# STUDIES ON THE ANTIGENIC DETERMINANTS OF PARAMYXOVIRUSES

By DAN YOUNG B.Sc.

Department of Biochemistry and Microbiology,
University of St. Andrews, March 1991

## ABSTRACT

Monoclonal antibodies (MAbs) were produced to study the antigenic relationships between SV5, PIV-2 and other paramyxoviruses, these MAbs had specificities for the HN, F, M, NP and P-proteins of SV5 and for the HN, NP and P-proteins of PIV-2. A minority of these MAbs reacted with Western blotted virus polypeptides. All anti-HN and anti-F MAbs neutralized virus infectivity. At least 4 and 3 antigenic sites were demonstrated on the HN-proteins of SV5 and PIV-2 respectively. Immunofluorescence revealed different patterns and distributions of virus proteins between SV5 and PIV-2. The majority (55 out of 60) of MAbs were specific for the virus against which they were raised, the minority that did cross-react between SV5 and PIV-2 had specificities for the M, NP and P-proteins and one of these MAbs, with specificity for the P-protein, also showed cross reaction with mumps virus. In contrast only minor differences were detected between canine, human and simian isolates of SV5. None of the MAbs showed cross-reactivity between the HN-proteins of SV5 and PIV-2 but, polyclonal sera raised against purified preparations of these proteins did show limited cross-reactivity. Anti-SV5 and anti-PIV-2 antibodies, in the same human sera, were shown to be a mixture of specific and cross-reacting species of antibody.

I, Dan Young, hereby certify that this thesis has been composed by myself, that it is a record of my own work (apart from the production of the hybridoma cell lines secreting monoclonal antibodies, which I carried out in conjunction with Dr. R. E. Randall), and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed

Date 22nd March 1991

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Randall, R. E. and Young, D. F. (1988). Comparison between Parainfluenza virus type 2 and Simian virus 5: Monoclonal antibodies reveal major antigenic differences. *Journal of General Virology* **69**, 2051-2060.

# CONTENTS

# INTRODUCTION

## Paramyxoviridae: Taxonomy and associated diseases

The paramyxovirus genus is one of three members of the family Paramyxoviridae (Kingsbury *et al.*, 1978), the other two being the morbilliviruses (type species: measles virus) and the pneumoviruses (type species: human respiratory syncytial virus). Members of the Paramyxoviridae are grouped together by virtue of having common morphology and path of transmission (which is normally via the respiratory route). The paramyxovirus genus includes viruses such as parainfluenza (PIV) types 1, 2, 3 and 4, mumps, Sendai, simian virus 5 (SV5) and Newcastle disease virus. Parainfluenza viruses are often associated with respiratory diseases, for example croup (laryngo-tracheobronchitis) in young children, and occasionally with mild upper respiratory tract infections in older children and adults (Wright, 1984). SV5 has not been associated with any acute disease in man but antibodies to SV5 in the human population have been reported (Hsuing, 1972; Goswami *et al.*, 1984). Although it was first isolated from monkey kidney cell lines (Hull *et al.*, 1956), the

precise origin of SV5 is unclear, antibodies to SV5 appear only to occur in monkeys that have come into contact with humans (Tribe, 1966). SV5 has also been isolated from dogs (Baumgärtner *et al.*, 1981; McCandlish *et al.*, 1978) and, on several occasions, from the bone marrows of patients with multiple sclerosis (Mitchell *et al.*, 1978, Goswami *et al.*, 1984). Indeed in dogs, SV5 commonly causes minor respiratory tract infections (kennel cough) and is known in veterinary circles as canine parainfluenza virus. SV5 and other members of the Paramyxoviridae, together with their principal characteristics and disease symptoms are listed in Table 1. Paramyxovirus infections are normally of limited duration with the immune response apparently eliminating the virus from the infected individual. However, in certain circumstances, paramyxoviruses are capable of establishing prolonged or persistent infections. Indeed a number of important human diseases have been shown to be either caused by (eg. SSPE, a fatal brain degenerative disease caused by persistent measles infection) or linked (eg. Paget's bone disease, autoimmune chronic active hepatitis and multiple sclerosis; for review see, Randall and Russell, 1991) with such persistent infections.

**Table 1.** *Paramyxoviridae*, hosts and disease producing potential.
(from Pringle, 1987)

Genus *Paramyxovirus:*

| Serotype | Primary host | Associated disease |
|---|---|---|
| PIV-1 | Man | URTI, rarely pneumonia in adults |
| | Mouse (Sendai) | Inapparent, latent in mice |
| PIV-2 | Man | URTI, croup mainly in children |
| | Monkey (SV5) | URTI |
| | Dog | URTI |
| PIV-3 | Man | URTI, laryngitis, bronchiolitis and pneumonia in children |
| | Cattle | Shipping fever |
| | Sheep | URTI |
| | Monkey | URTI, pneumonia |
| PIV-4 | Man | URTI |
| Mumps virus | Man | Parotitis, gastroenteritis, orchitis, oophoritis, pancreatitis and other conditions |
| Avian PIV-1 | Chicken (NDV) | Inapparent to lethal viscerotropic and neurotropic (Meningoencephalitis in mink; URTI, pneumonitis and encephalitis in man) |
| Avian PIV-2 | Several avian spp (Yucaipa) | Inapparent |
| Avian PIV-3 | Several avian spp | Inapparent |
| Avian PIV-4 | Duck, chicken, geese | Inapparent |
| Avian PIV-5 | Budgerigar (Kunitachi) | Inapparent |
| Avian PIV-6 | Duck and chicken | Inapparent |
| Ungrouped viruses | Several avian spp | Inapparent |

Genus *Morbillivirus:*

| Serotype | Primary host | Associated disease |
|---|---|---|
| Measles virus | Man | URTI, rash, otitis media, bronchopneumonia, rarely (1/2000) encephalitis, exceptionally ($1/10^6$) SSPE |
| Canine distemper virus | Dog and mustelids | URTI, skin eruptions, bronchopneumonitis, keratitis of the feet (hard pad), old dog encephalitis, demyelination of neural tissue |
| Rinderpest virus | Cattle, sheep, goats, pigs, buffalo | Mucosal lesions, diarrhoea, bronchopneumonia |
| Peste de petits ruminants | Sheep, goats | Mucosal disease, diarrhoea, bronchopneumonia, abortion |

