugates with the highest antigenic reactivity. A ratio of HRPO to immunoglobulin of 2,5:1 was found to be optimal, which is similar to the optimal ratio of 2,3:1 reported by Nakane (1979). While unconjugated IgG may reduce assay sensitivity by competing with labelled antibody for binding to antigen (Porstmann *et al*, 1981), the activity of the RVFV MAb conjugates was not increased by purification. A similar finding has been reported by Jeanson *et al* (1988) who carried out a comprehensive comparison of conjugation procedures for the preparation of MAb-enzyme conjugates.

In contrast to the amino group labelling procedures in which active conjugates were obtained for the majority of the MAbs, conjugation of the antibodies via their carbohydrate moieties was completely unsuccessful. Several other studies have reported that in the case of MAbs, this method of conjugation was inferior to conjugation via amino groups (Gretch *et al*, 1987; Jeanson *et al*, 1988). This in turn may possibly be due to the destruction of the sugar moieties of the antibodies caused by the treatment conditions being too severe (Jeanson *et al*, 1988).

The fact that antigenically reactive conjugates could not be prepared for six of the MAbs by any of the methods used suggests that the activity of these antibodies was lowered or destroyed by these coupling procedures. This loss of reactivity may have been due to the modification of amino acid residues involved in the antigen binding site (Ghose *et al*, 1983). Alternatively, since these particular MAbs all had low avidity, enzyme labelling may have further lowered their affinity for the antigen, resulting in increased antibody displacement from the antigen solid phase in the ELISA.

With regard to the CBA itself, while this type of assay is undoubtedly a powerful technique for epitope mapping (Heinz, 1986; Roehrig *et al*, 1982; Stone and Nowinski, 1980), interpretation of competitive binding data is not without some difficulties (Cepica *et al*, 1990; Henchal *et al*, 1985; Shaw *et al*, 1986). The definition of epitopes by this type of blocking assay is based on the assumption that a MAb binding to a specific site hinders the attachment of another antibody to the same or proximal site (Stone and Nowinski, 1980). However, the possibility that blocking may result from positionally unrelated steric hindrance cannot be disregarded. Moreover Stone and Nowinski (1980) have shown that if the CBA was performed with two antibodies of similar specificity, but of different avidities, the competition reactions invariably favoured the binding of the antibody with higher avidity. Taking this into consideration, the RVFV MAbs of low

avidity were not used in the CBA. Avidity effects were also minimized by preincubating the antigen for 3 h with an excess of competitor antibody before the addition of the labelled antibody.

Another difficulty in the interpretation of CBA results lies in the fact that there is no standard cut-off percentage for what is representative of significant competition. In this study competition was defined as complete, partial or negative if the unlabelled antibody prevented attachment of labelled antibody by >75%, 25-75% or <25% respectively (Arikawa *et al*, 1989) when the competitor was used at 100 μg/ml.

Analysis of the competitive binding data using the above criteria revealed the presence of four antigenic domains on both the G1 and G2 proteins of RVFV. In the case of the G1 protein, if it is assumed that the partial competition observed between the MAbs binding to G1 I with MAbs recognizing each of the other domains is due to steric hindrance, the results can be interpreted to indicate that the four G1 regions are overlapping or adjacent to each other, forming a single antigenic domain of interlinked epitopes. However, because of the possibility that partial or asymmetrical competition is due to allosteric prevention of binding, the domains might be located on various parts of the glycoprotein and share a functional spatial relationship by virtue of higher protein structure.

With regard to the epitopes comprising domain G2 I, if the asymmetrical competition observed between the MAbs defining sites G2 Ia, Ib and Ic is due to avidity differences, then these epitopes may be clustered and form part of a single large antigenic region on the G2 spike. The complete asymmetrical inhibition which occurred between epitopes G2 Ib and Ia, and Ib and Ic, but not Ia and Ic could then be interpreted to indicate that Ia and Ic are located on opposite sides of Ib.

The G2 domains II, III and IV, on the other hand, were defined by MAbs which displayed no reciprocal competition with any of the other antibodies. Based on the partial one-way competition exhibited by the MAb recognizing G2 Ic with all the other G2-specific MAbs, it might be argued that all four areas are linked to form a single antigenic domain similar to the G1 protein. However, this asymmetrical competition is most likely due to allosteric phenomena rather than steric hindrance, since this particular antibody was shown to also partially inhibit binding of some of the MAbs specific for G1. The observation that a limited number of pairs of G1 and G2-specific MAbs were capable of competing for binding is of interest, demonstrating that these antibodies can induce conformational changes in an entirely different viral protein. To our knowledge there is no published data regarding epitope mapping studies on the effect of binding of G1-specific MAbs on the G2 protein and *vice versa* for any other phleboviruses. Conformational changes in the PE2 protein induced by the binding of a MAb specific for the envelope E1 protein have nevertheless been shown for Sindbis virus (Clegg *et al*, 1983).

The unidirectional and bidirectional enhancement observed between various antibody pairs here has been reported for many other viruses (Boere *et al*, 1984; Burns *et al*, 1988; Cecilia *et al*, 1988; Heinz *et al*, 1984; Kingsford, 1984; Lubeck and Gerhard, 1981). This phenomenon is thought to be due to a change in the conformation of the antigen brought about by the binding of the first antibody (Lubeck and Gerhard, 1981). Antibody interaction with critical buried residues in one site, for example, may help to induce side-chain adjustments in adjoining sites (Getzoff *et al*, 1987), making the binding of additional antibodies more favourable (Heinz *et al*, 1984; Lubeck and Gerhard, 1982). Studies have furthermore shown that MAbs which enhanced the binding of other antibodies in the CBA system can have a synergistic effect on the neutralizing activity of such antibodies (Anderson *et al*, 1988; Chanas *et al*, 1982; Dubuisson *et al*, 1990; Kingsford, 1984; Russell, 1986; Van Drunen Littel-van den Hurk *et al*, 1985;

Volk *et al*, 1982). The increased antibody binding which occurred between various RVFV MAb pairs in the CBAs here may thus have important biological consequences in terms of neutralization of RVFV and will be further investigated.

By correlating the data of the competitive binding and serological assays, it was possible to assign functional properties to the mapped regions. The epitope recognized by MAb 3E5 (G1 Ia) appears to define a biologically important area on the G1 protein since it had very potent neutralizing and significant HI activity and was also associated with complete protection to otherwise lethally infected mice. The fact that the epitopes assigned to the G1 domains II and IV also exhibited virus neutralizing as well as haemagglutination activity, indicates that these sites, like G1 Ia, are accessible to antibody on the intact virion. These domains therefore are likely to be located in the hydrophilic portions on the outer surface of the folded G1 polypeptide as shown for the neutralizing sites on many other viral glycoproteins (Eisenberg *et al*, 1985; Nowak and Wengler, 1987; Pellet *et al*, 1985; Wiley and Skehel, 1987).

Of interest is the finding that the three MAbs earlier shown to cross-react with Gordil virus all mapped to closely adjacent sites (G1 IIc, IId and IIe), indicating that this particular region on the G1 protein is more highly conserved than the epitopes defined by the other antibodies.

As in the case of the G1 protein, the epitope G2 Ia defined by the G2-reactive MAb 9C4 exhibited very high neutralization as well as haemagglutination activity. Since this site was also involved in protection of mice from virulent RVFV infection, it must represent an immunologically significant area on the G2 protein, similar to the G1 determinant defined by MAb 3E5. In contrast to the G1 glycoprotein, however, only one domain (G2 I) was associated with significant neutralizing activity. As the remaining G2 domains only displayed low level C'-

dependent neutralization, these may be less accessible than G2 I for antibody binding.

These CBAs with peroxidase labelled RVFV MAbs have thus permitted the topographical analysis of ten of the G1 epitopes and seven of the G2 antigenic sites. Four heterogeneous antigenic regions which may be interlinked were identified on the G1 protein, while four distinct domains on the G2 were defined. The collinearity of the epitopes defined by the mapping studies, however, do not necessarily imply sequence collinearity on the G1 and G2 viral proteins. While the relationships between the epitopes may be linear, the antigenic sites may share spatial relationships by virtue of tertiary protein folding. To clarify this, further characterization will be undertaken by ELISA studies using denatured viral proteins.

4. STRUCTURAL ANALYSIS OF EPITOPES

4.1 Introduction

Since antigenic determinants in proteins are often complex conformations dependent on the tertiary folding of the protein chain (Crumpton, 1974; Getzoff *et al*, 1987; Geysen *et al*, 1987b; Sela, 1969), the linear arrangement of epitopes mapped by CBAs does not necessarily reflect sequence collinearity along the viral polypeptide chain (Coe and Meengeling, 1990). Both conformation-dependent as well as conformation-independent antigenic sites have been identified on the envelope proteins of various viruses (Cybinski *et al*, 1990; Eliot *et al*, 1990; Hall *et al*, 1990; Heinz *et al*, 1983; Niesters *et al*, 1987).

Furthermore, conditions which destroy the tertiary structure of the protein usually result in a large reduction in reactivity of antibodies to the native protein (Crumpton, 1974; Sela, 1969). MAbs against the glycoproteins of various viruses, for example, have revealed that certain neutralizing sites are found only on the native viral protein and not on the denatured protein (Brioen *et al*, 1982; Cybinski *et al*, 1990; Eliot *et al*, 1990; Heinz *et al*, 1983; Niesters *et al*, 1987). Thus not only the position but also the conformation of the antigenic determinant may be important for antibody binding and the expression of functional activities at the epitope level (Berzofsky and Berkower, 1989; Carter and ter Meulen, 1984; Hall *et al*, 1990; Jackson and Nestorowicz, 1985; Pollock *et al*, 1984).

In order to further characterize the structural properties of the RVFV G1 and G2 epitopes, the reactivity of the MAbs with viral antigen that has been denatured will be assessed to establish whether the antigenic determinants are linear or conformation-dependent. This will be investigated by firstly examining the sensitivity of MAb interactions to conformational changes generated by disruption of noncovalent bonds of the viral protein. Further conformational changes will then be generated by reducing disulphide bonds and determining the effect these

changes have on the antibody interactions. By combining the CBA data with the results of the conformational studies, a diagrammatic representation of the conformation and topography of the RVFV glycoprotein epitopes can be constructed. The structural analysis will also help to elucidate the role of conformation of the RVFV envelope proteins in antibody attachment and other functional activities.

4.2 Materials and Methods

4.2.1 MAb reactivity with heat denatured viral antigen

The structural properties of each epitope were assessed by analysing the effect of heat denaturation of the native viral protein on the antigenic reactivity of each MAb. The method used was based on that described by Eliot *et al* (1990). Purified stock viral glycoproteins (17 mg/ml, section 2.2.1.2) were diluted 1:50 in PBS and denatured by heating for 3 min at 100°C with or without 2,5% B-mercaptoethanol (ME). The treated antigens and untreated glycoprotein (control antigen) were diluted in carbonate-bicarbonate coating buffer (pH 9,6) (2.2.2.3) to yield a final dilution of 1:200 (85 µg/ml). Microtitre plates (Nunc Immuno II, As/Nunc, Roskilde, Denmark) were coated in parallel with 0,1 ml/well of the treated and control antigens. After overnight incubation at 4°C, the wells were saturated with 4% BSA for 30 min at 37°C. Serial dilutions of MAbs in ELISA diluent were added for 1 h at 37°C, followed by anti-mouse peroxidase conjugate and substrate as outlined earlier (2.2.2.3).

4.2.2 MAb reactivity with reduced and alkylated viral antigen

Reduction of the disulphide bonds in the viral glycoproteins was performed by the method of Kari *et al* (1986). Purified stock extracted viral glycoproteins (17 mg/ml; section 2.2.1.2) were prepared for reduction by diluting 0,05 ml in 5 ml TN buffer (0,02 M Tris-hydrochloride buffer, pH 7,8, containing 2,5 mM NaCl and 0,1% Nonidet P40) adjusted to pH 8,0, containing 8 M urea. Dithiothreitol (DTT; Sigma Chemicals) was added to the viral antigen to a final concentration

of 0,01M. The reaction was allowed to proceed at rt overnight with constant stirring. Alkylation was performed by adding 9 mg of iodoacetamide and allowing the reaction to proceed at rt for an additional 2 h. Samples were then dialyzed to remove excess reagents. The reduced and alkylated viral proteins were then diluted in carbonate-bicarbonate buffer (pH 9,6) to give a final concentration of 85 μg/ml. Untreated viral glycoproteins were used as control antigen at the same protein concentration as the treated antigen. ELISA plates were coated with the reduced and alkylated viral antigen in parallel with the control antigen and the reactivity of each MAb with the viral proteins was examined by ELISA as described above.

4.3 Results

4.3.1 Heat denatured antigen

With the exception of MAb 1E4, none of the G1-specific MAbs bound to the heat denatured antigen as indicated by the extremely low O.D. readings (< 0.200) obtained in the ELISA. With regard to the MAbs specific for the G2 glycoprotein, the reactivities of MAbs 8C2 and 7F1 (G2 Id and G2 II) were unaffected by heat or mercaptoethanol treatment (Table 16). The remaining anti-G2 MAbs did not react with antigen that had been denatured by heat.

4.3.2 Reduced and alkylated antigen

Of the three MAbs which recognized heat denatured viral glycoprotein (1E4, 8C2, 7F1), the G1-specific MAb 1E4 was unable to bind to antigen that had been subjected to conditions which disrupted the polypeptide disulphide bonds (Table 16). The reactivities of MAbs 8C2 and 7F1, on the other hand, were not affected by this treatment as evidenced by strong positive readings in the ELISA.

Table 16. Reactivity of MAbs in ELISA with RVFV antigen which had been denatured by various treatments

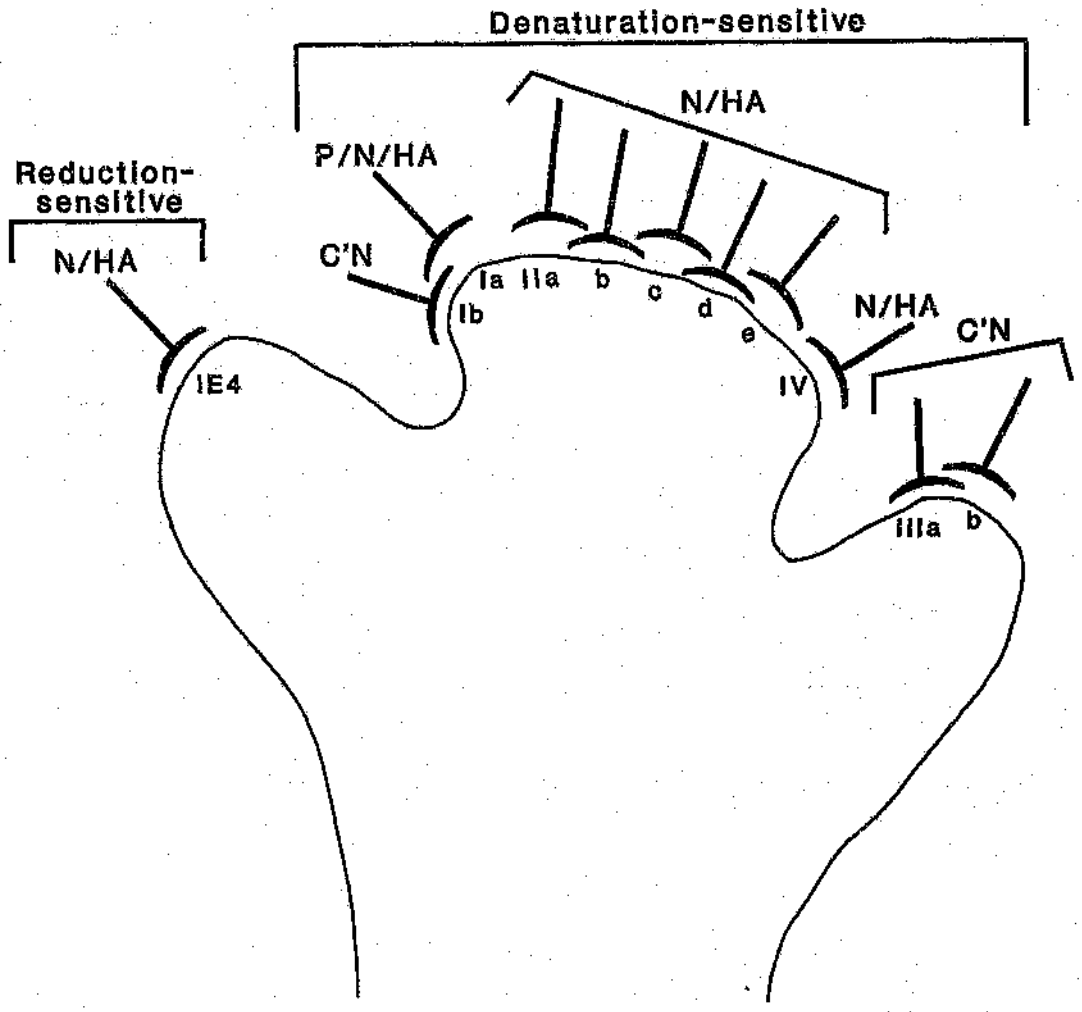
MAb	Domain	Heat	Heat/ME[1]	Reduction/ alkylation	Enhanced antibody binding
3E5	G1 Ia	S[2]	S		E[4]
5E1	Ib	S	S		
3D2	IIa	S	S		
3H1	IIb	S	S		
4B3	IIc	S	S		
5A1	IId	S	S		
9E4	IIe	S	S		I[5]
5E11	IIIa	S	S		E
8A3	IIIb	S	S		
5E9	IV	S	S		
5F2	?	S	S		
8G10	?	S	S		
1E4	?	R[3]	R	S	
5A6	?	S	S		
9C4	G2 Ia	S	S		I
8G2	Ib	S	S		E, I
7F2	Ic	S	S		
8C2	Id	R	R	R	E, I
7F1	II	R	R	R	E, I
4D10	III	S	S		E, I
8E6	IV	S	S		
6E10	?	S	S		
5C12	?	S	S		

[1] ME = B-mercaptoethanol
[2] S = epitope sensitive to denaturation
[3] R = epitope resistant to denaturation
[4] E = exhibits enhanced binding
[5] I = induces enhanced antibody binding to other sites

4.3.3 Diagrammatic representation of topography

By combining the data from the CBAs with the denaturation studies, it was possible to construct a diagrammatic representation of the conformation and topography of the G1 and G2 epitopes here (Figs 4 and 5). Fig. 4 clearly shows that the G1 domains I to IV are comprised of epitopes which are homogeneous regarding their sensitivity to denaturation and thus all map to a conformation-dependent portion of the protein. In contrast, the site defined by MAb 1E4 is not destroyed by heat denaturation and therefore has been depicted as lying in a different region of the glycoprotein.