Genus *Pneumovirus:*

| Serotype | Primary host | Associated disease |
|---|---|---|
| Respiratory syncytial virus | Man | URTI, bronchiolitis and pneumonia in infants, otitis media |
| | Cattle, sheep, goats | URTI, pneumonia, bronchiolitis and emphysema in calves |
| Murine pneumonia virus | Mouse, Syrian hamster, cotton rats | Inapparent, latent in mice, rarely disease producing in guinea pigs |

## Paramyxovirus structure

The paramyxoviruses are enveloped viruses of pleomorphic form and variable dimensions. They have six major structural proteins, the fusion (F), haemagglutinin-neuraminidase (HN), large (L), matrix (M), nucleocapsid (NP) and phospho- (P) proteins. Two other proteins (V and a small hydrophobic protein, SH) have been identified but their functions are as yet unclear. The HN and F-proteins are glycosylated and separately form spikes which protrude from the surface of the virus envelope (Fig. 1a). It is against these two components that neutralizing antibodies are directed. The F-protein is synthesized as a precursor polypeptide ($F_0$), this is activated by proteolytic cleavage into two fragments ($F_1$ and $F_2$) which are held together by a single disulphide bridge. The M-protein is located on the inner side of the virus envelope and this surrounds the helical nucleocapsid which is comprised of the L, NP and P-proteins complexed together with the single stranded, monomolecular, anti-sense RNA genome (Fig. 1a). The estimated length of the RNA genome is in the range 15,000 to 16,000 nucleotides, this is close to the upper size limit (17,600 nucleotides) set by Reanney (1984) for a genetically stable RNA genome.

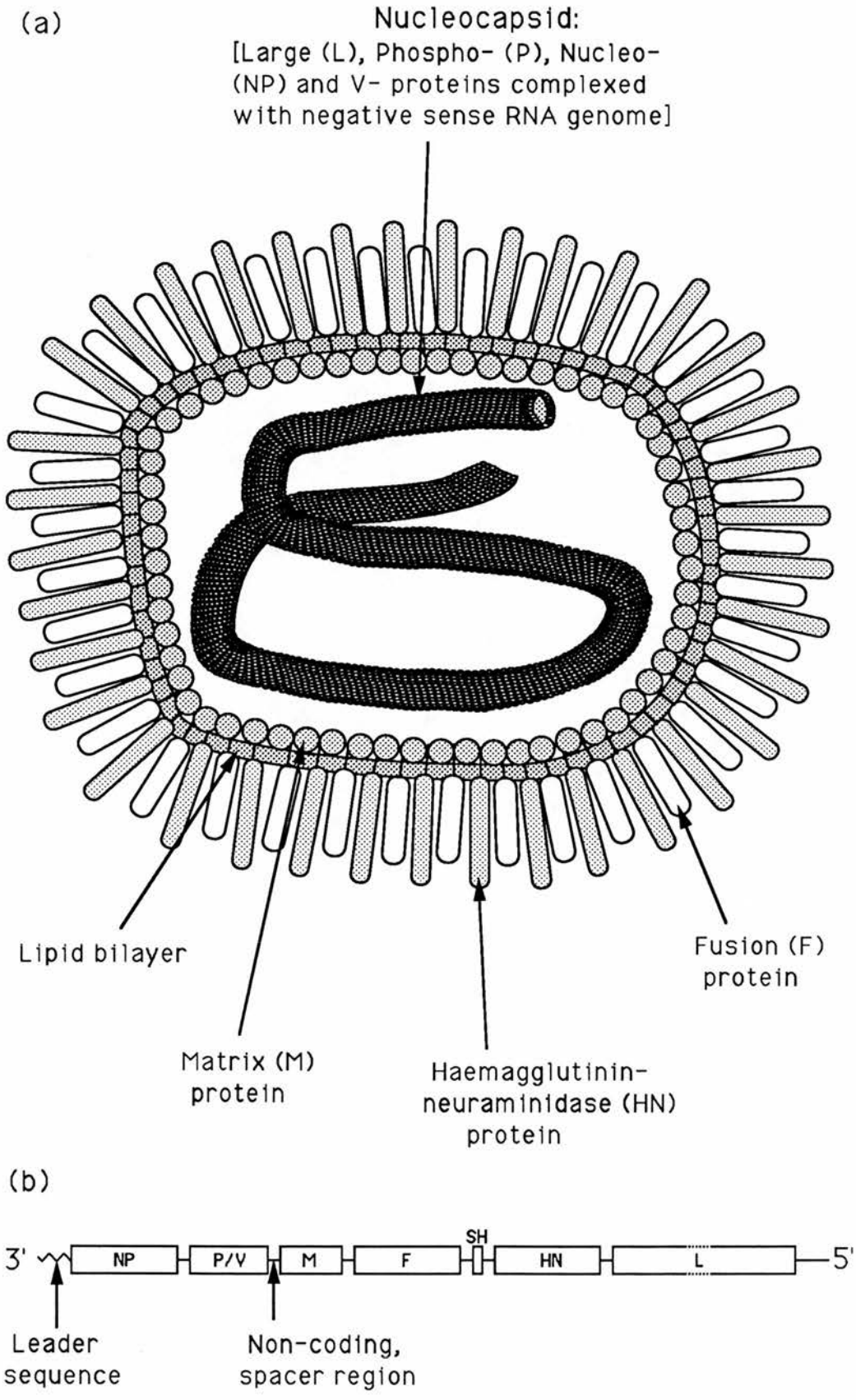



**Fig.1**. Schematic representations of (a) the structural components and (b) the genome arrangement of simian virus 5.

## Genome structure of Paramyxoviridae

All the Paramyxoviridae possess a single promoter site adjacent to the 3'-terminus of the genome, the linear array of genes being transcribed with decreasing frequency with increasing distance from the promoter site. A schematic representation of the genome structure of SV5 is shown in Fig. 1b. The morbilliviruses and paramyxoviruses exhibit the same basic pattern of genome structure whereas the pneumoviruses are quite distinct. For example the genome of the pneumovirus, respiratory syncytial (RS) virus, consists of ten genes compared to the six or seven of paramyxoviruses and morbilliviruses. In the case of the paramyxoviruses SV5, mumps and PIV-2, all but one of the genes encode a single protein. Transcription of the the P/V-gene however yields two mRNA species, V and P (Thomas *et al.*, 1988, Elliott *et al.*, 1990 and Southern *et al.*, 1990). Whilst V mRNA is transcribed faithfully from the genome, P mRNA arises from the insertion of two G residues (the mechanism for which is, as yet, unclear) at a specific point in the mRNA. This process introduces a frame-shift into the mRNA thereby allowing translation to proceed past the wouldbe termination codon utilized in V mRNA. Thus, approximately

70% of the V-protein (from its amino-terminus) is identical to the amino-terminus of the P-protein.

## Functions and roles of paramyxovirus surface glycoproteins

The surface glycoproteins, HN and F, dominate the biology of paramyxoviruses being involved in both attachment of the virus to host cell membranes and in penetration (Choppin and Scheid, 1980). The HN-protein is a type II glycoprotein ie, it is anchored in the viral membrane by the amino-terminus of the protein. The HN-protein possesses both neuraminidase (NA) and haemagglutinating (HA) activities and is present on the surface of the virus particle as a tetramer (formed by the non-covalent association of two disulphide-linked dimers; Ng *et al.*, 1989). The HA function in paramyxoviruses, in common with that of influenza-viruses, is believed to mediate attachment by binding to sialic acid-containing oligosaccharides (sialyloligosaccharides). These are a feature of many cell surface glycoproteins (and glycolipids) and removal of sialic acid abolishes binding of the attachment protein thereby preventing infection. Infectivity can be restored to de-sialated cells

by re-sialation (Paulson *et al.*, 1979) or addition of sialic acid-containing glycolipids (Bergelson *et al*, 1982 and Suzuki *et al.*, 1985). Neuraminidase removes sialic acid (N-acetyl-neuraminic acid) moieties from sialyloligosaccharides but the precise role of this function is as yet unclear. It is possible that neuraminidase functions to eliminate sialic acid residues from viral glycoproteins, thereby eliminating receptors on the virus particles themselves, thus promoting dissemination and preventing aggregation of virus particles. Evidence supporting this idea has come from studies using NA-defective mutants of influenza A viruses whose glycoproteins contained sialic acid. These viruses were prone to forming large aggregates since they contained both the receptor and its attachment protein (Palese *et al.*, 1974). The morbilliviruses, in contrast to paramyxoviruses, have no neuraminidase, and some pneumoviruses exhibit neither HA nor NA activities, although the H and G proteins of these respective genera are both involved with attachment.

The fusion (F) protein, in contrast to HN, is a type I glycoprotein, (anchored by the carboxy-terminus) and has been shown to be involved in virus penetration, haemolysis and cell fusion (Homma and Ohuchi, 1973; Scheid and Choppin, 1974). An extremely hydrophobic

domain which resides at the amino-terminus of the $F_1$ fragment of the F-protein (Paterson and Lamb, 1987) is responsible for the fusion activity. In the inactive precursor protein ($F_0$) this domain is immediately adjacent to a sequence of 1 to 5 basic amino acid residues (the cleavage/activation site). In this context the fusion domain remains "masked", unable to form stable interactions with lipid bilayers, until the nascent $F_0$-polypeptide is translocated across the plasma membrane of the rough endoplasmic reticulum (ER). Once in the lumen of the rough ER, host cell proteolytic enzymes cleave $F_0$, exposing the fusion domain of $F_1$ thereby activating the F-protein.

## Functions and roles of internal proteins of paramyxoviruses

The matrix (M) protein is involved with the processes of assembly and formation of new paramyxovirus virions during the infectious cycle. Envelopes are formed around the nucleocapsid by budding of the cellular membrane, this process being the result of the intimate adhesion of the nucleocapsid to the M-protein at the cytoplasmic

side of the cell membrane where the surface glycoproteins, HN and F, are embedded. This adhesion causes the membrane to curve into a protruding sphere surrounding the nucleocapsid. The M-protein adheres to the cytoplasmic side of the plasma membrane through hydrophobic domains and interacts with the cytoplasmic domains of the surface glycoproteins. The M-protein appears to gather the surface glycoproteins into discrete areas on the plasma membrane where the budding process occurs (Dulbecco, 1990). Interaction of the M-protein with actin-containing filaments seems to be important for budding.

The L, P, NP and possibly V-proteins all appear to play roles in transcription and replication of the RNA genome. The L-protein possesses the basic transcriptional functions, including poly-adenylation and methyl- and guanyl-transferase activities. The P-protein is also part of the polymerase complex and binds, via amino acid sequences at the carboxy-terminus (Ryan and Portner, 1990), to the NP-protein. Although the V-protein has an unknown function it has a highly conserved carboxy-terminal region which is cysteine-rich (Thomas *et al.*, 1988; Southern *et al.*, 1990). In proteins possessing similar domains it is thought that the binding of metal ions by such cysteine-rich regions may play an important part

in either the binding of nucleic acid by the protein or in mediation of protein-protein interactions (Thomas *et al.*, 1988). Paramyxoviruses need to generate three different RNA products during their infectious cycle, namely messenger and full length antigenomic and genomic RNAs. The viral polymerase responsible for mRNA synthesis is thought to bind to the template, at or near the 3' end of the genome in the non-coding leader sequence, and then to synthesize sequentially the leader and the NP, P/V, M, F, HN and L mRNAs by terminating and re-initiating at each of the non-coding spacer regions or gene junctions (see Fig. 1b). Distinct polar effects on transcription have been observed with the NP mRNA being more abundant than that for L (Glazier *et al.*, 1977; De *et al.*, 1990). This presumably occurs because the polymerase initially binds to the genome in the 3' leader sequence but then has an increasing chance of "falling off" the further it proceeds along the genome during transcription. However, it is possible that there are internal binding/initiation sites for the polymerase at the start of specific genes (De *et al.*, 1990; Ray and Fujinami, 1987).