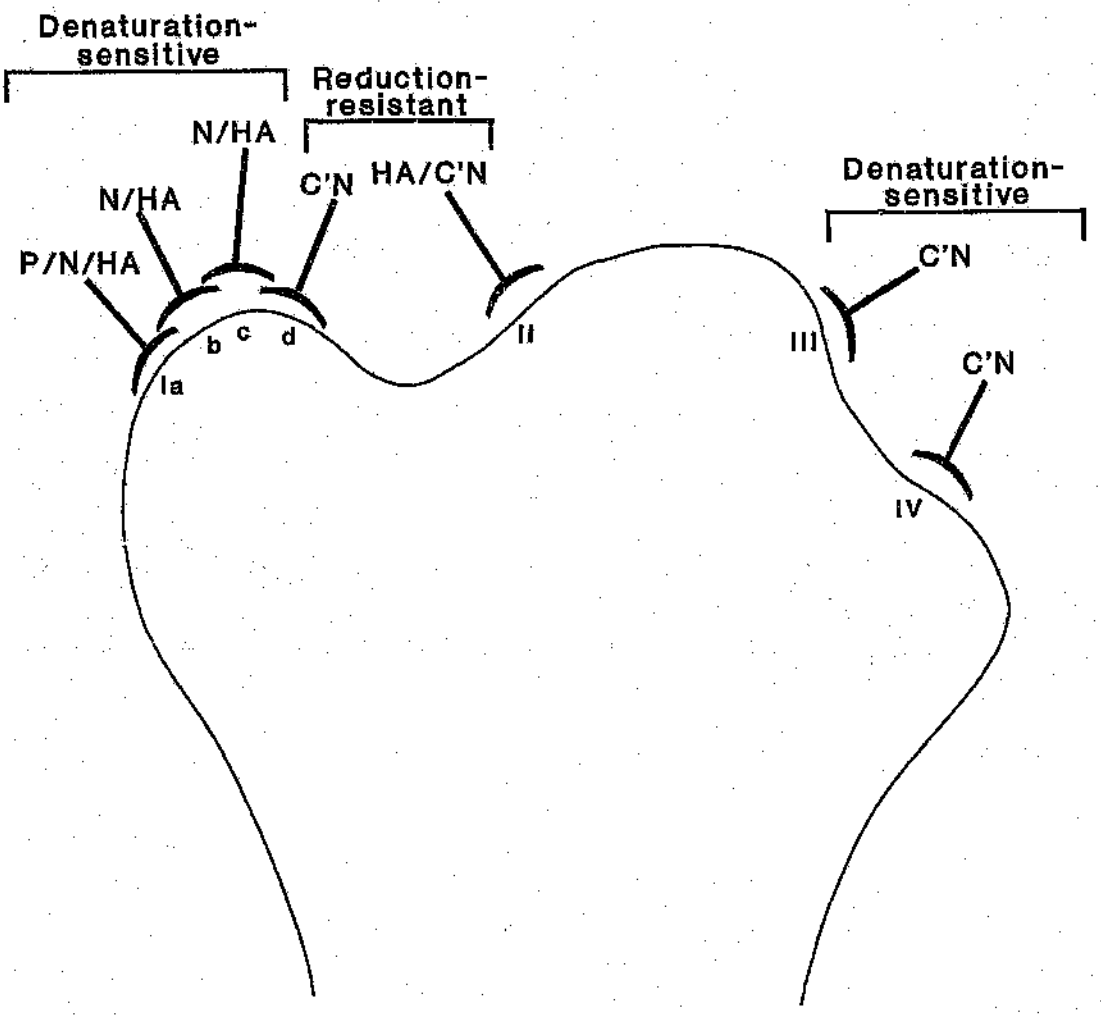
The majority of the G2 antigenic sites likewise map to a conformationally-dependent area on the envelope protein (Fig. 5). Epitopes G2 Id and G2 II, on the other hand, are completely resistant to denaturation and thus comprise a portion of the G2 protein that is conformationally more stable.



Epitopes associated with the following functions:

P = protection	HA = haemagglutination
N = neutralization	C'N = complement-dependent neutralization

Fig. 4. Diagrammatic model of the proposed spatial arrangement, conformational and biological properties of the epitopes on the RVFV G1 protein recognized by the MAbs. Overlapping or closely adjacent epitopes are indicated by overlapping lines in the figure, as defined by mutual inhibition of antibody binding.



Epitopes associated with the following functions:

P = protection	HA = haemagglutination
N = neutralization	C'N = complement-dependent neutralization

Fig. 5. Diagrammatic model of the proposed spatial arrangement, conformational and biological properties of the epitopes on the RVFV G2 protein recognized by the MAbs. Overlapping or closely adjacent epitopes are indicated by overlapping lines in the figure, as defined by mutual inhibition of antibody binding.

4.4 Discussion

All the antigenic sites comprising domains I, II, III and IV on the RVFV G1 glycoprotein appear to be strongly dependent on the native conformation of the protein as shown by their sensitivity to heat denaturation. These epitopes thus probably represent discontinuous determinants which are formed as loops or clefts in the folded native structure stabilized by hydrophobic interactions (Atassi, 1980; Crumpton, 1974). The fact that all the G1 domains I to IV are conformation-dependent supports the CBA data that these are situated in close proximity to each other on the G1 protein. The low level C'-dependent neutralization exhibited by epitopes G1 Ib, G1 IIIa and G1 IIIb in turn suggests that these sites are less accessible for antibody binding than those comprising the epitopes involved in haemagglutination and potent neutralization. These particular antigenic determinants (ie G1 Ib, G1 IIIa, G1 IIIb) may therefore be located in areas or pockets of the G1 polypeptide which are more folded than those comprising the more functionally active epitopes.

The G1-specific epitope defined by MAb 1E4, on the other hand, lies in a region of the protein that resists heat and mercaptoethanol denaturation. The involvement of disulphide bridges in stabilizing the native conformation of this antigenic site is suggested by the loss of antigenicity upon reduction and alkylation. While this epitope could not be mapped due to the weak avidity of MAb 1E4 in the ELISA, it probably corresponds to an independently folding region within the G1 compared to the domains I to IV.

As in the case of the G1 protein, most of the G2 determinants including the neutralizing epitopes G2 Ia, Ib and Ic, appear to be dependent on the tertiary protein structure. These antigenic sites thus map to an area of the G2 protein which is conformation-dependent, and most likely represent discontinuous determinants which are assembled from residues from several different portions of the polypeptide chain.

The G2 Id determinant, on the other hand, which is associated with C'-dependent neutralization, is resistant to denaturation. Epitope G2 II, although non-overlapping with G2 Id, similarly maintained its antigenic structure under conditions which destroy disulphide bonds. This could be interpreted to indicate that these epitopes represent conformation-independent, sequential antigenic sites. This is somewhat unexpected since the antibodies to these sites exhibited enhanced binding when mixed with certain MAbs. Synergistic binding of this nature is usually interpreted to be due to an allosteric rearrangement which permits easier access for the MAb to its conformational epitope (Heinz *et al*, 1984; Kingsford, 1984). A possible explanation for this phenomenon may be that although G2 Id and G2 II are linear sequential sites, they are partially hidden in the native structure of the protein by loops or folds comprising other sites. The enhanced binding which occurs in the presence of other specific antibodies may be due to allosteric changes which are induced in areas of the viral polypeptide close to the G2 Id and G2 II epitopes, which result in better exposure of these sites for antibody binding.

Alternatively, these antigenic sites may in fact be conformational, but owe their resistance to denaturation on the presence of the carbohydrate side chain which serves to shield these sites from the action of reducing agents. This has, for example, been shown for several epitopes on tick-borne encephalitis virus (Guirakhoo *et al*, 1989). Studies to determine whether the antigenic reactivity of these epitopes is abolished by deglycosylating the G2 protein might help to elucidate this. In contrast to the G2 Id and G2 II determinants, all the other epitopes which were involved in enhanced antibody binding appear to be discontinuous sites which is consistent with the general interpretation of this phenomenon.

With regard to the role of conformation of the RVFV envelope proteins in antibody attachment, it is evident that the native protein structure is required for

recognition and expression of functional activities of all the MAbs in this study which are involved in neutralization and haemagglutination-inhibition. Of interest is that despite the fact that extensive disulphide formation has been predicted for the RVFV envelope proteins (Ihara *et al*, 1985), only one epitope defined by these particular MAbs appears to be dependent on disulphide bridges for conformation.

Together, the CBA and structural analyses have permitted the probable spatial location and structure of these epitopes to be defined. Further localization of the epitopes will require other approaches, such as by the expression of small fragments of recombinant DNA clones, to map these sites to the gene coding regions and the corresponding predicted amino acid sequences on the polypeptide chain as has been performed by Keegan and Collett (1986). However, due to the discontinuous conformational nature of the majority of these determinants, mapping using the above method would be difficult. A possible alternative approach would be to analyse the amino acid changes associated with mutants (eg neutralizing escape mutants) which exhibit an altered pattern of reactivity with the MAbs. The precise localization of discontinuous epitopes nevertheless would require a knowledge of the complete three-dimensional structure of the RVFV envelope proteins, similar to that acquired for the influenza haemagglutinin (Wiley and Skehel, 1987; Wilson *et al*, 1981).

The topological studies have thus revealed not only that the spatial conformation of the RVFV glycoproteins plays a decisive role in determining the antigenic specificity of these particular sites, but that some MAb mixtures exhibit enhanced binding to certain conformational epitopes. As this may have important biological consequences in terms of virus neutralization, the possibility that such co-operative effects may potentiate the neutralizing activities of the MAbs will be investigated.

5. NEUTRALIZATION

5.1 Introduction

Glycoproteins of many enveloped viruses have been reported to play important roles in the adsorption and penetration of the virus (Choppin and Scheid, 1980) and are also the targets of the immune response to infections (Dimmock, 1987; Sarmiento *et al*, 1979). The neutralization of virus infectivity by antibodies has been proposed as a major mechanism of immunological defence against virus invasion (Dimmock, 1984; 1987; McCullough, 1986). Several mechanisms of virus neutralization have been described for other viruses, including cross-linking of virus particles by antibody (Emini *et al*, 1983), inhibition of virus binding, virus internalization and uncoating (reviewed by Dimmock, 1984; 1987; Iorio, 1988). Complete neutralization may moreover require synergistic enhancement of neutralization by pairs of antibodies to different antigenic sites (Gehrz *et al*, 1992; Kingsford, 1984; Lussenhop *et al*, 1988). It has recently become apparent that there is no mechanism of neutralization specific for all viruses (Kingsford *et al*, 1991; Outlaw and Dimmock, 1991).

With regard to RVFV, several neutralizing epitopes on the glycoproteins have been identified by monoclonal antibodies (Collett *et al*, 1987; Schmaljohn *et al*, 1989). The neutralizing antibodies have also been used to analyse antigenic determinants of vaccinia virus- or baculovirus-expressed RVFV glycoproteins (Collett *et al*, 1987; Dalrymple *et al*, 1989; Schmaljohn *et al*, 1989) and to investigate the potential efficacy of a recombinant vaccine (Saluzzo and Smith, 1990). However, there is little published information regarding the mechanisms of neutralization of RVFV.

The majority of the RVFV MAbs produced in this study exhibited neutralizing activity, suggesting that they will be useful for examining the mechanisms of

inhibition of virus infectivity. The MAbs were therefore used to analyse the mechanisms involved in the antibody-mediated neutralization of RVFV, including possible synergistic neutralization by mixtures of antibodies.

5.2 Materials and Methods

5.2.1 CPENT tests with mixed MAbs

MAb pairs were tested using both uninactivated and heat inactivated (56°C for 30 min) ascitic fluids. These were diluted 1:4 in Leibovitz medium supplemented with 5% FCS and 0,3% gentamicin antibiotic. Volumes of 50 µl of each MAb pair were added to the first well of a microtitre plate to give a 1:8 dilution for each antibody. Serial two-fold dilutions of each MAb mixture were then made, followed by the addition of an equal volume of virus (100 $TCID_{50}$) to yield final initial dilutions of 1:32 for each MAb. The plates were incubated for 2 h at 37°C in a humidified container, then seeded with cells and incubated as outlined in section 2.2.3.3 e. Each MAb pair was tested in duplicate and end points recorded as the mean reciprocal of the highest dilutions which prevented CPE.

5.2.2 PRNT tests with mixed MAbs

MAbs which did not neutralize individually but which neutralized when used in combination in the CPENT were further tested by PRNT. The method used was based on that of Lussenhop *et al* (1988) with the exception that MAb ascitic fluid was used instead of purified antibody preparations, and no C′ was added. Each combination was tested with one MAb at a fixed concentration, mixed with serial fourfold dilutions of the second MAb and *vice versa*. The dilution used for the MAb at fixed concentration was that which yielded a 50% PRNT titre. Assays were performed in duplicate on Vero cell monolayers in 24 well tissue culture plates essentially as described in section 2.2.3.3f. The neutralization titres were recorded as the reciprocals of the highest dilutions which gave an 80% reduction in the number of plaques relative to the virus controls.

5.2.3 Blocking of MAb neutralization

Those MAbs which competed for binding of the strongly neutralizing MAbs 3E5 and 9C4 to RVF antigen in the CBA (Section 3.3.3) were tested for their ability to inhibit the neutralizing activity of the latter two antibodies. This was carried out using a modification of the CPENT described above. Briefly, the competing MAbs at 10^{-1} and 10^{-2} in Leibovitz medium were preincubated with 100 $TCID_{50}$ virus for 1 h at 37°C before serial two-fold dilutions of MAbs 3E5 and 9C4 were added. The plates were incubated for a further hour at 37°C, followed by the addition of the cells. After 6 days at 37°C, the wells were examined for the presence of CPE.

5.2.4 Post-adsorption virus neutralization tests

Post-adsorption neutralization tests were performed as described by Highlander *et al* (1987) with slight modifications. RVFV was diluted to ± 800 PFU/ml in Leibovitz medium and aliquoted in 0,4 ml volumes. Dilutions for each MAb were prepared so that approximately 80% of the input virus would be neutralized. Vero cell monolayers in 24-well plates were prechilled for 1 h at 4°C. One set of virus samples was mixed with an equal volume of the MAbs for 2 h at 37°C. The second set of virus samples was added directly to the individual monolayers for 1 h at 4°C and unadsorbed virus was removed by washing the cells with phosphate buffered saline with magnesium chloride (PBS-$MgCl_2$). Bound virus was incubated with the individual MAbs for an additional 2 h at 4°C. Virus control samples were plated for 1 h at 4°C, washed and incubated an additional 2 h at 4°C without antibody. In all three cases, the monolayers were again washed twice, overlaid with agarose, stained, and the plaques counted as for the PRNTs. Neutralization before and after virus adsorption for each sample was compared to that of the virus control without antibody and expressed as a ratio. Post-adsorption neutralization assays were also carried out with C′-dependent MAbs; here C′ was added to the MAb dilutions to yield a 1:40 final concentration of C′.

5.3 Results

5.3.1 CPENT with MAb pairs

The effects on the neutralizing activity of MAbs 3E5 (G1 Ia) and 9C4 (G2 Ia) when mixed with other MAbs which were non-neutralizing in the CPENT are shown in Table 17. Only those MAb pairs which resulted in enhanced neutralization or binding in the CBA with either MAb 3E5 or 9C4 are shown.

The neutralizing activity of MAb 3E5 (G1 Ia) was increased two to almost eight-fold when mixed with MAbs binding to domains G1 II as well as MAbs 1E4 and 8G10. The sites recognized by the latter could not be mapped due to the weak avidity of these antibodies in the CBA. MAb 9E4, which resulted in augmented binding of 3E5 in the CBA, on the other hand, did not increase the neutralizing titre of 3E5. Of the two MAbs which caused increased binding of MAb 9C4 (G2 Ia), only MAb 6E10 brought about synergistic neutralization of 9C4. Enhanced neutralization of MAb 9C4 also occurred when mixed with another of the G2-specific MAbs (7F1, mapping to G2 II) as well as some antibodies specific for the G1 protein.

Tables 18, 19 and 20 show the results of mixing all the RVFV MAbs in various combinations in the CPENT. Many of the pairs of the G1-specific MAbs were able to neutralize the virus when tested as uninactivated ascitic fluid, but only four of these retained their neutralizing ability once inactivated (Table 18). None of these neutralizing antibody pairs exhibited augmented binding in the CBA as determined previously.

In contrast to the G1-specific MAb pairs, only one of the mixtures of the G2-reactive MAbs (8C2 and 7F1 mapping to G2 Id and II) resulted in synergistic neutralization (Table 19). This particular MAb pair had previously been shown to display bidirectional enhanced binding in the CBA. A number of the mixtures

Table 17. Effects on neutralizing activity of MAbs 3E5 and 9C4 by mixing with other MAbs

	Increased neutralization ratio[1] G1 protein								G2 protein			
CBA domain	Ia	IIa	IIb	IIc	IId	IIe	?	?	Ia	II	III	?
MAb	3E5	3D2	3H1	4B3	5A1	9E4	8G10	1E4	9C4	7F1	4D10	6E10
3E5	1	5.5	2	2.7	2.7	1	5.5	7.8	3.1	1	1	1
9C4	3.1	1	8	4	8	1	1	1	1	4	1	2.8

[1] Neutralization ratio = $\frac{\text{neutralization titre of mixtures}}{\text{mean of expected neutralization titre}}$

Table 18. Neutralizing activity of paired G1-specific MAbs

		Neutralization titre[1]											
CBA domain		Ib	IIa	IIb	IIc	IId	IIe	IIIa	IIIb	IV	?	?	?
	MAb	5E1	3D2	3H1	4B3	5A1	9E4	5E11	8A3	5E9	5F2	8G10	1E4
Ib	5E1	-[2]	-	128	-	-	-	-	-	-	-	-	-
IIa	3D2		-	-	-	-	-	-	-	-	-	-	-
IIb	3H1			-	128	-	-	-	-	256	-	128	-
IIc	4B3				-	128	-	128	-	-	128	256	-
IId	5A1					-	-	-	-	128	-	128	-
IIe	9E4						-	-	-	-	-	-	-
IIIa	5E11							-	-	128	-	128	-
IIIb	8A3								-	-	-	-	-
IV	5E9									-	128	32	-
?	5F2										-	128	-
?	8G10											-	-
?	1E4												-

[1] Neutralization titre in CPENT
[2] No neutralization
☐ Inactivated MAb ascitic fluid

Table 19. Neutralizing activity of paired G2-specific MAbs

		Neutralization titre[1]							
CBA domain		Ib	Ic	Id	II	III	IV	?	?
MAb		8G2	7F2	8C2	7F1	4D10	8E6	6E10	5C12
Ib	8G2	-[2]	-	-	-	-	-	-	-
Ic	7F2		-	-	-	-	-	-	-
Id	8C2			-	256	-	-	-	-
II	7F1				-	-	-	-	-
III	4D10					-	-	-	-
IV	8E6						-	-	-
?	6E10							-	-
?	5C12								-

[1] Neutralization titre in CPENT

[2] No neutralization

☐ Inactivated MAb ascitic fluid

of G1 and G2-specific MAbs neutralized when tested as uninactivated ascitic fluid, but upon inactivation only three pairs still exhibited synergistic neutralization (Table 20).

5.3.2 PRNT with mixed MAbs

The combinations of MAbs which exhibited synergistic neutralization in the CPENTs were further tested by PRNT. When the antibodies were tested individually, neutralizing activity was seen with G1-specific MAbs 3H1, 4B3, 5A1, 5E9, 8G10 and the G2-specific 7F2 at antibody concentrations ranging from 1:256 to 1:8000 using a 80% plaque reduction endpoint. MAbs 8C2 (G2 Id) and 7F1 (G2 II), on the other hand, did not neutralize RVFV at the 80% plaque reduction level even at a concentration of 1:128.