For replication to occur the polymerase must ignore the signals in the non-coding spacer regions. Anti-genomic RNAs (ie. positive sense RNAs synthesized using the genome as a template) destined

for translation or for encapsidation with the NP-protein, in the case of measles virus, appear to be distinguished by the absence or presence respectively of a leader sequence (Casteneda and Wong, 1990). Leaderless RNAs were found to be associated with poly-ribosomes, whereas RNAs (including anti-genomic and sub-genomic species) with the leader sequence were found exclusively in ribonucleoprotein complexes and were shown to be immunoprecipitable using sera containing antibodies to the NP-protein. Thus it appears that the NP-protein is closely involved with the mechanisms that control transcription and replication of the genomes of Paramyxoviridae.

## Vaccination against paramyxovirus infections

Protective immunity following natural virus infections involves the induction and interaction of specific antibody and cell mediated (T-cell) immune responses. The antibody response is concerned primarily with the inactivation, or neutralization of free virus, while T-cells inhibit virus replication through the release of lymphokines (such as $\gamma$ interferon) or cause the direct lysis of

infected cells (for review see; Randall and Souberbielle, 1990). Attempts have been made to vaccinate against paramyxovirus infections and notable successes have now been achieved with live attenuated vaccines against mumps, measles and canine distemper viruses (Norrby, 1991). Vaccination using inactivated preparations of paramyxoviruses has in some instances been successful (eg. Newcastle disease virus in birds; Pringle, 1987) but there have also been problems with this approach. It appears that in certain circumstances inactivation of virus for vaccination can lead to exacerbation of the disease rather than protection. Early formalin-inactivated vaccines used against measles, mumps and parainfluenza viruses appeared to induce an imbalanced immune response in vaccinees, this generated antibodies to the attachment protein (HN or H) but not to the fusion (F) protein. In the case of measles an atypical form of the disease appeared in some vaccinees upon natural infection with wild type virus. However, this was seen in cases where the natural infection occurred several years after immunization. One convenient explanation of this is that upon natural reinfection the virus was able to multiply initially because of the absence of both local immunity and anti-F antibodies (presence of anti-F antibodies would prevent cell fusion and thus the

spread of virus directly from one cell to another). The subsequent release of viral antigen could then induce a secondary immune response to the proteins other than F to which the host had been primed, possibly resulting in formation of immune complexes in the lungs, inflammation and antibody-dependent cytotoxic cell proliferation (Pringle, 1987). Similarly, using formalin-inactivated RS virus, vaccination resulted in exacerbation of the disease rather than protection in vaccinees exposed to natural RS infection (Pringle, 1987). However, in contrast to the situation with measles, this occurred within a few months rather than years of vaccination. Thus, it appears that successful schedules for vaccination against paramyxovirus infections must ensure the correct presentation and complement of viral components to ensure long-lasting immunity. Perhaps the most desirable paramyxovirus vaccines will be live attenuated strains of virus. To date these have been derived by adaptation of the virus to grow in non-natural host cells, this is necessarily a somewhat stochastic process. However techniques are now available whereby genetic information can be introduced, using cDNA's, into negative stranded RNA genomes (Ballart *et al.*, 1990), this should allow for much greater control over genetic manipulation of paramyxoviruses.

## Evolutionary relationships between paramyxoviruses as revealed by antigenic and sequence analysis

SV5 and PIV-2 have been shown to be antigenically related and have, on occasion, been grouped in the same serotype (Goswami and Russell, 1982). More recently antigenic and sequence analyses have begun to reveal varying degrees of evolutionary relatedness between paramyxoviruses. Ito *et al.* (1987), using a series of polyvalent antisera against virions and virus proteins, suggested that Sendai and human PIV-1 and 3 should be placed in one major antigenic group and that mumps, SV5 and human PIV-2 and 4 be placed in another. They also suggested a third group comprising mumps, and human PIV-1, 2, 3 and 4 viruses which overlapped the first two groups. Goswami and Russell (1982) concluded that SV5 and PIV-2 are antigenically related, as are PIV-1 and PIV-3. Örvell *et al.* (1986) also concluded, using monoclonal antibodies (MAbs), that Sendai and PIV-3 were antigenically related as were Sendai and mumps, however they found no antigenic cross-reaction between PIV-3 and mumps viruses. Rydbeck *et al.* (1988) using MAbs to PIV-2-HN found only limited cross-reaction between SV5 and PIV-2 and none

between PIV-2 and Sendai, PIV-3, mumps, Newcastle disease, measles or canine distemper viruses. Various workers have also used antigenic studies to investigate the extent of intratypic variation between individual strains of different viruses. Rydbeck *et al.* (1986) using MAbs found antigenic variation between strains of mumps virus isolated over a 10 to 15 year period. Coelingh *et al.* (1985), also using MAbs, found a range of variability between epitopes on the HN-protein of human PIV-3. In a more limited study using MAbs Rydbeck *et al.* (1987) showed only minor variations between isolates of human PIV-3 but extensive variation between human and bovine PIV-3 isolates.

Computer aided sequence analysis has revealed varying degrees of homology between members of the Paramyxoviridae. In general, studies have shown that the internal, NP and M-proteins tend to be more highly conserved than P and L (Sakai *et al.*, 1987). Of the surface glycoproteins, the F-protein appears to be more highly conserved than the HN-protein (Suzu *et al.*, 1987). Sequence data for all genes of most paramyxoviruses is, as yet, incomplete. However, nucleic acid sequences of the HN-genes of eight different paramyxoviruses have been determined and comparisons of the predicted amino acid sequences demonstrates the varying degrees of

**Table 2**. Proportion (as percentages) of identical amino acids between HN-proteins of paramyxoviruses.

| | PIV-4 | SV5 | Mumps | NDV | Sendai | Human PIV-3 | Bovine PIV-3 |
|---|---|---|---|---|---|---|---|
| PIV-2 | 41.6 | 48.3 | 42.6 | 34.5 | 21.7 | 22.9 | 23.3 |
| PIV-4 | | 39.4 | 38.1 | 31.8 | 21.9 | 21.9 | 22.7 |
| SV5 | | | 46.2 | 34.7 | 23.1 | 22.9 | 23.9 |
| Mumps | | | | 32.5 | 22.1 | 22.5 | 22.3 |
| NDV | | | | | 24.9 | 22.3 | 22.7 |
| Sendai | | | | | | 48.7 | 50.1 |
| Human PIV-3 | | | | | | | 77.5 |

From Kawano *et al.* (1990).

relatedness between those viruses (see Table 2). In addition to the overall homologies between proteins, patterns of homology have been shown to exist within some proteins. For instance, although overall homology between the F-proteins of SV5 and measles viruses is about 25%, homology within the protein varies from 76% at the N-terminus of the $F_2$-polypeptide to negligible homology at the extreme ends of the $F_0$-polypeptide (Buckland *et al.*, 1987). The M-protein shows the highest overall homology between proteins of Paramyxoviridae. The same type of analysis has shown that there is, in general, a high degree of conservation of sequences between different strain isolates of specific paramyxoviruses. However, small but significant differences have been revealed. For example, van Wyke Coelingh (1988) showed differences of between 1·2 and 3·8% in the amino acid residues of the HN-proteins of isolates of

PIV-3. Millar *et al.* (1988) demonstrated differences of 6% in the amino acid sequences of both the HN and F-proteins from the Beaudette C and Ulster strains of Newcastle disease virus.

## Aims of this study

When this study was undertaken, in 1986, there were two main aims: One of these was to determine more fully the extent to which SV5 and PIV-2 are related and to put this into the context of antigenic relationships between and with other paramyxoviruses. The other main aim was to examine the antigenic variations that exist between strains of SV5 isolated from different species and thereby, begin to address the question: Are such variations sufficient to prevent SV5 crossing species barriers or does SV5 move freely between species?

# METHODS

## Growth and maintenance of cells and viruses

Vero and BHK cells (Flow Laboratories) were grown as monolayers in 75 $cm^2$ tissue culture flasks or in rotating 80 oz Winchester bottles, in Glasgow's modification of Eagles tissue culture medium containing 10% newborn calf serum. Five human isolates (designated LN, RQ, DN, NR, MT; Goswami *et al.*,1984), three canine isolates (M7, CP1+ and CP1-; Cornwell *et al.*, 1976, Baumgärtner *et al.*, 1981 and Baumgärtner *et al.*, 1987 respectively) and a prototype simian isolate (Pr; obtained from Central Public Health Laboratory, Colindale, London) of SV5, two field isolates of PIV-2, a prototype isolate of PIV-2 and a prototype isolate of PIV-3 (all from C.P.H.L. Colindale, London) were used in these studies. These viruses, together with the Edmonston strain of measles and the Enders strain of mumps (kindly provided by Dr. B. K. Rima, Queen's University of Belfast, Northern Ireland), were grown and titrated under appropriate conditions in vero cells using medium containing 2% calf serum. All the virus stocks were plaque purified and the virus stocks obtained were free from mycoplasma contamination (when tested on

agar plates formulated to support the growth of mycoplasmas). Furthermore, special emphasis was placed on obtaining virus stocks free from high levels of defective interfering particles. This was achieved by infecting the monolayers with approximately 0·1 pfu/cell and harvesting the virus present in the culture medium when 70 to 80% of the cells were showing a cytopathic effect (cpe). All virus stocks were harvested before 30 hrs post infection.

## Purification of viruses

Monolayers of vero cells in rotating 80 oz Winchester bottles were infected with LN strain of SV5 or prototype strain of PIV-2 at 0·1 pfu/cell. When 10 to 20% of the cells showed a cpe the medium was decanted, the monolayers washed twice with warm phosphate-buffered saline (PBS) and the infected cells were reincubated at 37° C in medium without calf serum until 80% of the cells showed a cpe (6 to 12 hrs later). The medium was harvested and the small amount of cell debris present pelleted by low speed centrifugation (10,000 x g for 15 min). The cleared medium was then centrifuged at 70,000 x g for 3 hrs, the virus pellet collected and stored at -70°C until required.

## Immunization of mice, production and selection of antibody-secreting hybridomas and nomenclature for monoclonal antibodies

Inbred BALB/c mice were immunized intraperitoneally with purified preparations of the LN isolate of SV5 or the prototype isolate of PIV-2 (250μg of protein per immunization) precipitated in alum (Chase, 1967). At the same time the mice were immunized with a fixed and killed suspension of Bordetella pertussis ($2.10^9$ cells per mouse) to enhance the immune response (Kohler, 1981). After 28 days, mice were reimmunized intraperitoneally with virus only, and after a further 21 days they were boosted by both intraperitoneal and tail vein immunizations. Four days later the spleens were removed, the splenocytes fused with SP2/0-Ag-14 myeloma cells (Shulman *et al.*, 1978) and the hybrid cells were plated out in 96-well microtitre plates such that, on average, one colony of cells grew in every other well under selective growth conditions. Cell culture conditions were based on those previously described (Fazekas de St.Groth & Scheidegger, 1980; Kohler & Milstein, 1975). Hybridomas secreting antibodies specific for virus antigens were

differentiated from those secreting antibody to uninfected cell antigens using a method in which infected or uninfected cell antigens were bound to sheets of nitrocellulose, media from hybridoma cells were then incubated with the nitrocellulose sheets, and bound antibody was detected using $^{125}$I-labelled Protein A or by ELISA (enzyme-linked immunosorbent assay) using horse radish peroxidase conjugated anti-mouse immunoglobulin (SAPU, Carluke, U.K.). Full details of the technique have been published elsewhere, as have the methods for subcloning hybridomas secreting specific antibody to virus antigens and for the production of ascitic fluids (Randall *et al.*, 1984). Each MAb was originally given a number corresponding to the hybridoma colony tested. Subsequently when the reactivity of the MAbs had been established, they were given capital letters corresponding to the virus protein recognized, followed by trivial small letters as identifying labels for individual antibodies, eg. HN-a. Monoclonal antibodies that reacted with the HN-proteins of SV5 and PIV-2 were tested for their ability to compete with one another for binding to those proteins. If any competition between two antibodies was noted these antibodies were placed into a group which was given a number that was incorporated into the name of a particular antibody eg. HN-4a, HN-5b

etc. The full name of the antibody was then prefixed with SV5 or PIV-2 to denote the antibody was isolated from mice immunized with SV5 or PIV-2 respectively eg. SV5-HN-4a. It should be noted that the nomenclature for the two MAbs to the HN glycoprotein of SV5 previously isolated by Goswami and Russell (1983) has not been altered significantly ie. MAb HN-1 in their nomenclature is now referred to as MAb SV5-HN-1a. The procedures used above, for the isolation of hybridoma cell lines secreting monoclonal antibodies to SV5 and PIV-2, were carried out in collaboration with Dr. R. E. Randall.