By combining mutually enhancing pairs of antibodies eg 8C2 + 7F1, 8G10 + 7F1 and 4B3 + 7F2, strong neutralizing effect was seen. The combination of MAbs 8C2 + 7F1 (Fig. 6a), both of which were individually non-neutralizing at the 80% plaque reduction level, resulted in neutralization up to a dilution of 1:64000. The MAb pairs 8G10 + 7F1 (Fig. 6b), 4B3 + 7F2 (Fig. 6c) and 5E9 + 8G10 (Fig. 6d) all neutralized with an 80% PRNT titre of 1:128000. An 80% PRNT titre of 1:256000 was obtained for the MAb pairs 3H1 (G1 IIb) + 5E9 (G1 IV) (Fig. 6e) and 5A1 (G1 IId) + 8G10 (G1?) (Fig. 6f). The MAb combination of 4B3 (G1 IIc) + 8G10 (Fig. 6g) neutralized at the 80% plaque reduction level up to a dilution of 1:1024000, while the neutralizing titre for 5E9 (G1 IV) + 7F1 (G2 II) was 1:64000 (Fig. 6h). For each MAb pair, the synergistic neutralization obtained by mixing a fixed concentration of one antibody with serial dilutions of the second antibody, and *vice versa*, was almost identical. The only exception was the MAb pair 4B3 + 8G10, where mixing varying dilutions of 4B3 with a constant amount of 8G10 gave a titre of 1:1024000 compared to 1:32000 when the antibodies were mixed the other way around. The

Tabl. 20. Neutralizing activity of paired G1 and G2-specific MAbs

		Neutralizing titre [1]											
CEA domain		G1Ib	IIa	IIb	IIc	IId	IIe	IIIa	IIIb	IV	?	?	?
	MAb	5E1	3D2	3H1	4B3	5A1	9E4	5E11	8A3	5E9	5F2	8G10	1E4
G2 Ib	8G2	-[2]	-	-	-	128	-	-	-	-	32	-	-
Ic	7F2	-	-	128	512	-	-	-	-	-	128	-	-
Id	8C2	-	-	-	-	-	-	-	-	-	-	-	-
II	7F1	64	-	128	128	-	-	-	-	32	-	128	-
III	4D10	-	32	-	32	128	-	-	-	-	-	-	-
IV	8E6	-	32	-	64	-	-	-	-	-	-	-	-
?	6E10	-	-	-	-	32	-	-	-	-	-	-	-
?	5C12	-	-	-	-	-	-	-	-	-	-	-	-

[1] Neutralization titre in CPENT

[2] No neutralization

☐ Inactivated MAb ascitic fluid

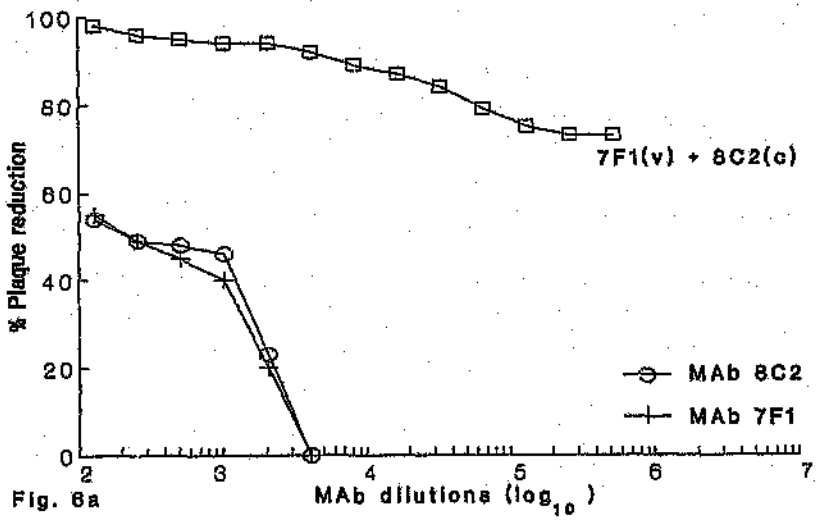


Fig. 6a

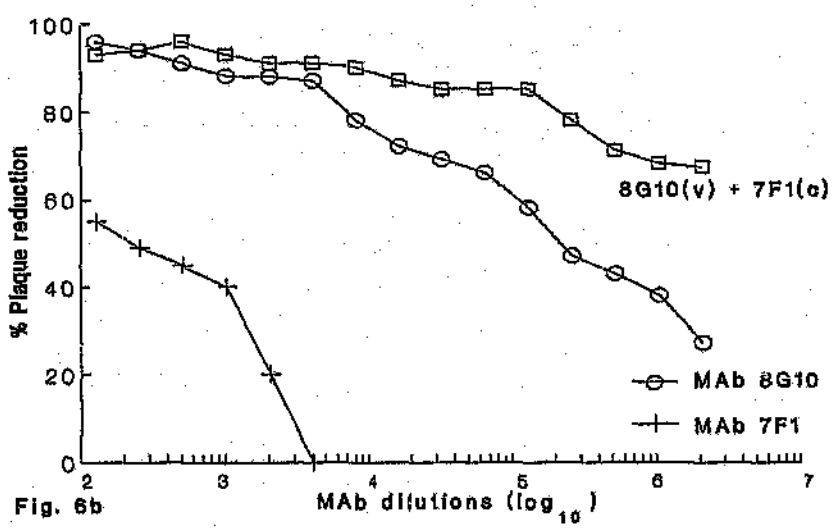


Fig. 6b

Fig. 6. Plaque reduction neutralization test (PRNT) using MAb pairs. **(a)** PRNT with a combination of two non-neutralizing G2-reactive MAbs (8C2 + 7F1) which exhibited reciprocal augmented binding in the CBA. The synergistic neutralization obtained by mixing a constant (*c*) amount of 8C2 (G2 Id) and varying (*v*) dilutions of 7F1 (G2 II) is shown. **(b)** PRNT with a mixture of G1 and G2-specific MAbs (8G10 + 7F1). The enhanced neutralization observed by mixing a constant (*c*) amount of 7F1 (G2 II) with varying (*v*) dilutions of 8G10 (G1 ?) is shown.

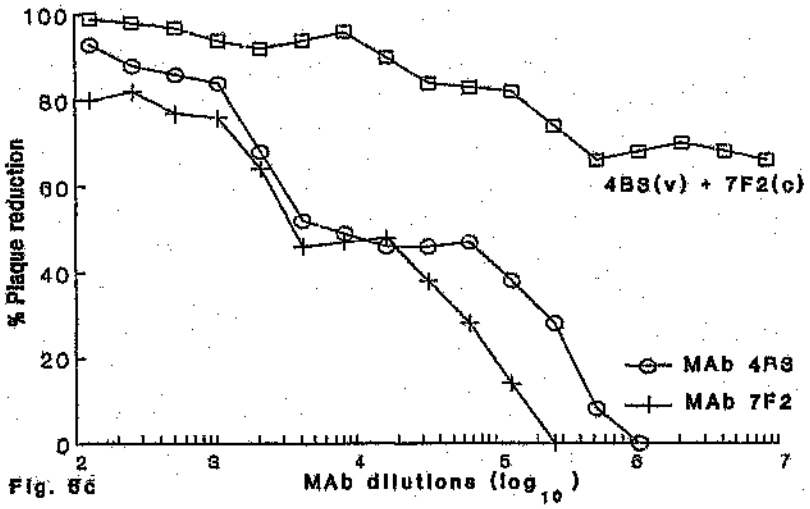


Fig. 6c

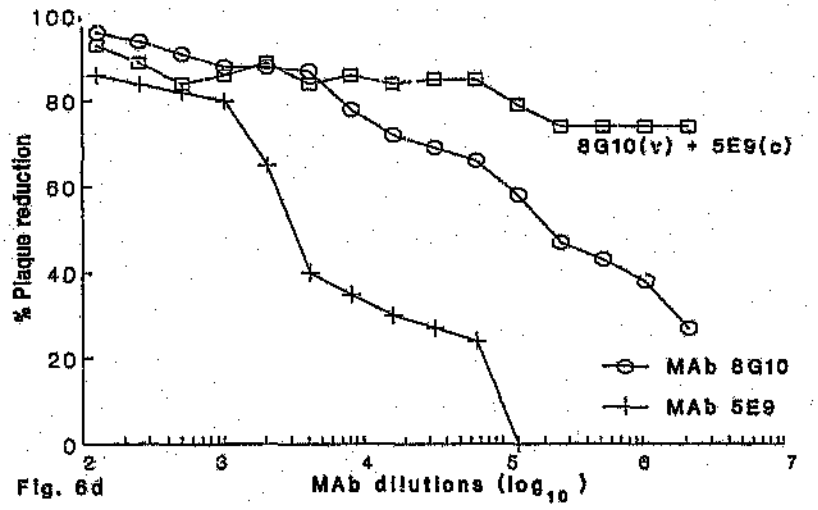


Fig. 6d

Fig. 6. Plaque reduction neutralization test (PRNT) using MAb pairs.
(c) Potentiation of the neutralizing activity of MAbs 4B3 (G1 IIc) and 7F2 (G2 Ic) when used in combination by mixing a constant (*c*) amount of 7F2 with varying (*v*) dilutions of 4B3. (d) The enhanced neutralization obtained by mixing a constant (*c*) amount of MAb 5E9 (G1 IV) with varying (*v*) dilutions of 8G10 (G1 ?).

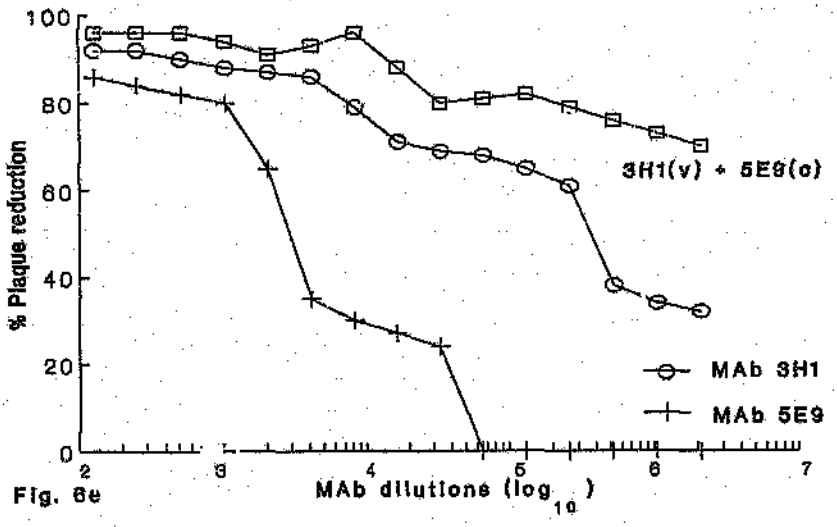


Fig. 6e

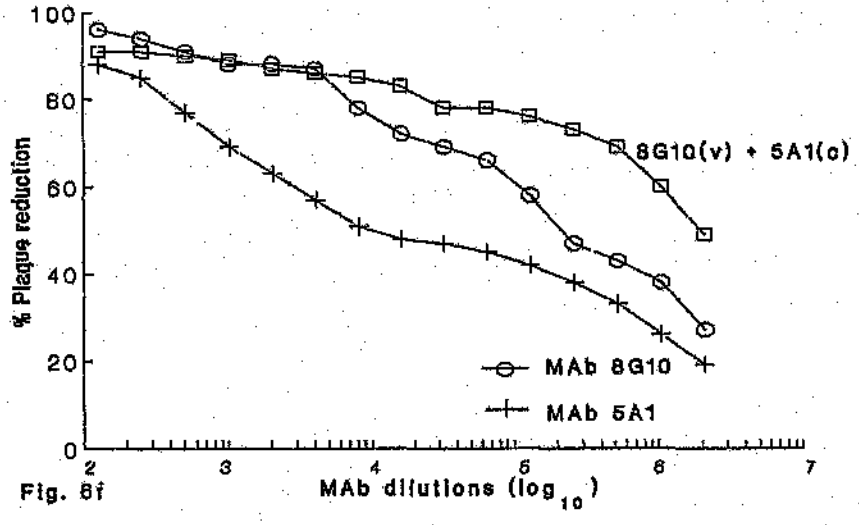


Fig. 6f

Fig. 6. Plaque reduction neutralization (PRNT) using MAb pairs.
(e) PRNT with a combination of two G1-reactive MAbs (3H1 + 5E9). The enhanced neutralization observed by mixing a constant (*c*) amount of 5E9 (G1 IV) with varying (*v*) dilutions of 3H1 (G1 IIb). (f) Potentiation of the neutralizing activity of MAbs 8G10 (G1 ?) and 5A1 (G1 IId) when used in combination by mixing a constant (*c*) amount of 5A1 with varying (*v*) dilutions of 8G10.

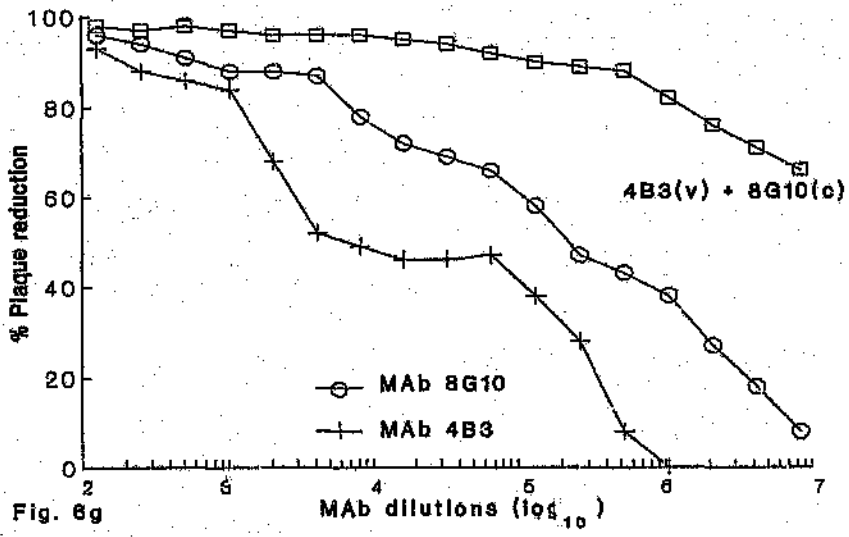


Fig. 6g

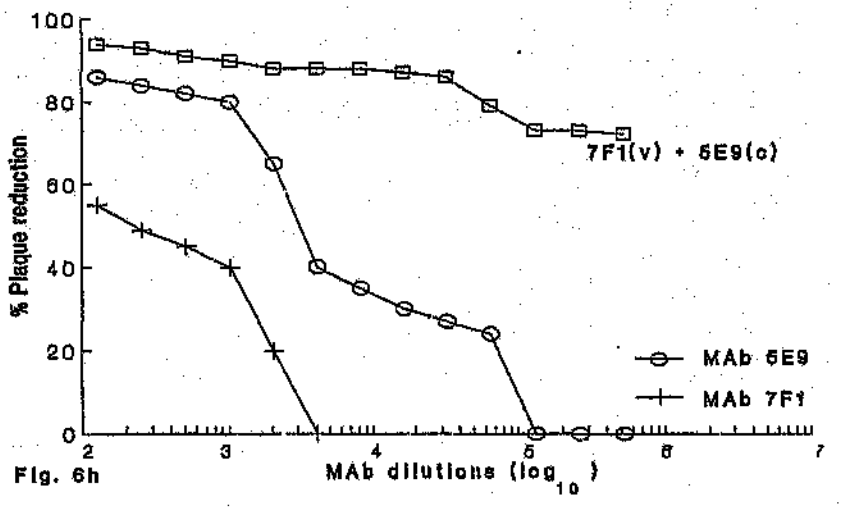


Fig. 6h

Fig. 6. Plaque reduction neutralization test (PRNT) using MAb pairs. **(g)** The synergistic neutralization obtained by mixing a constant (*c*) amount of 8G10 (G1 ?) with varying (*v*) dilutions of 4B3 (G1 IIc). **(h)** PRNT with a mixture of G1 and G2-specific MAbs where a constant (*c*) amount of 5E9 (G1 IV) was added to varying (*v*) dilutions of 7F1 (G2 II).

greatly enhanced percentage in plaque reduction achieved by combining the MAbs could not be explained by adding the percentage of plaque reduction contributed by each antibody tested individually.

5.3.3 Inhibition of neutralizing activity of MAbs

Those MAbs which had partially inhibited the binding of MAb 3E5 (G1 Ia) and 9C4 (G2 Ia) in the CBA were examined for the ability to inhibit the neutralizing activity of the latter two antibodies. None of the antibodies were found to affect the neutralizing titre of MAbs 3E5 and 9C4.

5.3.4 Post-adsorption neutralization tests

The MAbs which exhibited neutralization activity independent of C′ in the PRNT were tested for their ability to neutralize the virus once it had attached to the cells (Table 21). Some of the MAbs specific for G1 and G2 were found to neutralize adsorbed virus as efficiently as pre-adsorbed virus eg those mapping to G1 Ia, IIb, IId and G2 Ia. Several antibodies directed against G1 neutralized the adsorbed virus to lower titres than before virus adsorption (MAbs 4B3, 8G10, 5E9) while one of the anti-G1 MAbs, 9E4, and two of the anti-G2 MAbs (mapping to G2 Ib and G2 Ic) were unable to neutralize the attached virus. None of the MAbs requiring complement for neutralization were able to neutralize cell-adsorbed virus.

Table 21. Neutralization by RVFV MAb treatment of virus before or after adsorption

MAb	Antigenic domain	Neutralization[1]		Neutralization ratio[2]
		Preadsorption	Postadsorption	
3E5	G1 Ia	32 768	32 768	1.00
3H1	G1 IIb	1 024	1 024	1.00
4B3	G1 IIc	16 384	4 096	0.25
5A1	G1 IId	1 024	1 024	1.00
9E4	G1 IIe	1 024	-	0.00
5E9	G1 IV	1 024	128	0.125
1E4	G1 ?	4 096	512	0.125
8G10	G1 ?	4 096	512	0.125
9C4	G2 Ia	32 768	32 768	1.00
8G2	G2 Ib	2 048	-	0.00
7F2	G2 Ic	2 048	-	0.00

[1] 80% PRNT

[2] Ratio of neutralization after virus adsorption to neutralization before virus adsorption

5.4 Discussion

Studies on the neutralization of various enveloped viruses suggest that antibodies binding to different sites on a viral protein neutralize by different mechanisms and also that a single MAb can neutralize by a number of alternative procedures (Dietzschold *et al*, 1987; Dimmock, 1987; Dubuisson *et al*, 1990; Fuller *et al*, 1989; Gollins and Porterfield, 1986a; Kingsford *et al*, 1991; Okazaki *et al*, 1986; Roehrig *et al*, 1988; Taylor *et al*, 1987). Synergistic effects which potentiate the functional activity of MAbs have been demonstrated with many viruses, including La Crosse virus (Kingsford, 1984), Newcastle disease virus (Russell, 1986), respiratory syncytial virus (Anderson *et al*, 1988), bovine herpes virus (Dubuisson *et al*, 1990) and cytomegalovirus (Gehrz *et al*, 1992; Lussenhop *et al*, 1988).

The mechanisms involved in the antibody-mediated neutralization of RVFV were analysed here using the panel of MAbs. Since several of the MAbs were found to augment the binding of other antibodies in competitive binding assays, an important aspect of the study was examining the possibility that mixtures of the antibodies could have a synergistic effect on RVFV neutralization.

Individually, nine MAbs mapping to the G1 domains I, II and IV, and three MAbs specific for the G2 I domain, were able to neutralize RVFV *in vitro* without C′, while the remaining antibodies required C′ for neutralization. On mixing all combinations of these antibodies, synergistic neutralization was observed for many MAb pairs. The fact that some of these antibody pairs were no longer capable of achieving this synergy when tested after heat-inactivation emphasizes the important role of C′ in this particular aspect of RVFV neutralization.

However, even in the absence of C′, various different mixtures of MAbs exhibited enhanced neutralization; these could be divided into three categories. In the first instance, synergistic neutralization occurred for two of the MAb pairs

that showed augmented binding for G2 in the binding assay ie 9C4 + 6E10 and 8C2 + 7F1. The former pair comprised one neutralizing MAb (mapping to G2 Ia) and a non-neutralizing antibody. This probably presents an example of conformational changes by one MAb altering epitope expression by the virus so that the second MAb binds or neutralizes more efficiently (Clegg *et al*, 1983; Kingsford, 1984; Lubeck and Gerhard, 1982). The other MAb pair (8C2 + 7F1) which exhibited synergistic neutralization were both individually non-neutralizing at the 80% plaque reduction end-point. Here neutralization could be due to the combined mass of both antibodies binding which could interfere with the process of cellular attachment and fusion by steric hindrance as suggested for cytomegalovirus (Lussenhop *et al*, 1988). Alternatively, increased neutralization could be due to combined conformation changes brought about by both antibodies binding. The fact that this MAb pair displayed reciprocal enhanced binding in the competitive binding assay favours the latter explanation.

The second category of synergistic neutralization occurred between combinations of MAbs for which increased binding had not been detected, indicating that enhancement of RVFV neutralization must involve more than just an increase in the amount of neutralizing antibody bound to the virus. Synergy of this type was seen between MAbs directed against closely adjacent epitopes eg 3E5 (G1 Ia) and 3H1 (G1 IIb) as well as distant epitopes eg 3H1 (G1 IIb) and 5E9 (G1 IV). The enhanced neutralization in the latter case is most likely the result of conformational changes in sites critical for virus infectivity which do not lead to extra antibody binding to the virion surface as indicated by the binding assays. In contrast, the increased neutralizing activity obtained by mixing MAbs against overlapping or closely adjacent epitopes may either be due to a more efficient blocking of critical sites on the surface of the virion (Russell, 1986; Van Drunen Littel-Van den Hurk *et al*, 1985) or to allosteric phenomena (Gehrz *et al*, 1992; Kingsford 1984).

Lastly, mixtures of MAbs reacting with the G1 and G2 RVFV proteins were also capable of enhancing neutralization, supporting the findings in the binding studies that some antibodies can induce conformational changes in an entirely different viral protein.

The data from these in-vitro neutralization assays thus suggest that synergistic neutralization between various antibodies directed against different antigenic determinants on the RVFV G1 and G2 proteins is a common phenomenon. This may be of considerable immunological relevance with respect to the functional activity of a polyclonal immune response to infection with RVFV.

In order to further elucidate the mechanisms of antibody-mediated RVFV neutralization, post-adsorption neutralization tests were performed. If RVFV neutralization primarily involves the inhibition of virus attachment, adsorbed virions should be resistant to neutralization. In contrast, if the process involves the blocking of some steps subsequent to virus adsorption, the antibodies should neutralize cell surface-bound virions as shown for example for rabies virus (Dietzschold *et al*, 1987), herpes simplex virus (Fuller and Spear, 1985), transmissible gastroenteritis coronovirus (Sune *et al*, 1990) and bovine herpes virus (Okazaki *et al*, 1986).