**Preparation of radiolabelled antigen extracts, immune precipitation and SDS-PAGE**

Vero or BHK cell monolayers in 75 $cm^2$ tissue culture flasks or in rotating 80 oz Winchester bottles were infected with virus at 0·1 pfu/cell. After an adsorption period of 2 hrs at 37°C, the inoculum was removed and replaced with tissue culture medium containing 2% newborn calf serum. When 10 to 20% of the cells showed a cpe they were radioactively labelled for 4 to 6 hrs with L-[$^{35}$S]methionine (500 Ci/mmol; Amersham) in tissue culture medium containing

1/10th the normal concentration of non-radioactive methionine (ie. 1·5 mg/ml). At the end of the labelling period, the cells were washed in ice-cold PBS and lysed into immune precipitation buffer (20mM Tris-HCl pH 7·8, 5mM EDTA, 0·5% NP40, 0·65M NaCl and in some experiments 0·1% SDS; 4 to $10.10^6$ cells per ml of buffer) by sonication with an ultrasonic probe. Soluble antigen extracts were obtained after pelleting particulate material from these total cell extracts by centrifugation at 400,000g for 30 min. Immune complexes were formed by incubating 200µl samples of the soluble antigen fraction with an excess of antibody (1µl of undiluted ascitic fluid) for 2 hrs at 4°C. If the antibody involved bound protein A directly the immune complexes were isolated on an excess of a fixed and killed suspension (Kessler, 1975) of the Cowan A strain of *Staphylococcus aureus* (*S. aureus*; 20µl of a 10% w/v suspension per µl of ascitic fluid for 30 min at 4°C). If the antibody did not bind protein A directly, these complexes were isolated in an identical manner, except that preparations of fixed and killed *S. aureus* that had been previously saturated with sheep anti-mouse immunoglobulin (SAPU, Carluke, U.K.) were used (50µl of a 10% w/v suspension per µl of ascitic fluid). This latter method is an extremely simple way of precipitating antibodies that do not bind

protein A directly. The immune complexes on *S. aureus* were washed 3 times by suspension in and sedimentation (2,500g for 3 min) from immune precipitation buffer. The proteins in the immune complexes were dissociated by heating (100°C for 2 min) in gel electrophoresis sample buffer (50mM Tris-HCl pH 7·0, 2·0% SDS, 5% 2-mercaptoethanol and 2·5% glycerol), and analysed on 10 or 15% SDS-polyacrylamide slab gels: separating gels (10 or 15%w/v acrylamide:diallyltartardiamide [40:1w/w], 0·375M Tris-HCl pH 8·6, 0·1%w/v SDS, 0·035%w/v ammonium persulphate and 0·05% N,N,N',N'-Tetramethylethylenediamine [TEMED]) were overlayed with a stacking gel (5%w/v acrylamide:diallyltartardiamide [40:1 w/w], 0·12M Tris-HCl pH 7·0, 0·1%w/v ammonium persulphate and 0·06% TEMED). After electrophoresis, gels were either stained with PAGE-blue (BDH) and destained in acetic acid-methanol or were stained using a Bio-Rad silver staining kit and dried. Labelled polypeptides were visualized by autoradiography with Fuji X-ray film.

**Radioimmune competition assay**

Monoclonal antibodies were purified on a Protein A-Sepharose

column (Goswami & Russell, 1983) and radioactively labelled with $^{125}I$ using the chloramine-T method (Hunter, 1978). $^{125}I$-labelled MAbs were titrated against infected cell antigen bound to nitrocellulose using an assay that has been described elsewhere (Randall *et al.*, 1984) with the modification that the nitrocellulose sheet was sandwiched between 84-well Terasaki plates (10µl of diluted antibody per well). Unlabelled MAbs were then tested for their ability to compete with the binding of the $^{125}I$-labelled antibodies by making four-fold dilutions of ascitic fluids (starting at a 1/20 dilution) in an appropriate dilution of the labelled antibody in PBS containing 1% bovine serum albumin. The antibodies were then reacted with virus antigens bound to nitrocellulose as described previously (Randall *et al.*, 1984).

**Assay to ascertain the reactivity of MAbs to various isolates of SV5 and PIV-2 and to other paramyxoviruses**

Vero cells, grown as monolayers in 96-well microtitre plates, were infected with the different viruses. When the cells showed an 80% cpe (24-30 hrs post-infection) the cells were fixed in PBS containing 5% formaldehyde and 2% sucrose for 10 min at 20°C. The

monolayers were then washed three times with PBS, permeabilized with PBS containing 0·5% NP40 and 10% sucrose for 5 min at 20°C and washed three times in PBS containing 1% calf serum. The monolayers were reacted for 1 hr at 20°C with MAbs (as ascitic fluid diluted 1/500 in PBS containing 1% calf serum). The monolayers were washed four times with PBS containing 1% calf serum and incubated for 1 hr with $^{125}$I-labelled protein A (Amersham). If the Mabs did not bind protein A directly the washed monolayer was first treated for 1 hr at 20°C with rabbit anti-mouse immunoglobulin (Nordic Immunological Laboratories, Maidenhead, U.K: diluted 1/10,000 in PBS containing 1% calf serum) and washed four times with PBS containing 1% calf serum before the addition of $^{125}$I-labelled protein A. The monolayers were then washed once with PBS containing 0·1% NP40 and three times with PBS, dried and exposed to Fuji X-ray film with an intensifying screen at -70°C.

**Neutralization test**

100µl aliquots of 2-fold dilutions of MAbs (as ascitic fluids diluted in tissue culture medium containing 2% calf serum and virus at $1.10^5$ pfu/ml) were incubated at 37°C for 2 hrs. The

antibody-virus mixtures were then transferred to vero cell monolayers in 96-well plates and reincubated at 37°C for 3 to 4 days. They were then either fixed with 10% formol-saline and stained with crystal violet or, treated as in the assay described above for ascertaining reactivities of MAbs to various para-myxoviruses except that a pool of MAbs was used to detect viral antigens. The neutralization titre was taken as the highest dilution of ascitic fluid that gave a total reduction in either the cpe or the amount of SV5-specific antigen present in cells as judged by the radio-immune assay.

**"Western blot" tests**

Polypeptides separated on polyacrylamide gels were transferred to sheets of nitrocellulose by transverse electrophoresis using a semi-dry multi-gel blotter (Ancas Aps from Dako Ltd., High Wycombe, U.K.). After transfer the filters, bearing the electrophoretically separated polypeptides, were blocked with PBS containing 10% milk powder (Marvel) overnight at 4°C, incubated with antibody (1/500 dilution of ascitic fluid in PBS containing 0·1 % NP40) for 1 hr at 20°C and then washed three times with PBS

containing 0·1% NP40. The filters were incubated at 20°C for 1 hr with $^{125}$I-labelled protein A (Amersham; 0·5μCi/ml in PBS), washed four times with PBS, dried and exposed to X-ray film with an intensifying screen at -70°C.

## Production of antisera

Polyvalent antisera to purified preparations of SV5 and PIV-2 were raised in BALB/c mice using the immunization scheme described for the production of MAbs. Monospecific polyclonal anti-SV5-HN or PIV-2-HN sera were prepared in BALB/c mice by two intraperitoneal injections of 5-10μg of the HN protein, coupled to equimolar amounts of the MAbs SV5-HN-4a or PIV-2-HN-1a respectively, in a manner described in detail elsewhere (Randall and Young, 1988). Briefly, fixed and killed Staph A. as used for immune precipitation tests (described above) was saturated with the appropriate MAb by mixing equal volumes of Staph A. (10%w/v) and undiluted ascitic fluid, washed, then saturated with the corresponding antigen, by incubation of these MAb-Staph A complexes with soluble antigen extracts of SV5 or PIV-2 infected cells. The resulting complexes were washed and finally resuspended

in sterile PBS then frozen at -70°C until required.

## Immunofluorescence

Vero cells were grown as monolayers on 12 well multispot microscope slides (C.A. Hendley [Essex] Ltd., Loughton, U.K.), infected with SV5 or PIV-2 at 0·1-1·0 pfu/cell. 24 hrs post infection the monolayers were fixed, permeabilized and indirectly stained for immunofluorescence with specific MAbs as described by Randall and Dinwoodie (1986).

# RESULTS

## Production and characterization of monoclonal antibodies to SV5 and PIV-2

Fig. 2 shows a silver-stained profile of the polypeptides present in a sample of purified SV5 virus obtained using the simple and rapid purification technique detailed in Methods. Preparations of SV5 and PIV-2 of such purity were used to immunize mice in the production of monoclonal antibodies (MAbs). It was estimated that at least 70 percent of the protein present in such preparations was of viral origin. Anti-SV5 MAbs were characterized in terms of their specificities and their abilities to bind protein A, neutralize virus infectivity and react in Western blot tests.

Immune precipitation tests showed that the 54 MAbs raised against SV5 had specificities for the haemagglutinin-neuraminidase (HN), fusion (F), matrix (M), nucleocapsid (NP) and phospho- (P) proteins (Fig. 3). A summary of the specificities of all these MAbs is given in Table 3. The anti-F MAb SV5-F-1a immune precipitated both the precursor $F_0$-polypeptide and its processed form $F_1$ and $F_2$ which dissociate under reducing conditions used in SDS-PAGE. Anti-P MAbs

**Fig. 2** Analysis of the total polypeptide content of preparations of the SV5 isolate LN used to immunize mice for the production of hybridomas. The polypeptides were separated through a 10% SDS-polyacrylamide slab gel and were visualized by silver staining the gel. The positions of the viral polypeptides are shown in the left-hand column. The mol. wts. of the viral polypeptides, shown in the right-hand column, were estimated by comparison with the electrophoretic mobilities of proteins with known molecular weights (Bethesda Research Laboratories protein mol.wt. standards; data not shown).

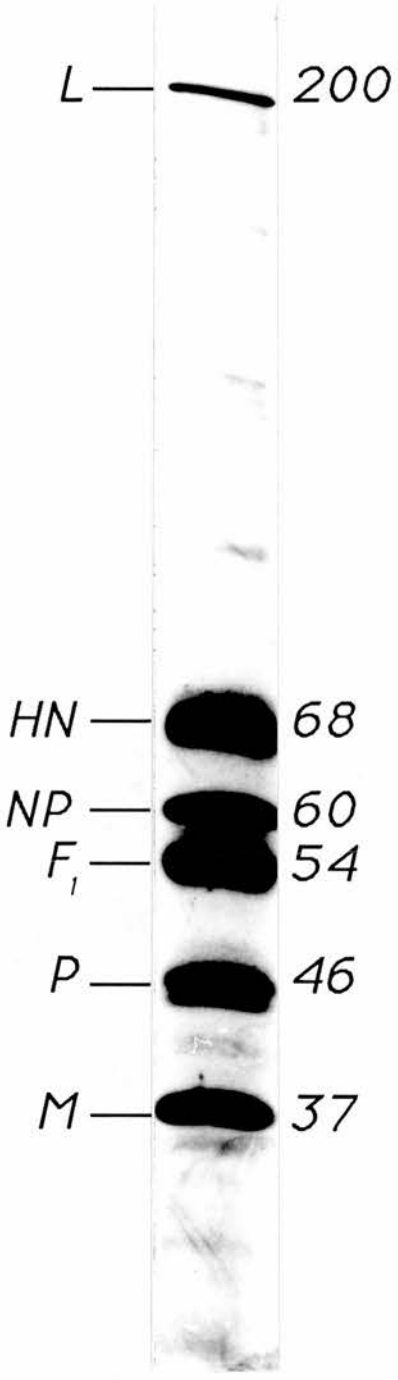

L
200
HN
68
NP
60
F1
54
P
46
M
37

**Fig. 3** Analysis of $^{35}$S-methionine-labelled polypeptides, separated by electrophoresis through a 15% SDS-polyacrylamide slab gel, from immune precipitates formed by the reaction of soluble antigen extracts of SV5(Pr)-infected BHK cells with either a mouse polyclonal anti-SV5 serum (lane 1), or with MAbs to the HN (lane 2), F (lane 3), NP (lane 4), P (lanes 5 and 7) and M (lane 6) polypeptides. The buffer used to prepare the soluble antigen extracts for the immune precipitates shown in lanes 1 to 6 contained both 0·1% SDS and 0·5% NP40 as detergents, whereas that used for the immune precipitate shown in lane 7 contained 0·5% NP40 but no SDS. The labelled polypeptides were visualized by autoradiography. The positions of the SV5 polypeptides are indicated at the left hand-side.