The results obtained revealed not only that the RVFV MAbs exhibited different mechanisms of neutralization, but that antibodies mapping to a single antigenic domain (eg G1 II) do not neutralize in an identical manner. Some of the antibodies specific for G1 and G2 neutralized cell-attached virus as efficiently as unadsorbed virus, thereby indicating that they can neutralize by inhibiting the infection process after virus attachment. Both the G1 and G2-specific MAbs previously shown to be highly protective *in vivo* (3E5 and 9C4) exhibited this type of neutralization.

While a relationship of both the RVFV envelope proteins to infectivity has been established (Battles and Dalrymple, 1988; Collett *et al*, 1987; Dalrymple *et al*, 1989; Keegan and Collett, 1986; Schmaljohn *et al*, 1989) the exact role played by either of the glycoproteins in viral penetration has not yet been directly demonstrated. The findings here that MAbs directed against both G1 and G2 neutralize virus that has already bound to cells strongly suggests that both the RVFV envelope proteins are responsible for penetration of the virus.

Other MAbs, eg those mapping to G1 IIe, G2 Ib and G2 Ic, on the other hand, did not exhibit any post-adsorption neutralizing ability, suggesting that these antibodies may neutralize by preventing virus attachment to cells. The RVFV MAbs requiring C' for virus neutralization likewise may effect neutralization by preventing virus attachment, possibly by enhancing aggregation of virus-antibody complexes (Oldstone *et al*, 1974).

The remaining MAbs specific for the G1 protein displayed a certain degree of post-adsorption neutralizing activity. These antibodies may be capable of inhibiting RVFV infectivity in several ways, as reported for certain poliovirus MAbs (Emini *et al*, 1983). The mechanism of neutralization may be partly dependent on the isotope of the antibody (Dimmock, 1984; 1987). In the case of the two IgM antibodies 1E4 and 8G10, neutralization is most likely effected by both prevention of virus attachment as well as virus internalization, as shown for IgM neutralization of influenza virus (Taylor and Dimmock, 1985). The IgG MAbs defining sites G1 IIc and G1 IV, on the other hand, could possibly neutralize by inhibiting any of the various stages of virus infectivity i.e. attachment, penetration, uncoating or replication.

The post-adsorption studies also help to further elucidate the mechanism underlying the enhanced neutralization which occurred between the pairs of MAbs which map to the closely adjacent G1 I and G1 II domains. The fact that, for

example, MAbs 3E5 (G1 Ia) and 3H1 (G1 IIb) can neutralize virus that is already cell-attached, indicates that the increased neutralization is not due to a more efficient blocking of sites involved in virus attachment as has been suggested for Newcastle disease virus (Russell, 1986).

The data obtained from these neutralization assays thus provide evidence for an interaction between the RVFV G1 and G2 proteins by showing that antibodies to one of the envelope glycoproteins can cause a conformation change resulting in an altered antigenicity in the other glycoprotein. The findings furthermore suggest that inhibition of virus attachment is not the principal means of antibody-mediated RVFV neutralization. Instead such neutralization is most likely the result of several different processes, including synergistic neutralization by combinations of different antibodies, prevention of virus attachment and inhibition of steps subsequent to virus adsorption. To clarify the neutralization mechanisms further, possible interference by the MAbs of various stages of virus infectivity such as virus attachment and penetration will be examined in more detail.

6. RVFV ATTACHMENT

6.1 Introduction

The early events in the infection process of RVFV and other members of the *Bunyaviridae* are not well defined (Schmaljohn and Patterson, 1990). As with other enveloped viruses, attachment of RVFV to host cells is a critical first step to infection mediated by the interaction between one or both of the viral envelope glycoproteins and cell surface receptors (Choppin and Scheid, 1980; Lentz, 1990). While the cell receptors involved in binding have not been identified thus far for any member of the *Bunyaviridae* family (Wang *et al*, 1991), the viral attachment proteins have been examined indirectly using MAbs (Arikawa *et al*, 1989; Dantas *et al*, 1986; Gonzalez-Scarano *et al*, 1982; Grady *et al*, 1983; Keegan and Collett, 1986; Kingsford *et al*, 1983; 1991; Ludwig *et al*, 1989; 1991; Pifat *et al*, 1988).

Antibodies against the region of the viral coat proteins thought to be involved in binding have been shown to block the attachment of virus (Dietzschold *et al*, 1987; Dimmock, 1987; Dubuisson *et al*, 1990; Massey and Schochetman, 1991; McCullough, 1986; Roehrig *et al*, 1988). Inhibition of virus binding by antibodies in fact represents one of the classic explanations of virus neutralization (Mandel, 1967). Blocking of virus attachment may be effected through the binding of antibody at or near the cell attachment site on the virus, "bridging" of this cell binding site through the MAb reacting with epitopes on either side of it, saturation of the virion surface by antibody, or aggregation of virus particles to form virus/antibody complexes (reviewed by McCullough, 1986). In addition to these inhibitory effects on virus binding, some MAbs have been identified which neutralize by preventing internalization of the adsorbed virus (Armstrong *et al*, 1990; Suné *et al*, 1990; Taylor and Dimmock, 1985).

With regard to the *Bunyaviridae* family, virus attachment and MAbs which inhibit such binding have recently been examined in some detail for the bunyavirus La

Crosse virus (LACV)(Kingsford *et al*, 1991; Ludwig *et al*, 1991). The findings of these and earlier studies (Gonzalez-Scarano *et al*, 1982; Kingsford and Hill, 1981; 1983; Kingsford *et al*, 1983) suggest that the LACV G1 protein is more actively involved in binding to vertebrate host cells than G2.

Unlike LACV, comprehensive analyses on the viral proteins involved in attachment of RVFV have not been performed to date. In contrast to the situation for LACV, however, the presence of neutralizing and haemagglutination sites on both the RVFV G1 and G2 proteins (Keegan and Collett, 1986) indicates that both envelope glycoproteins may be involved in attachment, either directly or by conformational requirements (Schmaljohn and Patterson, 1990).

The neutralization studies undertaken here have revealed that some MAbs were unable to inhibit RVFV infectivity once virus had already attached to cells. This suggests that these particular antibodies may effect neutralization by either preventing virus from binding to cells or by inhibiting the adsorbed virus from entering the cell. To further identify the stage of the virus cycle interrupted by the neutralizing antibodies, the effect of the MAbs on virus binding and internalization will thus be examined. An investigation of this nature may also help to identify the site(s) on the RVFV G1 and G2 glycoproteins which are involved in virus attachment.

6.2 Materials and Methods

6.2.1 Preparation of purified radiolabelled virus

[^{35}S] Methionine-labelled virus was prepared as outlined below. Vero cell monolayers in 75 cm^3 flasks were infected with RVFV (TC3) at a multiplicity of infection (m.o.i.) of 10. After adsorption of virus for 1 h at 37°C, 10 ml of serum-free EMEM was added to each flask for a further 2 h at 37°C. The medium was then removed and replaced with 10 ml of methionine-free EMEM (Flow Laboratories, Irvine, U.K.). Following incubation for 1 h at 37°C, [^{35}S]

methionine (sp.act 1000 Ci mmol, Amersham International, U.K.) was added to give a final concentration of 50 μCi/ml. Virus was harvested at 48 h post-infection and clarified by centrifugation at 7000 g for 30 min at 4°C in a Beckman J2-21 centrifuge.

Radiolabelled virus was purified based on the method of Kingsford and Hill (1983) with the exception that DMSO was not included in the gradient. Clarified virus (20 ml) was layered onto a discontinuous sucrose gradient comprising 20% (w/v) sucrose in 0,01 M Tris, 0,1 mM EDTA and a 65% sucrose cushion in the same buffer. The tubes were spun for 2½ h at 26 000 rpm at 4°C in a SW 28 rotor in a Beckman L8-60 M ultracentrifuge. Fractions of 0,5 ml from the sucrose-sucrose interphase were harvested and aliquots assayed for infectivity by titration in 96 well microtitre plates as described in 2.2.1.1. Infectious fractions with the same titres were pooled, aliquoted and stored at -70°C. Prior to use in the attachment assays, one amp from each batch of labelled virus was thawed in order to determine the PFU/ml by means of the plaque assay as described in section 2.2.3.3f, using 24 well plates.

6.2.2 Efficiency of virus attachment

Several control experiments were initially carried out in parallel to assess the efficiency of virus attachment. In the first instance, virus inoculated cells were incubated for 6 days to check for CPE as evidence for productive infection. Purified [^{35}S] methionine-labelled virus (6,5 x 10^7 PFU/ml) was diluted 1:20, 40, 100 and 500 in PBS-$MgCl_2$ and chilled on ice for 15 min. Aliquots of 0,4 ml were then added in triplicate to confluent Vero monolayers (1 x 10^6 cells/well) in 6 well plates (Cel-Cult, Sterilin Ltd., Hounslow, U.K.). Following adsorption for 2 h at 4°C with gentle rocking, the inoculum was removed, the cells washed and medium replaced with Leibovitz containing 5% FCS and antibiotics (2.2.1.1). The plates were incubated at 37°C for 6 days, then read for CPE.

Secondly, the amount of infectious virus which had not bound to the cells was examined by titrating the supernatant inoculum after a 2 h adsorption period. Labelled virus at the same concentrations as above was adsorbed to cell monolayers for 2 h at 4°C. The inoculum was removed and used for titration in 96 well microtitre plates as described in section 2.2.1.1. The cells were washed three times with cold PBS and lysed with 1 ml of 1% SDS in distilled water. Cell lysates were transferred to liquid scintillation vials containing 5 ml of scintillation fluid (Ultima-Gold XR, Packard Instruments, U.S.A.). Radioactivity of these samples was determined by liquid scintillation spectrometry using an LKB 1217 Rackbeta liquid scintillation counter.

6.2.3 Inhibition of virus attachment

The effect of MAbs on virus attachment was determined based on the procedure of Dietzschold *et al* (1987) with some modifications. Purified RVFV was preincubated with various dilutions of MAb ascitic fluids for 2 h at 37°C. The final concentration of virus was 5 x 10^6 PFU/ml. The mixtures were then chilled on ice prior to addition to the cells. Confluent Vero monolayers in 6 well plates were washed three times with cold PBS and incubated with 1 ml/well of 1% ovalbumin (Sigma Chemicals) in PBS for 15 min at 4°C to prevent non-specific binding. The ovalbumin was then removed and aliquots (0,4 ml containing 2 x 10^6 PFU with an activity of 10 000-15 000 c.p.m.) of the virus-antibody mixtures were added to the monolayers. Following incubation for 2 h at 4°C on ice with gentle rocking, the cells were washed, lysed and the cell-associated radioactivity determined as in 6.2.2.

The fraction of input counts for each sample of virus specifically bound to cells in the presence of antibody was then compared with the average amount bound when no antibody treatment was given. As a negative control, a MAb directed against West Nile virus (Besselaar and Blackburn, 1988) was used. RVFV hyperimmune ascitic fluid was included as a control for polyclonal immune IgG.

6.2.4 Effect of temperature on virus binding

Confluent monolayers of cells in three sets of six well plates were inoculated with the same concentrations of virus-antibody mixtures as in 6.2.3. One set of plates was incubated at 4°C on ice for 2 h with gentle rocking. The remaining plates were incubated in parallel at rt under the same conditions. The cells from the plates kept at 4°C and one of the sets held at rt were washed, lysed and the bound radioactivity determined as outlined above. The remaining plates were used to test for neutralization of virus infectivity by the PRNT method as described in section 2.2.2.3f.

6.2.5 Distinguishing between cell bound and internalized virus by proteolytic digestion

The method used to distinguish between virions adherent to the cell surface and endocytosed virus was modified from that of Gollins and Porterfield (1984) using the proteolytic enzyme proteinase K. Binding and internalization assays were all done using Vero cell suspensions (2 x 10^6 cells/ml) in sterile centrifuge tubes (Falcon, Bector Dickinson & Co, New Jersey, U.S.A.). Binding studies were carried out in a cold room, while internalization assays were performed in a 37°C incubator. Leibovitz medium containing 0,5% BSA, pH 7,0, was used as the assay medium.

a) Determination of total cell-associated radioactivity

To determine the binding of labelled virus to cells in the absence of the proteolytic enzyme, 0,5 ml of two different titred batches of [^{35}S] methionine-labelled purified RVFV (6.2.1) was added to 0,5 ml volumes of the cell suspensions to yield a m.o.i. of 2 and 0,02 respectively. The tubes were incubated at 4°C with gentle shaking for 2 h, and the cells were then washed using the following standard procedure: 3 ml of assay medium was added to each tube and the cells pelleted at 1000 rpm for 6 min in a refrigerated centrifuge

(Beckman J-6B). The cells were then washed twice more with 2 ml assay medium.

For determining cell-associated radioactive c.p.m., the final cell pellet was resuspended in 0,5 ml PBS and added to scintillation vials containing 0,1 ml 10% SDS in distilled water. Five ml of Ultima Gold XR scintillation fluid was then added and the radioactivity determined as in 6.2.2 to give a value for total virus-specific radioactivity (i.e. protease-resistant + protease-removable c.p.m.).

b) Determination of protease-resistant radioactivity

To determine irreversibly cell-bound virus, the protease-resistant radioactivity associated with cells was measured by means of virus binding assays followed by treatment with proteinase K. These experiments were carried out in parallel with the above virus attachment assays in which no enzyme was added. Virus was allowed to adsorb to the cells as above. The effect of the MAbs was tested by preincubating the virus with hybridoma ascitic fluids at a final dilution of 1:100 for 2 h at 37°C. The virus-antibody mixtures were then chilled on ice for 15 min prior to adding to the cell suspensions for 2 h at 4°C. Following washing of the cells by the standard procedure, the cells were resuspended in 5 ml PBS and 0,125 ml of a stock solution of proteinase K (Boehringer, Mannheim, Germany) in PBS added. The protease stock solution was prepared fresh prior to use, at a concentration of 0,5 mg/ml. The mixture was then incubated for 40 min on ice with constant gentle shaking. The cells were washed once in PBS containing 10% horse serum and twice in PBS with 0,2% BSA. The final cell pellet was lysed with 10% SDS as above and the c.p.m. determined. This was taken to represent the protease-resistant radioactivity, which in turn was considered to reflect either irreversibly cell-bound virus or endocytosed virus. Two replicates were included for each experimental point and all experiments were performed at least twice. With scintillation determinations, the average c.p.m. was calculated for replicate vials.

6.2.6 Effect of MAbs on virus internalization

The possible inhibition of virus internalization by MAbs was examined by shifting the cell-attached virus to 37°C to allow substantial penetration to occur prior to protease degradation.

Synchronized internalization of RVFV at 37°C was performed by prebinding labelled virus to Vero cells at 4°C in the presence or absence of the MAbs under identical conditions as in 6.2.5b, with the exception that the cell suspensions were shifted to 37°C for the second hour. Following the temperature shift, the cells were washed and proteinase K added as described earlier to remove any bound virus that had not yet been endocytosed. The virus-specific counts bound in the absence of antibody were taken to represent 100% internalization of the virus, and the counts obtained with the antibody-treated virus were calculated relative to this.

To control for any possible inhibition of binding, the proportion of virus bound in the presence of the MAbs after 2 h at 4°C without enzyme treatment was determined in parallel to the internalization assays.

6.3 Results

6.3.1 Efficiency of virus attachment

Monolayers which had been infected with purified labelled virus and incubated for 6 days at 37°C showed CPE at all the virus concentrations used. The fact that productive infection had occurred suggested that the virus had attached efficiently.

The results of the titration of virus present in the supernatant inoculum following the 2 h adsorption period are shown in Table 22. The extremely low titres of infectious virus remaining in the supernatant after adsorption confirmed the above findings that the majority of the added virus had bound to the cells at this time.

Table 22. Titre of infectious RVFV remaining in the supernatant following virus adsorption to cells for 2 h at 4°C

Virus concentration in inoculum[1]	Titre of infectious virus in supernatant[2]	Cell-associated radioactivity (c.p.m.)[3]
10×10^5	$2,5 \times 10^1$	24 240
5×10^5	$2,5 \times 10^1$	10 746
2×10^5	$1,0 \times 10^1$	4 500
$0,4 \times 10^5$	-	786

[1] Concentration of [^{35}S] methionine labelled RVFV added per 10^6 cells

[2] Titre of infectious RVFV remaining in the supernatant after adsorption of virus to cells for 2 h at 4°C

[3] Radioactivity associated with the cells after removal of the inoculum

6.3.2 Effect of MAbs on virus attachment

Initial experiments were performed using radiolabelled RVFV preincubated with the West Nile virus MAb at concentrations of 1:4 to 1:500 of the hybridoma ascitic fluid to determine if any non-specific inhibition of virus attachment occurred with non-immune antibody. Table 23 shows the percent inhibition by this negative control, as well as by the RVFV polyclonal HAF. Non-specific inhibition of virus binding by the unrelated MAb was observed at a concentration of 1:50 and below. At high concentrations the RVF HAF prevented virus adsorption substantially but at a 1:500 dilution there was only an 18% reduction in bound virus. In the presence of 20% FCS, the amount of bound virus was reduced by 52%.

Table 23. Effect of different concentrations of immune and non-immune antibody on the attachment of RVFV to Vero cells

Antibody[1] dilution	% Inhibition of virus [2] attachment	
	WN MAb	RVF HAF[3]
1:5	63	70
1:10	56	68
1:20	49	65
1:50	30	42
1:100	0	22
1:500	0	18
1:1000	0	7

[1] Ascitic fluid

[2] Purified [^{35}S] methionine labelled RVFV (5×10^6 PFU/ml with an activity of $\pm$15 000 c.p.m per well)

[3] RVFV polyclonal hyperimmune ascitic fluid

Based on these results, the effect of the RVFV MAbs on virus attachment was examined using the hybridoma ascitic fluids at a final dilution of 1:100. Some of the MAbs were also tested at a final dilution of 1:500. All the MAbs exhibited negligible inhibitory effects on virus binding when the virus was used at 5×10^6 PFU/ml (Table 24). Since this corresponded to a virus concentration $\pm$ 6×10^3 times greater than that used for the neutralization tests, attachment assays with lower titred virus (1500 PFU/ml) were subsequently performed to determine if the MAbs could prevent virus binding under these circumstances. The results showed that even at low virus concentration, none of the MAbs were able to significantly inhibit attachment (Table 24). The PRNTs performed concurrently nevertheless confirmed that the antibodies were neutralizing the virus.

Table 24. Effect of neutralizing MAbs on attachment of RVFV at a high and low multiplicity of infection

MAb[1]	Antigenic domain	% inhibition of attachment		% neutralization (PRNT)[4]
		High m.o.i.[2]	Low m.o.i.[3]	
Virus only	-	0	0	0
3E5	G1 Ia	0	2	99,0
3H1	G1 IIb	1	1	98,7
4B3	G1 IIc	8	9	96,5
5A1	G1 IId	12	11	96,5
9E4	G1 IIe	5	10	96,3
5E9	G1 IV	3	8	98,7
1E4	G1 ?	0	8	95,8
8G10	G1 ?	5	7	98,0
9C4	G2 Ia	6	5	99,9
8G2	G2 Ib	2	5	93,8
7F2	G2 Ic	3	4	97,0
Controls:				
5E11	G1 IIIa	nt[5]	25	< 50,0
8A3	G1 IIIb	0	0	< 50,0
8C2	G2 Id	2	8	< 50,0
4D10	G2 III	nt	26	< 50,0
8G10 + C'	G1 ?	77	71	99,3
RVFV HAF	-	18	22	99,9
WN MAb	-	0	0	0

[1] MAbs used at 1:100
[2] Purified labelled RVFV (5 x 10^6 PFU/ml)
Purified labelled RVFV (1500 PFU/ml)
[4] PRNT performed in parallel to attachment assays with RVFV at low m.o.i.
[5] Not tested

6.3.3 Effect of temperature on virus attachment

With the lower titred virus, the bound radioactivity measured at 4°C was relatively low (± 6000 c.p.m. per well). Attachment studies were therefore also carried out in parallel at rt in order to increase the efficiency of virus binding. At room temperature, the radioactivity specifically bound to the cells in the absence of antibody approximately doubled compared to that obtained for virus binding at 4°C. No significant blocking of virus attachment by the MAbs was observed in these experiments.

6.3.4 Effect of MAbs on stability of virus binding at 4°C

To distinguish between virions adherent to the cell surface and endocytosed virus, inoculated cell cultures were treated with the proteolytic enzyme proteinase K to remove putatively attached virus. By this means it was possible firstly to determine if preincubation of RVFV with the MAbs at 4°C influenced the stability of virus binding to the cell surface. Secondly, by shifting the inoculated cultures to 37°C, it was possible to examine if antibody-coated bound virus was prevented from penetrating the cell (6.3.5).

To determine the optimum concentration of protease to use for the removal of cell bound virus, two different concentrations of proteinase K were initially tested (Table 25). In the absence of antibody, both concentrations of enzyme removed approximately 81% of the c.p.m. bound to the cells which had been incubated with labelled virus at 4°C for 2 h. The remaining c.p.m. (19%) were considered irreversibly bound and probably represented virus which had fused to the cell surfaces and was no longer susceptible to the enzymatic activity of the proteinase K. As proteinase K concentrations greater than 0,5 mg/ml are reportedly toxic to cells (Gollins and Porterfield, 1984), the protease was used at 0,5 mg/ml in subsequent experiments to avoid any possible detrimental effects on the cultures.

Table 25. Percentage irreversibly bound virus to cells after digestion with different concentrations of the proteinase K enzyme

Input virus[1] (m.o.i.)	% binding[2] at 4°C		
	No PK[3]	+ PK (0,5 mg/ml)	+ PK (0,25 mg/ml)
0,02	100	19,1	18
2,0	100	18,4	nd

[1] Purified [^{35}S] methionine-labelled RVFV
[2] Based on cell-associated radioactivity
[3] Proteinase K

In the presence of RVF hyperimmune ascitic fluid, a greater proportion of cell-bound virus was removed by protease digestion relative to the virus without antibody (Table 26). In contrast, preincubation of the virus with MAbs 8A3 (G1 IIIb) and 8C2 (G2 Id), which served as controls for non-neutralizing antibodies in the absence of C′, had no effect on the amount of virus that remained cell bound.

In the case of the neutralizing G1-specific MAbs 3E5 (G1 Ia), 3H1 (G1 IIb), 4B3 (G1 IIc) and 9E4 (G1 IIe), approximately the same proportion of antibody-treated virus was removed from the cell surface as uncoated virus. In the presence of the other neutralizing G1-reactive MAbs (5A1, 5E9, 1E4, 8G10) and the MAbs defining the neutralizing G2 1 domain (9C4, 8G2, 7F2), on the other hand, more cell-bound virus was removed compared to the untreated virus as shown by the reduction in cell-associated radioactivity.

Table 26. Percentage irreversibly bound virus after 2 h adsorption to cells at 4°C and proteolytic digestion with proteinase K

Antibody	Antigenic domain	% irreversibly bound virus	
Virus[1] only (-PK)[2]		100[3]	Na[4]
Virus only(+PK)		19	100[5]
3E5	G1 Ia	19,4	102
3H1	G1 IIb	18,2	96
4B3	G1 IIc	17,9	94
5A1	GI IId	14,9	78
9E4	G1 IIe	18,0	95
8A3	G1 IIIb	19,7	104
5E9	G1 IV	9,9	55
1E4	G1?	9,4	49
8G10	G1?	11,0	58
9C4	G2 Ia	11,4	60
8G2	G2 Ib	10,3	54
7F2	G2 Ic	12,0	63
8C2	G2 Id	19,7	103
RVFV HAF	-	12,8	67

[1] Input virus = purified [^{35}S] methionine labelled RVFV (3 x 10^3PFU/ml)

[2] Proteinase K

[3] Virus specific counts in the absence of protease were taken to represent 100% attached virus and the counts obtained with the protease-digested virus were calculated relative to this

[4] Not applicable

[5] The non-neutralized virus-specific counts after protease treatment were taken to represent 100% irreversibly bound virus and the counts obtained with the antibody-treated virus were calculated relative to this

6.3.5 Effect of neutralizing MAbs on virus internalization at 37°C

Synchronized internalization of RVFV at 37°C was performed by prebinding labelled virus to Vero cells at 4°C in the presence or absence of the MAbs, then shifting the cell-bound virus to 37°C for 1 h. This was followed by digestion with proteinase K to remove any remaining putatively attached virus, thereby permitting the exclusive determination of penetrated virus only. For the purposes of comparison of antibody-coated virus with untreated virus, the total amount of radioactivity internalized for virus prebound in the absence of antibody was taken to represent 100% penetration.

The data for the binding assays (2 h at 4°C without enzyme digestion) which were carried out in parallel to the internalization studies are shown in Table 27. The results were similar to those obtained for virus attachment to cell monolayers (6.3.1) and confirmed that none of the MAbs significantly prevented binding.

The effect of the MAbs on the internalization of RVFV is also shown in Table 27. Premixing the virus with the polyclonal hyperimmune ascitic fluid prevented 18% of the virus from binding and resulted in an overall reduction of 29% internalized virus. This indicated that a proportion of the virus that managed to attach was prevented from penetrating the cell. Preincubation of the virus with the control MAbs 8A3 and 8C2, both of which are non-neutralizing without C′, had no affect on the amount of internalized radioactivity.

In contrast to the non-neutralizing MAbs, the amount of internalized virus-specific radioactivity was reduced considerably when virus was treated with the anti-G1 neutralizing MAbs 5A1, 1E4 and 8G10, and the G2-specific MAbs 9C4, 8G2 and 7F2. In these cases, therefore, a substantial proportion of the attached virus was prevented from entering the cell. Preincubation of the virus with the neutralizing

Table 27. Effect of neutralizing MAbs on virus internalization at 37°C, determined after protease removal of exposed virus

Antibody	Antigenic domain	% virus-specific counts at time after shift to 37°C	
		0 min (-PK)[1]	60 min (+PK)[2]
Virus[3] only	-	100[4]	100[5]
3E5	G1 Ia	104	102
3H1	G1 IIb	102	98
4B3	G1 IIc	98	94
5A1	G1 IId	86	66
9E4	G1 IIe	98	95
8A3	G1 IIIb	103	105
5E9	G1 IV	103	87
1E4	G1 ?	105	60
8G10	G1 ?	94	64
9C4	G2 Ia	103	60
8G2	G2 Ib	97	68
7F2	G2 Ib	96	70
8C2	G2 Ic	104	102
RVFV HAF	-	82	71

[1] No proteinase K digestion

[2] Proteinase K digestion after temperature shift

[3] Input virus = purified [^{35}S] methionine labelled RVFV (3 x 10^3PFU/ml)

[4] The non-neutralized virus-specific counts at 4°C were taken to represent 100% binding to cell and the counts obtained with the antibody-treated virus were calculated relative to this

[5] The non-neutralized virus-specific counts at 37°C were taken to represent 100% penetration and the counts obtained with the antibody-treated virus were calculated relative to this

MAbs binding to the G1 domains I and II (3E5, 3H1, 4B3, 9E4), on the other hand, did not affect the percentage of internalized virus-specific counts, indicating that virus penetration was not being blocked by these antibodies.

6.4 Discussion

Prior to examining the possible inhibition of virus attachment by the RVFV MAbs, various experimental parameters were initially assessed. The concentrations of both antibody and virus suitable for use in the binding studies were investigated, as well as the efficiency of virus attachment. As the inhibition of RVFV infectivity by neutralization mechanisms independent of C′ were of particular interest here, the attachment assays were performed using heat-inactivated antibodies.

Firstly, since immunoglobulin at high concentrations may interfere non-specifically with virus binding as has been demonstrated using non-immune antibody (Fuller and Spear, 1985; Kingsford *et al*, 1991) it was important to establish the concentration of antibody which would reflect only specific inhibition of attachment. The resulting data revealed that non-specific reduction in virus binding did indeed occur at high antibody concentration, thereby necessitating using a minimum dilution of 1:100 of hybridoma ascitic fluid.

Another important aspect of antibody concentration is that the density of neutralizing antibodies on the virus surface may influence virus attachment (reviewed by Outlaw and Dimmock, 1991). It has, for example, been shown for influenza virus that higher concentrations of IgG result in a closer packing of IgG molecules on the virus surface, thereby reducing the mobility of the antibodies which is necessary to prevent virus attachment to cells (Armstrong *et al*, 1990; Taylor and Dimmock, 1985). The relatively small decrease in the proportion of bound virus which was seen with the RVF polyclonal hyperimmune ascitic fluid at a 1:100 dilution was unlikely to have been due to too high a density of

antibodies on the virus surface, however, since lower antibody concentrations did not have an increased inhibitory effect.

Unlike the situation with the immune antibodies, virus attachment was considerably reduced in the presence of a high concentration of FCS. A similar phenomenon has been reported by Fassi-Fihri *et al* (1990) for concentrations of FCS of 5% or greater. The decreased binding in the presence of high serum concentrations might be due to the formation of large viral aggregates which are more easily removed from the cell surface during the washing procedure than single viral particles.

With regard to the optimum virus concentration, virus attachment is traditionally examined using high multiplicities of infection. In the present study, no inhibition of virus attachment by the heat-inactivated MAbs was observed using a high input multiplicity of virus. Since this was possibly due to the fact that the latter virus concentration was far greater than that used in the PRNTs, attachment assays were also carried out with lower titred virus. In these experiments, virus binding was shown to be highly efficient as indicated by the low level of infectious virus remaining unattached after the adsorption period, as well as by the subsequent productive infection of the inoculated cell monolayers.

With the lower virus concentration, inhibition of attachment by the MAbs was still negligible, even though virus infectivity was neutralized by 94 to 99,9%. Despite their large size, the two IgM MAbs 1E4 and 8G10 did not reduce the binding of neutralized virus, suggesting that the prevention of virus attachment by steric hindrance does not play an essential role in the neutralization mechanisms involved in these particular instances.

In contrast to the lack of inhibition which occurred with MAb 8G10 in the absence of C', virus attachment was reduced significantly when the virus was

mixed with this antibody with added C′. The inhibitory effect in these circumstances may have been due to aggregation of the antibody-coated virions (sensitized virus), resulting in a reduction in the number of infectious units (Oldstone *et al*, 1974). Alternatively, the C′ may have 'blanketed' or coated the sensitized virus, thereby interfering with binding to the cellular receptors (Beebe *et al*, 1983).

Unlike the situation with the neutralizing MAbs, RVFV attachment was blocked to a certain degree by two of the MAbs which are non-neutralizing in the absence of C′ under normal conditions of PRNT assays i.e. 5E11 (G1 IIIa) and 4D10 (G2 III). Several non-neutralizing MAbs directed against the LACV G1 protein have also been found to reduce virus binding (Kingsford *et al*, 1991). This phenomenon may be due to lack of stability between the antibody-coated virus and the cell receptor site, and thus removal of the treated virus by the extensive washing procedures used in the assay.

The fact that the neutralizing MAbs specific for the G1 domains I, II and IV and the G2 I domain inhibit virus haemagglutination, yet do not prevent binding of virus, strongly suggests that the epitopes they define are spatially separate from the site(s) responsible for virus attachment to the cellular receptor. These antibodies thus must neutralize by mechanisms other than inhibition of attachment. As one of the possible mechanisms may be the prevention of virus entry into the cell, it was important to determine whether or not the neutralized virus was being internalized. This was measured by resistance of the virus to removal from the cells by the proteolytic enzyme proteinase K.

The resulting data revealed that certain of the neutralizing MAbs directed against both RVFV envelope proteins prevented virus internalization substantially. The fact that these particular MAbs also caused more of the putatively attached virus at 4°C to be proteolytically removed from the cell surface than uncoated virus

suggests that they interfere with the stability of virus binding. Thus the apparent interference by these MAbs with the internalization of virus at 37°C may partly be due to the inability of the virus to bind as firmly to the cell surface as it would in the absence of such antibodies.

While all the neutralizing MAbs directed against the G2 I domain (9C4, 8G2, 7F2) exhibited this type of inhibition, the G1-specific MAbs that prevented virus entry mapped to various different sites i.e. G1 IId, GI IV and the unmapped epitopes recognized by MAbs 1E4 and 8G10. The fact that only one of the five MAbs defining the G1 II domain interfered with virus internalization while the others had no affect, supports the earlier post-adsorption findings that the MAbs binding to this domain neutralize by different mechanisms.

In all cases, however, virus internalization by these MAbs was not totally prevented. The fact that the remaining bound virus entered the cell, and yet was still almost completely neutralized, indicates that blocking of virus infectivity must also occur at another stage of the virus life-cycle.

Of some interest was the finding that RVFV antibodies of both the IgM and IgG isotype appeared to neutralize in a similar fashion i.e. by inhibiting internalization of a proportion of the attached virus. The situation here is thus different to that reported for influenza virus where the mechanism of neutralization appears to be partly dependent on the isotype of antibody (Armstrong *et al*, 1990; Dimmock, 1984; 1987; Taylor and Dimmock, 1985).

In contrast to the various MAbs which substantially blocked virus uptake into the cell, with the exception of 5A1, all the MAbs directed against the neutralizing epitopes comprising the G1 I and G1 II domains had no influence on virus internalization, indicating that the inhibition of virus infectivity must be effected at an intracellular stage of viral replication. Neutralization of this nature may be

due to conformational changes induced by antibody binding which prevent the virus from undergoing a subsequent replication step.

Thus, although the RVFV G1 and G2 antigenic sites involved in virus attachment could not be identified with these particular MAbs, the data from the binding and internalization assays nevertheless have helped to elucidate the mechanisms of antibody-mediated neutralization of the virus. The results indicate that while one of the mechanisms involves the inhibition of virus internalization, additional modes of action must involve some step(s) later in the virus infectious cycle such as interference with the rate of virus penetration or neutralization of virus after penetration. As the virion-cell fusion event leading to the release of the viral genome may be particularly sensitive to inhibition by neutralizing MAbs (Fuller and Spear, 1987) the sensitivity of the MAbs to pH-induced conformational changes in the RVFV glycoproteins also requires investigation.

7. PENETRATION

7.1 Introduction

As with all viruses, infection of host cells by RVFV is largely dependent on the ability of virus to penetrate the cell and release the genome into the cytoplasm (Choppin and Scheid, 1980; Dimmock, 1982; Lenard and Miller, 1982; Marsh and Helenius, 1980; 1989; Schmaljohn and Patterson, 1990; White *et al*, 1983). With enveloped viruses, this process occurs by fusion of the virus and lipid membranes of the target cells, induced by the viral envelope proteins (Marsh, 1984; Marsh and Helenius, 1980; 1989; White *et al*; 1981; 1983).

Depending on the pH threshold, the membrane fusion event may either occur on the plasma membrane, or in the acidic vacuoles of the endocytic pathway (Lenard and Miller, 1982; White, 1990; White *et al*, 1983.) In the latter case, the mildly acidic pH (pH 5,0 - 6,0) in the endosomal compartment (Tycko and Maxfield, 1982) triggers a conformational change in the glycoproteins on the virion surface, causing exposure of a hydrophobic portion of a virion surface protein (Subbarao *et al*, 1987; reviewed in White, 1990). This hydrophobic region promotes fusion between the virion envelope and the vesicle membrane (Marsh, 1984; Matlin *et al*, 1981; White, 1990; White *et al*, 1981; 1983). Such acid-induced changes in the tertiary structure of the envelope proteins are believed to be an important part of the mechanism by which the viral nucleocapsid is discharged into cell cytoplasm, thereby initiating the process of replication (Lenard and Miller, 1982; Marsh, 1984; Marsh and Helenius, 1989; Matlin *et al*, 1981; White *et al*, 1981; 1983).

With regard to RVFV, very little is currently known about the actual mechanisms by which the virus gains access to the cell cytoplasm (Ellis *et al*, 1988; Schmaljohn and Patterson, 1990). Studies on some of the other *Bunyaviridae* have shown that at acidic pH values, the envelope glycoproteins undergo a

conformational change and induce cell fusion (Arikawa *et al*, 1985; Gonzalez-Scarano, 1985; Gonzalez-Scarano *et al*, 1984; Wang *et al*, 1991). This pH-dependent membrane fusion suggests that they rely on endocytic uptake and that their fusion activity is triggered by the low pH of the intracellular vesicles (Gonzalez-Scarano *et al*, 1984; Marsh, 1984; Marsh and Helenius, 1980; 1989). Direct evidence for this process with RVFV, however, has not yet been obtained (Schmaljohn and Patterson, 1990). It is furthermore unclear at this stage whether one or both of the envelope glycoproteins are necessary for fusion.

As a step towards the understanding of the fusion mechanism of RVFV, the MAbs will be used to investigate the sensitivity of the viral glycoprotein epitopes to pH-induced conformational changes. Since the fusion event presents a point at which penetration can be blocked (Dietzschold *et al*, 1987; Gollins and Porterfield, 1986a; Marsh and Helenius, 1989) these studies may also indirectly help to ascertain if any of the MAbs which appear to neutralize intracellularly do so by interfering with this step.

The route of RVFV entry and penetration will be examined further by testing the possible inhibition of viral uncoating by the lysosomotropic agent ammonium chloride which acts to raise endosomal pH. In addition, the kinetics of resistance of penetrated virus to neutralization by the MAbs will also be investigated.

7.2 Materials and Methods

7.2.1 MAb reactivity with antigen exposed to different pH's

Analysis of the antigenic reactivity of the MAbs with virus preparations exposed to different pH's was carried out using the method of Guirakhoo *et al* (1989). Stock RVFV extracted glycoproteins (17 mg/ml; section 2.2.1.2) were diluted 1:100 in TAN buffer (0,5 M triethanolamine, 0,1 M NaCl) which had been previously adjusted to pH's ranging from 7,5 to 4,0 using HCl. After incubation at rt for 30 min, the pH's of the antigen samples were all readjusted to pH 8,0

with 1,0 M NaOH. The samples were then diluted in carbonate-bicarbonate ELISA buffer (pH 9,6) to yield a final concentration of 80 μg/ml. Volumes of 0,1 ml were added to wells of ELISA microtitre plates and incubated overnight at 4°C. The plates were washed and blocked with 4% BSA as described in section 2.2.2.3a. Reactivity of the MAbs with the acidified antigens was determined by adding 0,1 ml aliquots of each MAb (diluted 1:200 in ELISA diluent) to duplicate wells against each of the different pH-treated antigens. After incubation for 2 h at 37°C, the wells were washed and incubated with peroxidase conjugated anti-mouse immunoglobulins (2.2.2.3a) for a further hour at 37°C. The colour reaction was measured at 405 nm following the addition of ABTS substrate. The unmapped G1-specific MAbs 1E4, 8G10, 5F2, 5A6 and G2-specific MAb 5C12 were not tested due to their very weak avidities in the ELISA.

7.2.2 Determination of virus uptake and uncoating

The uncoating of virus was determined by measuring the appearance of acid-soluble material in supernatant fluids following shift of infected cells from 4°C to 37°C based on the method of Kennedy-Stoskopf and Narayan (1986). The possible inhibition of virus uncoating by raising endosomal pH was examined using ammonium chloride at a final concentration of 0,05 M as described by Gollins and Porterfield (1984).

Confluent monolayers of Vero cells in 6-well tissue culture plates (Cel-Cult; Sterilin Ltd, Hounslow, U.K.) were incubated with 0,4 ml [^{35}S] methionine-labelled RVFV (250 PFU/ml; 15 x 10^3 c.p.m./ml) for 2 h at 4°C. Cultures were then washed three times with cold PBS and replenished with 0,5 ml of Leibovitz medium (pH 8,0) in the presence or absence of 0,05 M ammonium chloride. The plates were shifted to 37°C for various incubation times. At each stage, the tissue culture supernatants were collected and the cells washed with PBS, then lysed with 1 ml of 1% SDS. The cell lysates were transferred to scintillation vials containing 5 ml Ultima-Gold XR and analysed by liquid scintillation spectrometry.

To determine the acid-soluble radioactive material, 0,1 ml of the collected tissue culture supernatant was precipitated with perchloric acid. This was carried out by adding 1 ml of 0,01 M sodium pyrophosphate and 1 ml of 3,5% perchloric acid in 0,01 M sodium pyrophosphate sequentially to each tissue culture supernatant aliquot at 4°C. The samples were mixed by vortexing and incubated on ice for 30 to 50 min. The precipitates were collected by vacuum on glass-fibre filters using a Millipore sampling manifold unit. The precipitates were rinsed with 5 ml of 0,01 M sodium pyrophosphate and 0,1 M HCl, then with 95% ethanol to facilitate drying. Filters were dried and placed in scintillation vials to which 3 ml of Ultima-Gold XR was added. Counts quantified in the liquid scintillation counter represented acid-precipitable radioactivity. Acid-soluble radioactivity was determined by subtracting acid-precipitable c.p.m. from the total radioactivity of a 0,1 ml sample in liquid scintillation fluid. All experiments were performed in duplicate.

7.2.3 Neutralization of penetrated virus

The method used to determine whether the MAbs mediated virus neutralization when virus had penetrated cells at various time intervals was based on that of Roehrig *et al* (1988) with some modifications. RVFV was diluted in Leibovitz medium and 0,4 ml (200 PFU) was adsorbed onto Vero cell monolayers in six well plates (Cel-Cult; Sterilin Ltd., Hounslow, UK) for 1 h on ice at 4°C. Each plate was independently shifted to 37°C for 1, 5, 10, 25, 45 and 60 min. After the appropriate time interval, the cells were shifted back to 4°C, washed and incubated in the presence of 0,4 ml of MAb diluted in Leibovitz medium for 15 min. Each MAb was tested at the antibody concentration corresponding to the 80% PRNT titre obtained in the post adsorption PRNTs (5.3.4).

The medium was removed and the cultures either overlaid directly with agarose as described in 2.2.3.3f, or were washed three times with PBS-$MgCl_2$ prior to the overlay. No antibody was added to the virus controls; instead, the extracellular

virus was inactivated with citrate buffer (0,04 M citric acid, 0,01 M KCl, 0,135 M NaCl, pH 3,0) for 1 min, followed by three washes with PBS-$MgCl_2$. The medium was removed and the cultures overlaid with agarose. The number of viral PFU surviving acid treatment after a 60 min time shift to 37°C was considered to represent 100% penetration, and the fraction of intracellular virus at any given time was calculated relative to this.

7.3 Results

7.3.1 MAb reactivity with RVFV exposed to low pH

The reactivities of the MAbs with RVFV glycoprotein antigens treated at different pH's as measured by the ELISA are depicted in Figs 7a to 7f. One of the anti-G1 MAbs (3E5) exhibited comparable levels of binding to acidified viral antigen compared to the untreated control antigen as indicated by the relatively constant O.D. levels over the entire pH range tested (Fig. 7a).

MAbs 5E1 (G1 Ib) and 5E11 (G1 IIa) showed a marked increase in binding to viral antigen exposed to pH values ranging from 6,5 to 5,5 relative to the untreated control (Figs 7a, 7c). Maximum binding occurred at pH 5,5. At pH 5,0, the increased binding was no longer evident, as reflected by the similar absorbance levels obtained at this pH compared to that at pH 7,5.

The remaining MAbs directed against the G1 protein showed a reduction in binding to antigen that had been exposed to pH values of 5,0 and lower (Figs 7a-c). The greatest reduction in reactivity occurred with MAb 9E4, corresponding to a 77% decrease in binding compared to that obtained with antigen at neutral pH. Over the mildly acidic pH range ie pH 5,5 and pH 6,0, however, there was only a slight reduction in the proportion of bound antibody. Unlike the situation with the G1 protein, the activity of the G2-specific MAbs was not affected substantially by low pH exposure of the virus (Figs 7d to 7f).

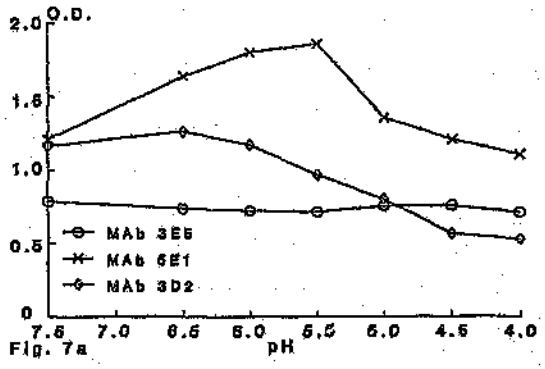


Fig. 7a

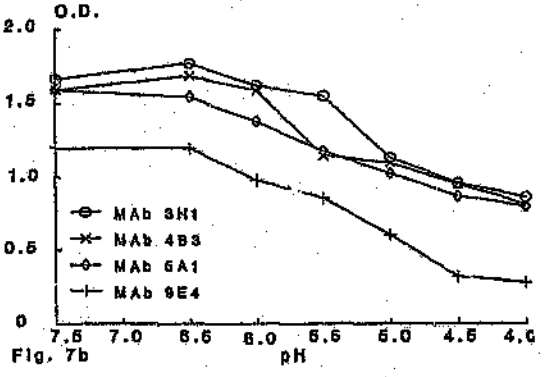


Fig. 7b

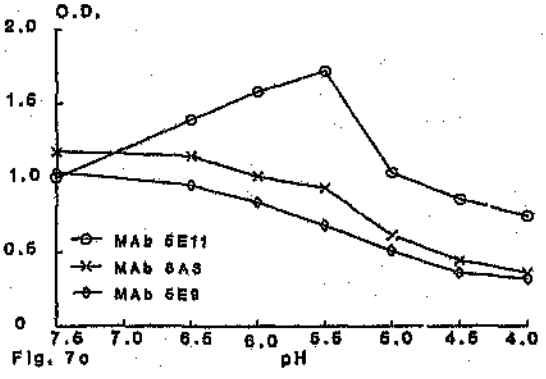


Fig. 7c

Fig. 7. MAb reactivity with RVFV glycoprotein antigens treated at different pHs as measured by the ELISA. Optical density (O.D.) was read at 405 nm.
(a) Reactivity of MAbs 3E5 (G1 Ia), 5E1 (G1 Ib) and 3D2 (G1 IIa).
(b) Reactivity of MAbs 3H1 (G1 IIb), 4B3 (G1 IIc), 5A1 (G1 IId) and 9E4 (G1 IIe).
(c) Reactivity of MAbs 5E11 (G1 IIIa), 8A3 (G1 IIIb) and 5E9 (G1 IV).

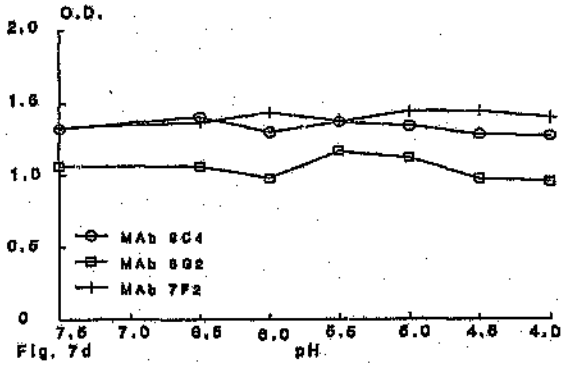


Fig. 7d

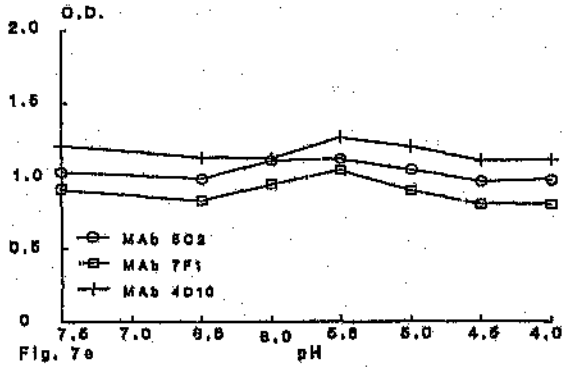


Fig. 7e

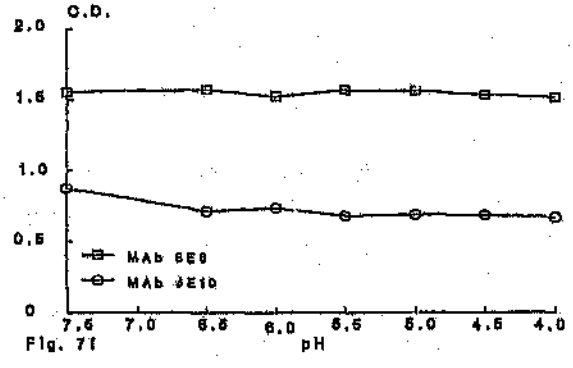


Fig. 7f

Fig. 7. MAb reactivity with RVFV glycoprotein antigens treated at different pHs as measured by the ELISA. **(d)** Reactivity of MAbs 9C4 (G2 Ia), 8G2 (G2 Ib) and 7F2 (G2 Ic). **(e)** Reactivity of MAbs 8C2 (G2 Id), 7F1 (G2 II) and 4D10 (G2 III). **(f)** Reactivity of MAbs 8E6 (G2 IV) and 6E10 (G2 ?).

The sensitivities of the individual glycoprotein epitopes to mildly acidic pH was examined by comparing the reactivities of all the MAbs with RVFV antigen exposed to pH 5,5. The latter pH was chosen as this firstly corresponds to the mid-pH value found within endosomes (Tycko and Maxfield, 1982) and secondly was the pH at which maximum changes in antibody reactivity with several G1 epitopes (G1 Ib and G1 IIIa) were seen. The percentage binding of the G1- and G2-specific MAbs to their corresponding epitopes which occurred on antigen exposure to pH 5,5 is shown in Figs 8a and 8b respectively. The maximum level of each antibody which reacted with antigen at pH 7,5 was taken to represent 100% binding, and the amount of antibody which bound to antigen treated at pH 5,5 was calculated relative to this.

The ɔ epitopes comprising the G1 I domain exhibited widely varying sensitivities to mildly acid pH as reflected by the different reactivities of the antibodies which defined these sites (Fig. 8a). While comparable levels of antibody attachment to G1 Ia occurred at both pH 5,5 and pH 7,5, enhanced binding corresponding to 153% was seen for epitope G1 Ib at the lower pH.

With regard to the G1 II domain, the G1 IIa and G1 IIb epitopes were relatively resistant to exposure to pH 5,5, while the remaining three determinants appeared to be slightly more sensitive as seen by the lowered reactivities of the antibodies recognizing these sites.

As in the case of the G1 I domain, at mildly low pH, increased antibody binding (172%) occurred for one of the G1 III epitopes (G1 IIIa), while the other determinant, G1 IIIb, was fairly resistant to pH treatment. The antigenic reactivity of the G1 IV domain was adversely affected at mildly acidic pH as indicated by the 35% reduction in bound MAb 5E9.

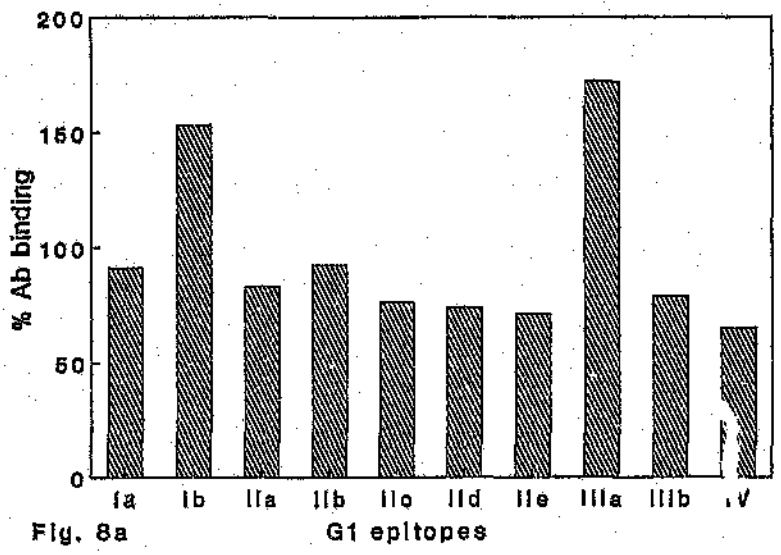


Fig. 8a

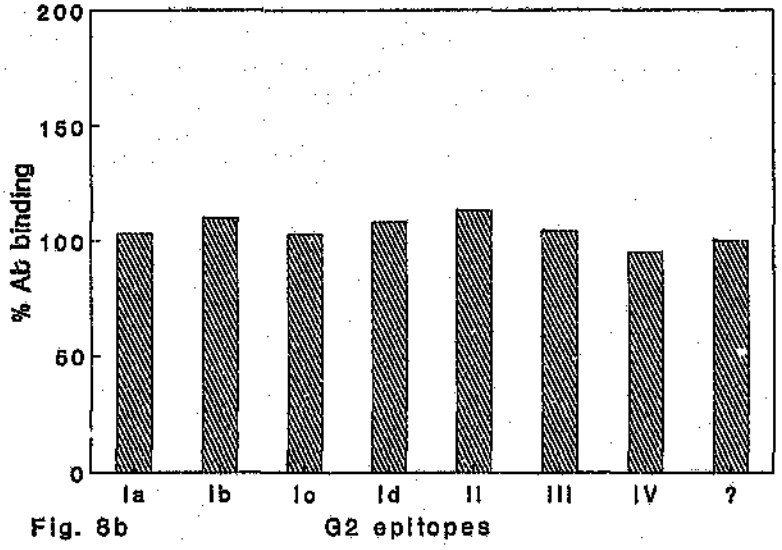


Fig. 8b

Fig. 8. Sensitivity of the RVFV glycoprotein epitopes to exposure to pH 5,5 as measured by the percentage binding of the respective MAbs. The maximum level of each antibody which reacted with antigen at pH 7,5 was taken to represent 100% binding, and the amount of antibody which bound to antigen treated at pH 5,5 was calculated relative to this. **(a)** Percentage binding of the G1-specific MAbs. **(b)** Percentage binding of the G2-specific MAbs.

With regard to the G2 domains, all the epitopes were relatively resistant to exposure to low pH as shown by the comparable antibody binding levels obtained with antigen at both pH values (Fig. 8b).

7.3.2 Effect of ammonium chloride on viral uncoating

In the absence of ammonium chloride, shift of the [^{35}S] methionine-labelled RVFV inoculated cells from 4°C to 37°C resulted in a steady decline in the amount of cell-associated radioactivity over the 2½ h period (Fig. 9a). The percentage of cell-associated c.p.m. measured at the 2½h shift interval in the presence of ammonium chloride, on the other hand, was much higher ie 74% compared to only 48% for the untreated RVFV.

Fig. 9b shows the rise in acid-soluble c.p.m. which occurred shortly after shift of the inoculated cells to 37°C. In the absence of ammonium chloride, there was a sharp increase in acid-soluble counts between a penetration time of 15 to 60 min, after which the c.p.m. levelled off. In the presence of ammonium chloride, however, the acid-soluble radioactivity measured in the supernatant at each given time interval was markedly lower than that of the control virus.

The results of the PRNTs performed in parallel to the uncoating experiments revealed a 79% reduction in the number of viral plaques formed when RVFV virus was treated with ammonium chloride compared to untreated virus.

7.3.3 Ability of MAbs to neutralize penetrated virus

The kinetics of penetration of RVFV in the absence of antibody were examined by infecting cells at 4°C, followed by a shift to 37°C to allow virus to penetrate cell membranes. Inactivation of the extracellular virus at different times after

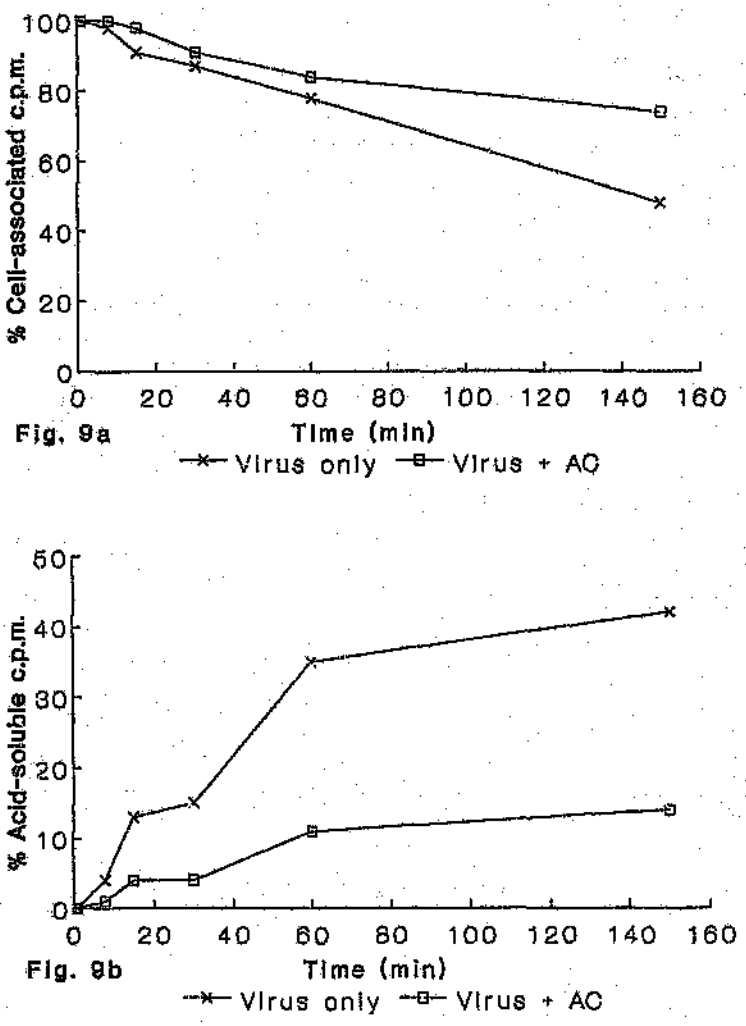


Fig. 9a

Fig. 9b

Fig. 9. Effect of raising endosomal pH with ammonium chloride (AC) on the fate of [^{35}S] methionine labelled RVFV after synchronized infection of Vero cells. Labelled RVFV (250 PFU/ml; 15 x 10^3 c.p.m./ml) was bound to cell monolayers for 2 h at 4°C. Cultures were washed with PBS and replenished with Leibovitz medium (pH 8,0) in the presence or absence of 0,05M ammonium chloride. The cells were shifted to 37°C for various times to allow virus penetration to occur. At each time interval, the cell-associated radioactivity and acid-soluble counts were measured. **(a)** The percentage radioactivity associated with the cells determined for each time point were 100% represents the total c.p.m. bound initially to the cells prior to virus internalization. **(b)** The acid-soluble radioactivity present in the supernatant determined by subtracting acid-precipitable c.p.m. from the total radioactivity of an aliquot of supernatant.

shift with a low-pH buffer permitted the exclusive determination of intracellular virus. In these experiments, penetration was operationally defined as the loss of sensitivity to acid inactivation, where the number of viral plaques which formed subsequent to a 60 min incubation period at 37°C represented 100% penetration or infectivity. The fraction of intracellular virus at any given time shift was calculated relative to this. The kinetics of resistance of penetrated virus to antibody-mediated neutralization was examined in parallel by treating the infected cells after each given time shift with various neutralizing MAbs prior to addition of the agarose overlay.

The penetration kinetics of RVFV in the absence of antibody as measured by plaque formation are shown in Fig. 10. The proportion of virus that entered the cell increased rapidly after a shift period of 10 min at 37°C. After 45 min, the rate of viral penetration decreased as shown by the levelling off of the curve.

The results of the resistance of penetrated virus to neutralization by the MAbs were similar for all the neutralizing antibodies tested. No significant reduction in infectivity was observed when the antibodies were added after viral penetration at each time period and subsequently removed prior to the overlay (Fig. 10a). No neutralization occurred even when immunoglobulin concentrations greatly exceeding the 80% PRNT titres under normal PRNT conditions were tested.

Those MAbs which had previously been shown in the post-adsorption neutralization tests to efficiently neutralize cell-bound virus, ie MAbs 3E5, 3H1, 5A1 and 9C4, were also tested for their ability to inhibit the infectivity of penetrated virus if they were allowed to remain in contact with the infected cells. The antibodies were used at the dilutions corresponding to the 80% PRNT titres obtained in the post-adsorption neutralization assays.

The results of these experiments are shown in Figs 10a and 10b. A 60% reduction in viral infectivity was observed with MAb 3E5 (G1 Ia) even after 60 min internalization of RVFV prior to exposure to the antibody (Fig. 10a). By increasing the immunoglobulin concentration, neutralization of penetrated virus was more efficient eg the addition of MAb 3E5 at a dilution of 1:1024 after 60 min virus internalization resulted in an 83% reduction in plaques (data not shown). The latter antibody concentration represented a 32-fold increase over the antibody dilution which gave an 80% neutralization titre under normal PRNT conditions.

The neutralizing MAb 3H1 (G1 IIb) substantially inhibited plaque development of RVFV that had internalized at any given interval (Fig. 10b). Even after a penetration period of 60 min, there was a 69% reduction in plaques which formed. In contrast, while RVFV infectivity was reduced to a certain extent on exposure to MAb 5A1 (G1 IId) during the early stages of viral internalization, once the virus had penetrated the cells for 25 min, it was resistant to neutralization (Fig. 10b). The G2-specific neutralizing MAb 9C4 (G2 Ia) had no effect on virus infectivity at any stage of viral penetration.

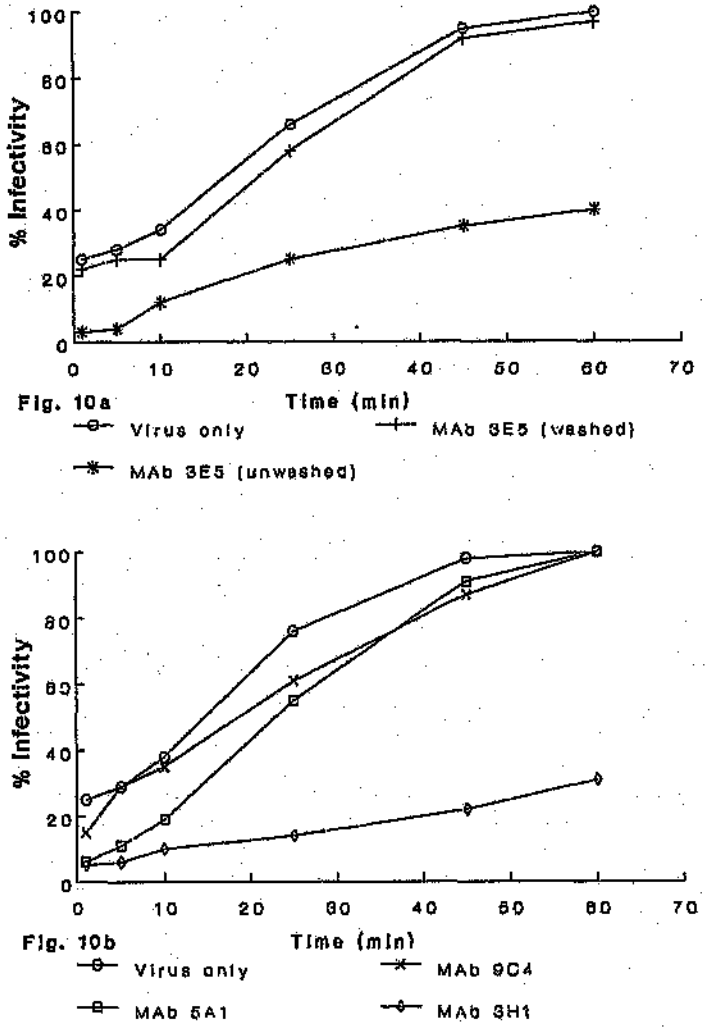


Fig. 10a

Fig. 10b

Fig. 10. Kinetics of resistance of penetrated RVFV to neutralization by MAbs. Vero cell monolayers were infected with RVFV at 4°C, then shifted to 37°C for various times to allow synchronized viral penetration to occur. After each shift interval, the cells were treated with a low-pH buffer to inactivate any extracellular virus (virus controls) or were treated with the neutralizing MAbs at the concentrations which corresponded to the previously determined 80% post-adsorption PRNT titres. The number of plaques which formed following viral penetration for 60 min in the virus control wells represented 100% infectivity. **(a)** Percentage infectivity following treatment with MAb 3E5 (G1 Ia) which was either washed off after 15 min or left in contact with the cells (unwashed) prior to the addition of the agarose overlay. **(b)** Percentage infectivity after the addition of MAbs 3H1 (G1 IIb), 5A1 (G1 IIc) and 9C4 (G2 Ia) which were left in contact with the infected cells.

7.4 Discussion

While the entry process of RVFV into cells has been studied at the ultrastructural level by electron microscopy (Ellis *et al*, 1988), the actual mechanisms involved in RVFV penetration have not been clearly defined to date. Studies on several of the other *Bunyaviridae* members suggest an entry route involving pH-dependent fusion with acidic intracellular vesicles, which is associated with concomitant specific conformation changes in one or both of the viral envelope proteins (Arikawa *et al*, 1985; Gonzalez-Scarano, 1985; Gonzalez-Scarano *et al*, 1984). The various methods used to examine fusion directly all have several drawbacks (Gallaher *et al*, 1980). Detailed study of viral fusion with intracellular membranes by electron microscopy (Matlin *et al*, 1982), for example, is impractical as the fusion process is so rapid (Pobjecky *et al*, 1986). Demonstration of the fusion function by fusing cells that are undergoing an active infection by exposing them to acidic pH (fusion from within) or adsorbing virus to cells and then acidifying them before infection (fusion from without) is very laborious. Not only do both techniques necessitate the counting of large numbers of nuclei within the fused cells, but the latter method also requires an extremely high m.o.i. (± 2000 PFU/cell) for optimal fusion (Gonzalez-Scarano *et al*, 1984; Pobjecky *et al*, 1986; Summers *et al*, 1989).

Since activation of the fusion function is accompanied by conformational changes of the fusion protein (Marsh, 1984; Matlin *et al*, 1981; White, 1990; White *et al*, 1981; 1983) an alternative means to obtain insight into the viral fusion mechanism is to use MAbs to investigate the location of any changes induced by acidic pH (Gonzalez-Scarano, 1985; Guirakhoo *et al*, 1989). The MAbs were therefore used in this manner to examine the possibility that entry of RVFV and penetration of the viral nucleocapsid into the cytoplasm involves an acid-catalyzed fusion reaction in the intracellular compartments.

Exposure of RVFV to low pH appears to induce a conformational change in the G1 surface protein as revealed by the altered binding of some of the G1-specific MAbs to acidified antigen. The fact that the changes in antibody binding persisted, in spite of readjusting the pH to neutral after acidification, shows that the conformational changes are irreversible once established.

The increased binding by MAbs 5E1 and 5E11 to their respective epitopes G1 Ib and G1 IIIa at mildly acidic pH suggests that at neutral pH these sites may be partially hidden on the virion surface. This is consistent with the earlier suggestion that these determinants are not as accessible for antibody binding as epitopes which are involved in C′-independent neutralization and haemagglutination. The enhanced antibody binding of MAbs 5E1 and 5E11 at mildly acidic pH is probably due to a rearrangement of the G1 polypeptide which results in greater exposure of these particular sites. A similar phenomenon has been reported for viruses such as Sindbis virus (Schmaljohn *et al*, 1983) and the well-studied influenza virus (Jackson and Nestorowicz, 1988; Kostolansky *et al*, 1988). These results are in keeping with the idea that treatment of a surface fusion protein to mildly acid pH results in the subsequent exposure of a hydrophobic face (Subbarao *et al*, 1987) which may play an important role in the initiation of the fusion reaction (reviewed in White, 1990). The biological significance of the pH-dependent irreversible conformational changes in these G1 epitopes thus may well lie in the proposed endosomal route of RVFV entry into the host cell cytoplasm.

Unlike the RVFV G1 protein, the G2 glycoprotein epitopes appear to be resistant to low pH. This is similar to the situation for LACV, where the G1 undergoes a conformational change at acidic pH (Gonzalez-Scarano *et al*, 1984) while the G2 protein is resistant to low pH (Gonzalez-Scarano, 1985; Kingsford and Hill, 1981). Since the induction of membrane fusion may involve multiple interactions between various virus components (Fuller and Spear, 1987), the conformational

change undergone by RVFV G1 at acidic pH may possibly expose other sites on the G1 molecule which comprise the actual fusogenic sites. Alternatively, the allosteric changes in the G1 protein may serve to expose the G2 protein, thereby facilitating involvement of the G2 protein in the physical event of RVFV fusion, as has been suggested for LACV (Ludwig *et al*, 1989; Pobjecky *et al*, 1989). Thus, while the actual fusogenic determinants have not been conclusively identified here, the pH-induced changes in the RVFV G1 protein implicate involvement of acidic endosomes in the entry process of the virus.

The dependence on endocytosis and subsequent exposure to acid conditions for viral fusion and release of the nucleocapsid into the cytoplasm presents a point at which penetration can be blocked (Dimmock, 1987; Marsh and Helenius, 1989; Outlaw and Dimmock, 1990). Neutralization of West Nile and rabies virus by antiviral antibody, for example, has been shown to be due to the inhibition of the intraendosomal acid-catalyzed fusion step (Dietzschold *et al*, 1987; Gollins and Porterfield, 1986a).

Analysis of the neutralizing activities of the RVFV MAbs earlier suggested that, although neutralization can be mediated by inhibition of virus entry, other mechanisms involving interference at an intracellular stage must also be involved. While the inhibition of the fusion step by these MAbs has not been directly examined, the fact that none of the antibodies exhibited drastically reduced binding to antigen exposed to mildly acidic pH suggests that they do not neutralize by binding to sites directly involved in the fusion event. Nevertheless, binding of certain of these antibodies to their respective epitopes could conceivably inhibit fusion by inducing a conformational constraint within the glycoprotein, thereby preventing the allosteric change necessary to expose the actual hydrophobic fusogenic region.

The means by which RVFV penetrates cells was further investigated by examining the sensitivity of the early stages of RVFV infection to the effects of the lysosomotropic agent ammonium chloride. The latter, which is a weak base, acts by raising the pH of acidic intracellular vesicles (Poole and Okhuma, 1981), and if they contain virus entering by endocytosis, it blocks the stage of membrane fusion required for viral nucleocapsid release into the cytoplasm (Marsh and Helenius, 1989; Marsh *et al*, 1983; White *et al*, 1983). Consequently, progression of viral synthesis is prevented.

The postulated acid-catalized fusion of RVFV with acidic intracellular vesicles was supported by the results of the uncoating experiments performed with ammonium chloride. In the present study, ammonium chloride was used at 50 mM, as concentrations between 10-100 mM have been shown by other workers to inhibit viral replication without any direct virucidal activity or effect on virus adsorption to cells (Glushakova *et al*, 1989; 1990; Gollins and Porterfield, 1986b; Helenius *et al*, 1982).

In the absence of ammonium chloride, the sharp rise in acid-soluble radioactivity present in the cell supernatant indicated that the labelled RVFV surface proteins had undergone degradation to acid-soluble material via the lysosomal compartments (Gollins and Porterfield, 1984; Kennedy-Stoskopf and Narayan, 1986). As the percentage of degraded protein released into the supernatant fluids increased rapidly after only 15 minutes at 37°C, uncoating of the RVF virion appears to occur shortly after viral internalization. The kinetics of RVFV uncoating thus seem to be similar to those of the alphavirus Semliki forest virus, where uncoating was shown to take place within 5-7 minutes of entry and passage to lysosomes was observed after 15 minutes (Marsh and Helenius, 1980).

In contrast to the above situation, the marked reduction in the acid-soluble material which occurred when RVFV was treated with ammonium chloride,

clearly showed inhibition of proteolytic digestion of the viral proteins, which in turn indicates that viral uncoating had been prevented. This finding was supported by the demonstration that RVFV replication, as measured by reduction in plaque formation, was inhibited by treatment with ammonium chloride. The data from these experiments thus confirm the role of endosomal uptake and pH-dependent fusion for release of the RVFV genome and subsequent replication.

With regard to the kinetics of resistance of penetrated RVFV, the infectivity of virus that had penetrated at any given time point was not reduced by subsequent brief exposure to any of the neutralizing MAbs. Interaction between antibody and virus prior to internalization is therefore nec[illegible]r for intracellular neutralization to occur. Since it had earlier bee[illegible]blishe[illegible]t virus infectivity can be efficiently inhibited by some of the antibodies if the latter are added after virus was already cell-bound, such an interaction would appear to have to take place at the cell surface prior to viral entry.

Of interest were the differences in neutrali[illegible]ion of penetrated RVFV which resulted when the MAbs were allowed to remain in contact with the infected cells. The uncoating studies had earlier shown that extensive proteolytic degradation of the viral glycoproteins had taken place following the shift of RVFV inoculated cells for 1 hour at 37°C. Thus any reduction in plaque formation which occurred after a 1 hour penetration period prior to the addition of immunoglobulin must be due to recognition and binding of the antibodies with newly synthesized RVFV glycoproteins.

In the case of viruses which mature at the plasma membrane, neutralization can occur as a result of the binding of specific antibodies directed against a viral antigen expressed on the surface of infected cells (Fujinami and Oldstone, 1979; Navarro *et al*, 1992; Oldstone *et al*, 1980; Roth and Compans, 1980). Fujinami and Oldstone (1979), for example, showed that measles antibody can bind to viral

proteins expressed at the cell membrane and not only strip off the surface determinants, but also alter viral polypeptides found inside the cell not associated with the plasma membrane.

The site of virus maturation in the *Bunyaviridae* family takes place predominantly at the Golgi complex, with release of mature virions at the cell surface by exocytosis (Matsuoko *et al*, 1991; Murphy *et al*, 1983; Smith and Pifat, 1982). In the case of RVFV, however, it has been suggested that the site of viral maturation varies as a function of both the strain of virus and the cell type used to support the replication of the virus (Anderson and Smith, 1987). The Lunyo strain of RVFV, for example, was shown by these workers to mature at the plasma membrane of infected Vero cells as well as internally. Furthermore, in hepatocytes, replication of both the ZH501 and Lunyo strains was associated with maturation at cellular surface membranes in addition to the intracellular locations. In a more recent study, the glycoproteins of recombinant RVF viruses were found to be present both intracellularly as well as on the surface of infected *Spodoptera frugiperda* insect cells (Takehara *et al*, 1990). With regard to the AN 1830 strain of RVFV, where the site of maturation in infected cells has not been studied to date, it is thus possible that in addition to intracellular maturation, some surface expression of the viral glycoproteins may occur at the plasma membrane.

As a result of these different possible sites of virus maturation, the reduction in RVFV plaque formation by the neutralizing MAbs 3E5 (G1 Ia) and 3H1 (G1 IIb) may reflect neutralization of virus which has been released from the cells and/or the interaction with cell surface-expressed G1 glycoprotein. In the former instance, such neutralization may be effected by the coating of virus by the antiviral antibodies present in the intercellular spaces as has been demonstrated by electron microscopic studies with several phleboviruses (Smith and Pifat, 1982). In this way, the RVFV MAbs would prevent spread of virus from infected to neighbouring uninfected cells, a process which would normally give rise to the

development of localized foci of lysed cells. On the other hand, should some RVFV maturation occur at the cell surface, neutralization may also possibly occur as a result of the binding of these specific antibodies to their respective epitopes on plasma membrane-expressed RVFV G1 protein.

The inability of MAbs 5A1 (G1 IId) and 9C4 (G2 Ia) to inhibit plaque development once virus had already penetrated is of particular interest. The results confirm the earlier data that these antibodies neutralize RVFV by a different mechanism to that of MAbs 3E5 (G1 Ia) and 3H1 (G1 IIb). Unlike the latter antibodies, MAbs 5A1 and 9C4 had previously been found to substantially block viral entry into the cell. These findings are thus similar to those for the unrelated herpes simplex virus (HSV) type 1, where some MAbs directed against the surface glycoprotein B interfered with plaque development by preventing cell-to-cell spread of the virus while several others that blocked viral entry did not inhibit plaque formation (Navarro *et al*, 1992).

The actual mechanism underlying the resistance of newly released virus to neutralization by MAbs 5A1 and 9C4 is unclear, but is conceivably related to the fact that they are directed against epitopes which are involved in viral entry into the cell. Should these particular determinants occur in a relatively high concentration on the RVFV glycoproteins, antibody concentrations which would neutralize 80% of input virus under normal PRNT conditions may not be sufficient to bind to the numbers of progeny virus released from the infected cells. Moreover, should some surface expression of the viral glycoproteins occur in addition to intracellular maturation, a proportion of the antibodies may recognize and bind to the epitopes on the surface expressed glycoproteins. This could serve to further reduce the number of antibodies available for interacting with the mature virions which are subsequently released from the cells. While the mechanisms outlined above are speculative, the differences in the ability of the various MAbs to neutralize penetrated RVFV suggest that although the processes

of cell-to-cell spread of RVFV and viral internalization are related, they are not identical.

In summary, therefore, specific conformational changes in the RVFV G1 protein were shown to take place at endosomal pH values. Such alterations are irreversible once established and result in the increased accessibility of two antigenic sites which may in turn play an important role in the initiation of the viral fusion reaction. These pH-dependent allosteric changes which occur in the G1 glycoprotein, together with the results of the uncoating assays, strongly suggest that the route of entry and penetration of RVFV into the host cell involves the endosomal pathway.

With regard to the further analysis of antibody-mediated neutralization of RVFV, cell-penetrated virus was found to be resistant to neutralization by several G1 and G2-specific MAbs which had earlier been shown to block virus internalization. The fact that other antibodies inhibited the infectivity of penetrated virus by preventing the spread of progeny virus to neighbouring cells suggests that while the processes of cell-to-cell spread of RVFV and viral entry are related, they are not identical. The data moreover supports the earlier suggestion that certain antigenic sites on both the RVFV G1 and G2 proteins which are involved in virus entry into the cell are important in viral pathogenesis.

8. GENERAL DISCUSSION

8.1 MAb production and functional activities

Knowledge of the structural, functional and antigenic properties of the envelope proteins is an essential requirement for the understanding of RVFV infection as well as the host immune response to such infection (Keegan and Collett, 1986; Parsonson and McPhee, 1985). At the time this study was undertaken, the antigenic determinants on the RVFV G1 and G2 glycoproteins involved in viral pathogenesis had not been fully characterized. Since the analysis of viral epitopes can be exquisitely performed by means of monoclonal antibodies (Carter and ter Meulen, 1984; Heinz, 1986), this was considered to be an ideal approach for such an investigation. MAbs were therefore generated specifically against the surface glycoproteins and used to analyse the topological and functional properties of the epitopes on the G1 and G2 proteins. Furthermore, in order to gain a better understanding of the role of the RVFV envelope proteins in infection and pathogenesis, the mechanisms of antibody-mediated neutralization of the virus were examined, as well as the function of the glycoproteins in viral attachment and penetration.

The successful generation of twenty three hybridomas which excreted antibodies specific for the RVFV envelope proteins was due in part to the use of a purified glycoprotein preparation as the immunogen. Another contributing factor was the development of an antibody screening assay that permitted the rapid detection of antibodies directed against the viral surface proteins.

Serological characterization of the resulting MAbs permitted the functional activities of the corresponding epitopes on the RVF viral surface proteins to be defined. While it is common knowledge that the RVFV envelope proteins elicit neutralizing and haemagglutination-inhibition antibodies (Battles and Dalrymple, 1988; Keegan and Collett, 1986; Shope *et al*, 1980), there has been no published

data regarding the possible correlation of these two functions for the several neutralizing epitopes identified by other workers (Collett *et al*, 1987; Schmaljohn *et al*, 1989). The present findings that all the G1 and G2 neutralizing sites also demonstrated a haemagglutination function are similar to those reported for the phlebovirus Punta Toro, where all except one of the neutralizing MAbs also had haemagglutination-inhibition activity (Pifat *et al*, 1988).

Several antibodies directed against both the G1 and G2 glycoproteins were not only associated with neutralization and haemagglutination-inhibition, but also with protection of mice against virulent RVFV infection. The demonstration that antibodies to a single epitope on each of the RVFV glycoproteins can provide complete protection to otherwise lethally infected mice suggests that both envelope proteins play a critical role in virus infectivity and pathogenesis. This finding is of particular significance, since previously only anti-G2 or a mixture of anti-G1 and G2 antibodies had been shown to be protective *in vivo* (Battles and Dalrymple, 1988).

8.2 Topographical, structural and functional analyses of epitopes

The topological relationship of the antigenic determinants to each other on the envelope proteins was examined using competitive binding assays as this method has been widely used for many viruses, including some members of the *Bunyaviridae* (Arikawa *et al*, 1989; Dantas *et al*, 1986; Gonzalez-Scarano *et al*, 1982; Kingsford *et al*, 1983; Pifat *et al*, 1988). Having taken into consideration all the various factors regarding possible misinterpretation of the competitive binding data (Cepica *et al*, 1990; Henchal *et al*, 1985; Shaw *et al*, 1986; Stone and Nowinski, 1980), the topological localization of ten of the G1 epitopes and seven of the G2 antigenic sites was achieved. As one of the major drawbacks of the CBA is that antibodies with weak avidity are unsuitable for use in this method (Stone and Nowinski, 1980; Yewdell and Gerhard, 1981), the epitopes recognized by the remaining six low avidity MAbs could not be mapped.

With regard to the RVFV G1 protein, four antigenic domains comprising clusters of overlapping or closely adjacent epitopes were identified. Based on the competitive binding patterns, these domains appear to be adjacent to each other and may even be interlinked to form one large antigenic region. The premise that these are in close proximity to each other on the G1 protein is further supported by the fact that they all appear to comprise an area on the glycoprotein that is highly conformational. In contrast, the unmapped G1-specific determinant defined by MAb 1E4, which was more resistant to denaturation, probably corresponds to an independently folding region within the G1 protein. It is of some interest that although extensive disulphide formation has been predicted for the RVFV envelope proteins (Ihara *et al*, 1985), this was the only determinant recognized by the MAbs which appears to be dependent on disulphide bridges for conformation.

Correlation of the biological activities and epitope specificities of the MAbs against G1 showed that the antigenic domains I, II and IV were involved in virus neutralization and haemagglutination, indicating they are accessible to antibody on the intact virion. These domains, therefore, are likely to be located in the hydrophilic portions on the outer surface of the folded G1 polypeptide as shown for the neutralizing sites on many other viral glycoproteins (Eisenberg *et al*, 1985; Novak and Wengler, 1987; Pellett *et al*, 1985; Wiley and Skehel, 1987). Since one of the epitopes within the G1 I domain (G1 Ia) was also associated with complete protection of mice against virulent RVFV infection, this region in particular appears to represent a biologically important area. In contrast, the low level C′-dependent neutralization exhibited by the epitopes comprising the G1 III domain suggests that this region is less accessible for antibody binding than those involved in haemagglutination and neutralization and may therefore be located in areas or pockets on the G1 polypeptide which are more folded.

In the case of the G2 protein, four antigenic domains were identified, but unlike the closely adjacent G1 domains, these appear to be spatially distinct. One of the

domains (G2 I) comprising a cluster of four sites was associated with significant neutralizing and haemagglutination activity. In addition to these functions, the epitope G2 Ia was shown to be a protective determinant, suggesting that it represents an immunologically important area on the G2 protein. Domain G2 II, on the other hand, was involved in weak haemagglutination and C′-dependent neutralization, while the other two G2 regions had no haemagglutination function and neutralized to a low level only in the presence of C′. These regions may therefore be less accessible for antibody binding than the G2 I domain.

The observation that several of the antibodies directed against the G2 protein were also capable of competing with G1-specific MAbs for attachment to certain sites on the G1 protein demonstrates that these antibodies can induce conformation changes in an entirely different protein. This finding is of particular interest since there have been no other reports regarding epitope mapping studies on the effect of binding of G1-specific MAbs on the G2 protein and *vice versa* for any other phleboviruses.

While the combined data from the topographical and structural analyses permitted the probable spatial location and structure of these determinants to be defined, actual epitope arrangements will require physical mapping to the G1 and G2 genome sequences and the corresponding regions on the polypeptide chains. The only mapping data published to date for RVFV utilizing the latter technique is that of Keegan and Collett (1986) for three neutralizing and one non-neutralizing determinants on the G2 protein of the Egyptian ZH 5 in. The discontinuous nature of the determinants comprising the neutralizing G2 I domain defined here suggests that they occur on a different area of the G2 protein to the sequential and continuous neutralizing sites identified by Keegan and Collett (1986). As the latter were shown to map to amino acid sequences 258-291 (epitope I), 280-299 (epitope IV) and 382-392 (epitope II), the epitopes in this study are most likely comprised of amino acid residues on the G2 polypeptide other than these.

Determination of the actual gene coding regions of the discontinuous antigenic determinants in the present study would require an alternative approach to that used for the mapping of the sequential G2 sites by Keegan and Collett (1986). Moreover, since discontinuous epitopes cannot be predicted by computer analysis (Becker, 1992), use of the hydrophilicity profiles of the RVFV glycoproteins (Collett *et al*, 1985) to localize the probable regions on the G1 and G2 polypeptides that these epitopes map to would also not be effective. The precise localization of the discontinuous epitopes would in fact require a combination of various complex approaches (Burnens *et al*, 1987; Geysen *et al*, 1987a; Horsfall *et al*, 1991) and knowledge of the complete three-dimensional structure of the RVFV envelope proteins, similar to that acquired for the influenza haemagglutinin (Wiley and Skehel, 1987; Wilson *et al*, 1981).

Another aspect of the conformational nature of the RVFV determinants is that the native protein structure of both the G1 and G2 proteins is clearly required for the recognition and expression of the functional activities of all the antibodies in this study. The finding that the protective determinants G1 Ia and G2 Ia are discontinuous has considerable implications with regard to the possible development of a sub-unit vaccine. While knowledge of the positions of protective epitopes could contribute significantly to the development of a RVFV sub-unit vaccine (Keegan and Collett, 1986), the construction of synthetic immunogens having a structure resembling the G1 Ia and G2 Ia topological determinants would represent a difficult immunochemical problem. T! could nevertheless possibly be approached by the 'surface simulation synthesis' technique as outlined by Atassi (1980) and Geysen *et al* (1987a).

5.3 Antibody-mediated neutralization mechanisms

In addition to the functional and topographical analyses of the RVFV envelope proteins, the mechanisms involved in antibody-mediated neutralization were examined. The C′-enhanced and C′-dependent neutralization exhibited by the

MAbs, while emphasizing the important role of C′ in the inhibition of RVFV infectivity, is a phenomenon that has been well-documented for many other viruses (reviewed by McCullough, 1986). Of particular concern in this study were the modes of action of the MAbs which neutralized the virus efficiently in the absence of C′.

An important mechanism of neutralization of RVFV appears to be that of synergistic neutralization between various antibodies directed against specific antigenic determinants on the G1 and G2 proteins. Such enhanced neutralization does not seem to be directly correlated with increased binding of the antibodies to their respective epitopes, but rather appears to be the result of complex conformational changes in the glycoproteins induced by antibody attachment. A similar phenomenon has been reported for LACV (Kingsford, 1984). Furthermore, the increased neutralization which occurred with certain combinations of anti-G1 and G2 MAbs supports the evidence from the competitive binding assays for an interaction between the two surface proteins of RVFV.

The demonstration that synergistic neutralization could even take place on combining two antibodies which were non-neutralizing individually suggests that the various types of co-operative effects between antibodies seen here may well have considerable relevance with respect to a polyclonal immune response to RVF infection.

A further neutralization strategy employed by certain MAbs was that of inhibition of virus entry into the cell. Antibodies directed against the G1 IId, G1 IV and the unmapped G1 epitopes defined by MAbs 1E4 and 8G10 displayed this type of neutralizing activity. The G2 specific neutralizing MAbs mapping to G2 Ia, Ib and Ic likewise substantially prevented virus internalization. However, as viral entry was not completely blocked, and yet subsequent plaque formation was

inhibited, interference by these antibodies at a later stage in the viral life cycle must also be involved.

In addition to these antibodies which appear to neutralize by two different mechanisms, some MAbs had no effect on viral attachment or internalization, but still inhibited viral plaque development effectively. These antibodies, which included the strongly neutralizing and protective MAb mapping to site G1 Ia, must thus neutralize solely at an intracellular stage. The fact that the MAb defining the G1 IId epitope substantially prevented viral internalization while the antibodies recognizing the closely adjacent G1 IIb, IIc and IIe sites had no effect on virus entry indicates that neutralizing antibodies mapping to a specific domain do not necessarily inhibit infectivity in an identical manner. Considering that the phlebovirus cross-reactive epitopes G1 IIc, IId and IIe are highly conserved, the heterogeneous neutralization mechanisms displayed by the antibodies recognizing these determinants comprising the G1 II domain is even more interesting.

One of the intracellular stages which may be particularly sensitive to inhibition by neutralizing antibodies is that of the virion-cell fusion event leading to the release of the viral genome (Dietzschold *et al*, 1987; Dimmock, 1987; Marsh and Helenius, 1989). Gonzalez-Scarano (1985) has furthermore shown that at the pH that activates the fusion function of LACV, the G1 protein undergoes a conformational change which results in the reduced binding of several neutralizing MAbs. Thus, while the inhibition of the RVFV fusion step by the MAbs was not examined directly, such interference was looked at indirectly by ascertaining if the attachment of any of these antibodies to acidified antigen was adversely affected, similar to the situation reported for the LACV G1 glycoprotein. The fact that none of the antibodies exhibited drastically reduced binding to RVFV antigen exposed to mildly acidic pH suggests that they do not neutralize by binding to sites directly involved in the fusion event. Nevertheless, binding of certain of

these antibodies to their respective epitopes could conceivably inhibit fusion indirectly by preventing the allosteric change necessary to expose the actual fusogenic region of the glycoprotein(s). Additional modes of action may involve a step later in the virus infectious cycle such as interference with viral uncoating as shown for West Nile virus (Gollins and Porterfield, 1986a) or by blocking viral transcription as reported for influenza virus (Rigg *et al*, 1989).

8.4 Role of the RVFV glycoprotein epitopes in virus attachment and penetration

While a relationship of both the RVFV envelope proteins to infectivity has been established (Battles and Dalrymple, 1988; Collett *et al*, 1987; Dalrymple *et al*, 1989; Keegan and Collett, 1986; Schmaljohn *et al*, 1989), the exact role played by either of the glycoproteins in viral attachment and penetration has not been directly demonstrated.

Based on the fact that both RVFV envelope proteins elicit neutralizing and haemagglutination-inhibition antibodies, it has been suggested that the glycoproteins may both be involved in virus attachment, either directly or by conformational requirements (Schmaljohn and Patterson, 1990). In the present study, the concordance of neutralizing and haemagglutination activities associated particularly with the G1 I and G2 I domains would seem to implicate these as the possible regions which bind to cellular receptors. However, since none of the MAbs defining the epitopes comprising these domains interfered with the attachment of virus to cells, it is evident that these antibodies are not directed against the viral binding site(s). This, in turn, suggests that the viral attachment determinant(s) may be located in a different area on the RVFV envelope proteins to the epitopes defined by these antibodies.

Certain epitopes involved in viral entry into the cell, on the other hand, were identified. The fact that both G1 and G2-specific MAbs were capable of inhibiting virus internalization clearly demonstrates that both envelope glycoproteins have an important function in entry of RVFV into the host cell. This finding is of considerable significance as it is the first time that the involvement of both envelope proteins in RVFV entry into the cell have been definitively shown.

The actual mechanisms involved in entry and penetration of RVFV into the host cell cytoplasm have not been clearly defined to date. Studies on several of the other *Bunyaviridae* members suggest an entry route involving pH-dependent fusion with acidic intracellular vesicles, which is associated with concomitant specific conformational changes in one or both of the viral envelope proteins (Arikawa *et al*, 1985; Gonzalez-Scarano, 1985). To obtain insight into the RVF viral fusion mechanism, the MAbs were therefore used to investigate the location of any changes in the surface glycoproteins induced by acidic pH.

Exposure of RVFV to low pH was shown to induce an apparent irreversible allosteric change in the G1 surface protein as revealed by the increased binding of several of the G1-specific MAbs to acidified antigen. Such a rearrangement of the G1 polypeptide which results in the greater exposure of certain sites is in keeping with the idea that treatment of a surface fusion protein to mildly acid pH results in the subsequent exposure of a hydrophobic face (Subbarao *et al*, 1987) which may play an important role in the initiation of the fusion reaction. The biological significance of the pH-dependent irreversible conformational changes in these G1 epitopes thus may well lie in the proposed endosomal route of RVFV entry into the host cell cytoplasm.

Unlike the RVFV G1 protein, the G2 glycoprotein epitopes appear to be resistant to low pH. Since the induction of membrane fusion may involve multiple

interactions between various virus components (Fuller and Spear, 1987), the conformation change undergone by RVFV G1 at acidic pH may possibly expose other sites on the G1 molecule which comprise the actual fusogenic sites. Alternatively, the allosteric changes in the G1 protein may serve to expose the G2 protein, thereby facilitating involvement of the latter protein in the physical event of RVFV fusion, as has been suggested for LACV (Ludwig *et al*, 1989; Pobjecky *et al*, 1989). Thus, while the actual fusogenic determinants have not been conclusively identified here, the pH-induced changes in the RVFV G1 protein strongly implicate involvement of acidic endosomes in the entry process of the virus. The postulated endocytic uptake and pH-dependent fusion for RVFV penetration and release of the viral genome was further supported by the demonstration that viral uncoating and subsequent replication was inhibited by raising endosomal pH with the lysosomotrophic agent ammonium chloride.

8.5 Concluding remarks

The possibility that any particular panel of hybridoma antibodies is able to discern only a portion of the total antigenicity must always be considered. However, although additional sites may be involved in the natural immune response to the RVFV glycoproteins, the antigenic determinants identified here conceivably account for a significant portion of the immunologically relevant domains of these envelope proteins. By correlating the biological activities of the MAbs with epitope specificities, antigenic sites with a neutralizing/haemagglutination function, as well as those associated with C′-dependent neutralization, were identified for both proteins. Protective determinants were shown to occur on both G1 and G2, indicating that both RVFV envelope proteins are important in viral pathogenesis. The neutralization studies, in turn, revealed that the inhibition of virus attachment is not the principal means of antibody-mediated neutralization of RVFV. Instead, such neutralization appears to be the result of several different processes, including synergistic neutralization by combinations of different antibodies, prevention of virus binding, virus internalization and the blocking of

the viral life cycle at an intracellular stage. Further insight into RVFV infectivity was obtained by showing that both glycoproteins are involved in virus entry into the host cell. Finally, the present findings strongly support an endosomal route of entry and penetration for RVFV, associated with concomitant allosteric changes in the G1 protein.

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APPENDIX A: ELISA buffers

1. ELISA coating buffer

   15mM sodium carbonate and 35 mM sodium hydrogen carbonate, pH 9,6.

2. ELISA wash buffer

   Phosphate buffered saline (PBS) containing 0,01% Tween-20.

3. ELISA blocking solution

   4% Bovine serum albumin in PBS.

4. ELISA diluent

   PBS with 5% foetal calf serum.

APPENDIX B: SDS-polyacrylamide slab gel electrophoresis (Laemmli, 1970; Smith and Brown, 1977).

Stock solutions:

1. Acrylamide-Diallyltartramide (DATD)
   Prepared in the ratio 30:1,6 and stored in a dark bottle.

2. Resolving gel buffer
   1M Tris-HCl pH 8,8.

3. Stacking gel buffer
   1M Tris-HCl pH 6,8.

4. Electrophoresis buffer (10 x)
   0,25 M Tris-HCl pH 8,3; 1,92 M glycine; 1% (w/v) SDS.

5. Dissociation buffer
   10% (w/v) SDS; 10% (v/v) B-mercaptoethanol; 15% (v/v) glycerol; 0,02% (v/v) bromophenol blue in 1 M Tris-HCl pH 6,8.

All the above solutions were kept at 4°C.

Sample preparation:

Dissociation buffer was added to each sample at a 1:5 ratio and boiled for 5 min prior to loading wells.

Slab gel formulations:

Resolving gel

Stock solution	12% final acrylamide gel concentration
	(ml)
Acrylamide-DATD	32,0
Resolving gel buffer	30,0
Distilled water	13,2
10% SDS (w/v)	0,8
1,5% fresh $NH_4S_2O^1_8$ (w/v)	4,0
Temed (μl)	20
Total volume	80

[1]$NH_4S_2O_8$ = ammonium persulphate

Stacking gel

Stock solution	4% acrylamide
	(ml)
Acrylamide-DATD	2,0
Stacking gel buffer	1,9
Distilled water	9,25
10% SDS (w/v)	0,15
87% glycerol	1,0
1,5% fresh $NH_4S_2O^1_8$ (w/v)	0,7
Temed (μl)	20
Total volume	15

[1] $NH_4S_2O_8$ = ammonium persulphate

Electrophoretic separation:

Electrophoresis was performed at 16 mA until the bromophenol blue tracking dye reached the bottom of the resolving gel (± 16 h).

Processing of gels for autoradiography

(a) Fixation of proteins in gel

Gels were immersed in 45% methanol/ 7% acetic acid solutions for 1 h after electrophoresis, then soaked in Amplify reagent (Amersham International, U.K.) containing 5% glycerol for 30 min.

(b) Gel hydration

The fixed gels were placed on sheets of Whatmann No 3 MM filter paper, covered with clingwrap plastic and dried under vacuum at 70°C for 2 h.

(c) Autoradiography

The dried gels were taped into X-ray cassettes and β-max autoradiographic films (Amersham International, U.K.) were placed over the gels. The cassettes were stored in the dark at -70°C and after the required exposure, the autoradiographic films were developed according to the manufacturers instructions.

APPENDIX C: Potassium tartrate-glycerol gradients (Objeski *et al*, 1974)

1. TE buffer

   2mM EDTA in 2mM Tris-HCl pH 7,4.

2. Solution A

   60% (w/w) sodium potassium tartrate (Sigma) in TE buffer.

3. Solution G

   30% (w/w) glycerol in TE buffer.

4. Solution B - F

   These were prepared by mixing soluuions A and G in the following ratios:

Solution	Solution A	Solution G
B	50g	10g
C	40g	20g
D	30g	30g
E	20g	40g
F	10g	50g

Gradients were prepared by hand by successively layering 1,5 ml of solutions A - G in a Beckman Ultra-Clear centrifuge tube.

APPENDIX D: Animal Ethics Committee approval

All experimental work involving the use of mice was approved by the Animal Ethics Screening Committee, University of the Witwatersrand. This included the:

1) Production of MAbs.
2) Production of hybridoma ascitic fluid*.
3) In-vivo protection studies.

Copies of the certificates are included.

* Since 23 hybridomas were produced instead of the estimated 20, it was necessary to use several extra mice for the production of hybridoma ascitic fluid. The subsequent use of mice for only four virus particle dilutions (10^{-3} to 10^{-6}) instead of the allocated seven dilutions (10^{-1} to 10^{-7}) for the determination of the virus LD_{50} in the in-vivo protection experiments nevertheless ensured that the total number of mice approved for the study was not exceeded.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:	89	52	6

APPLICANT: Ms TG Besselaar

DEPARTMENT: Virology, Medical School

PROJECT TITLE: Study of the surface glycoproteins of Rift Valley fever virus using monoclonal antibodies.

SPECIES	NUMBER	DATE OF EXPIRY
MICE	75	JUNE 1991

The use of these animals is subject to AESC Guidelines for the use and care of animals, to the procedures specified in the application form, and to:

The conditions itemised in the Chairman's letter of 31/5/89 and the secretary's letter of 28/4/89.

Signed G. Erasmus

(Chairman: Animal Ethics Screening Committee)

Date 3/5/89

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:

91	47.	6

APPLICANT: Ms T G Besselaar

DEPARTMENT: National Institute for Virology, Virology, MEDICAL SCHOOL

PROJECT TITLE: Study of the surface glycoproteins of rift valley fever virus using monoclonal antibodies

SPECIES	NUMBER	DATE OF EXPIRY
Mice	35	June 1993

The use of these animals is subject to AESC Guidelines for the use and care of animals, to the procedures specified in the application form, and to:

For the purpose of testing the remaining 7 MAbs. Noted that in the previous application animals were allocated for the purpose of determining the LD50 of RVFV. The committee assumes, therefore, that the researcher has established the average length of time it takes for death to occur after innoculation.

To protect the animals from unnecessary suffering the committee requires that after innoculation the animals are euthanased an appropriate time before the predicted time of death.

SIGNED G. Schumann

(Chairman: Animal Ethics Screening Committee)

DATE 9/4/91

APPENDIX E: List of abbreviations

BABS	borate buffered saline with 0,4% bovine albumin
BBS	borate buffered saline
BSA	bovine serum albumin
C′	complement
CBA	competitive binding assay
CF	complement fixation
CPE	cytopathic effect
c.p.m.	counts per minute
CsC1	caesium chloride
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EMEM	Earle's minimum essential medium
FCS	foetal calf serum
HA	haemagglutination
HAF	hyperimmune ascitic fluid
HAT	hypoxanthine aminopterin thymidine
HI	haemagglutination-inhibition
HMEM	Hank's minimum essential medium
HRPO	horseradish peroxidase
HT	hypoxanthine thymidine
i.c.	intracerebral
IFA	indirect immunofluorescence antibody assay
i.p.	intraperitoneal
LACV	La Crosse virus
LD_{50}	50% lethal dose
MAb	monoclonal antibody

APPENDIX E: List of abbreviations

m.o.i.	multiplicity of infection
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PFU	plaque forming units
PRNT	plaque reduction neutralization test
RIPA	radioimmune precipitation assay
rpm	revolutions per minute
rt	room temperature
RVFV	Rift Valley fever virus
SDS	sodium dodecyl sulphate
TCID	tissue culture infective dose
Tris	Tris(hydroxymethyl)aminomethane
VBS	veronal buffered saline
WN	West Nile