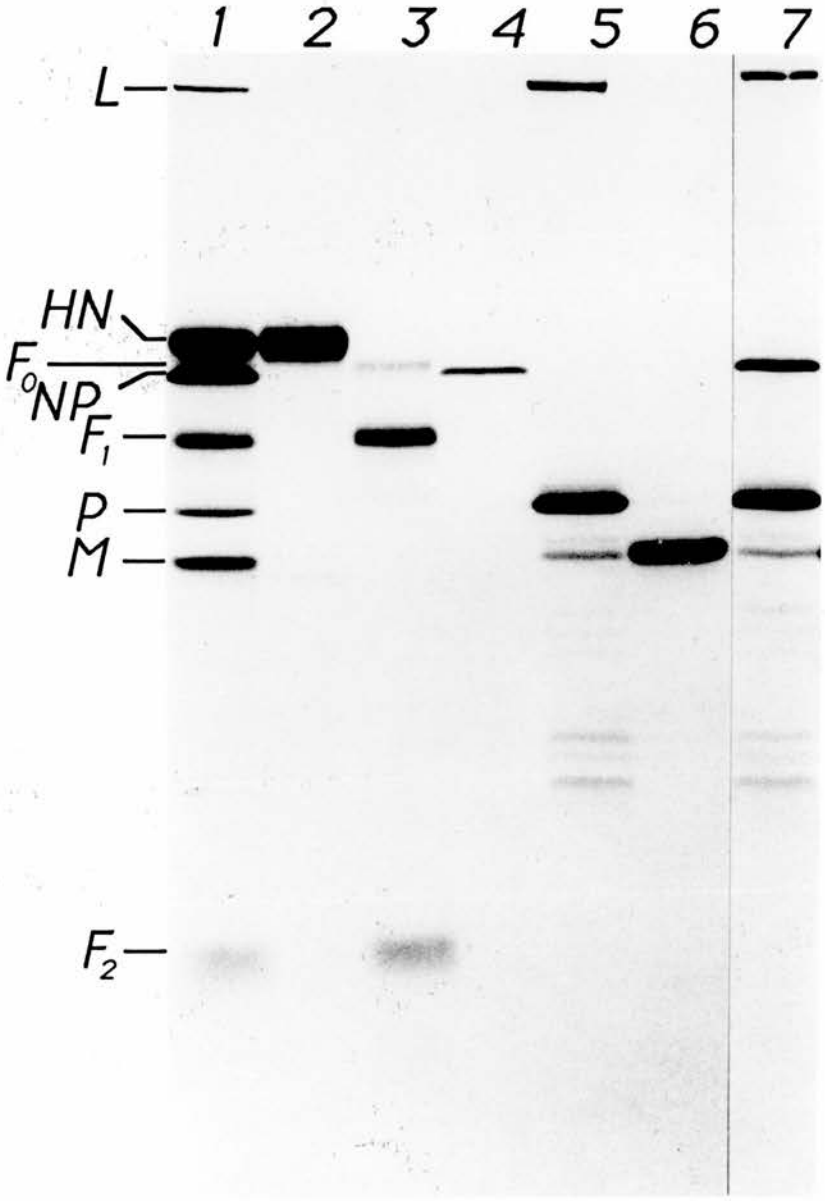

1
2
3
4
5
6
7
L
HN
$F_0$
NP
$F_1$
P
M
$F_2$

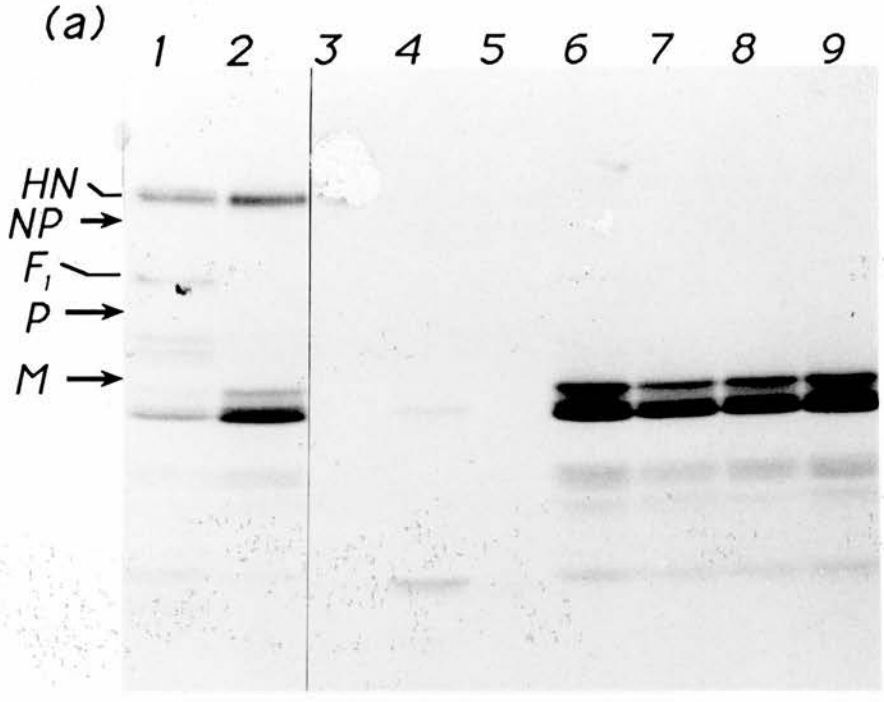
**Table 2.** Summary of the characteristics of monoclonal antibodies raised against SV5 and PIV-2.

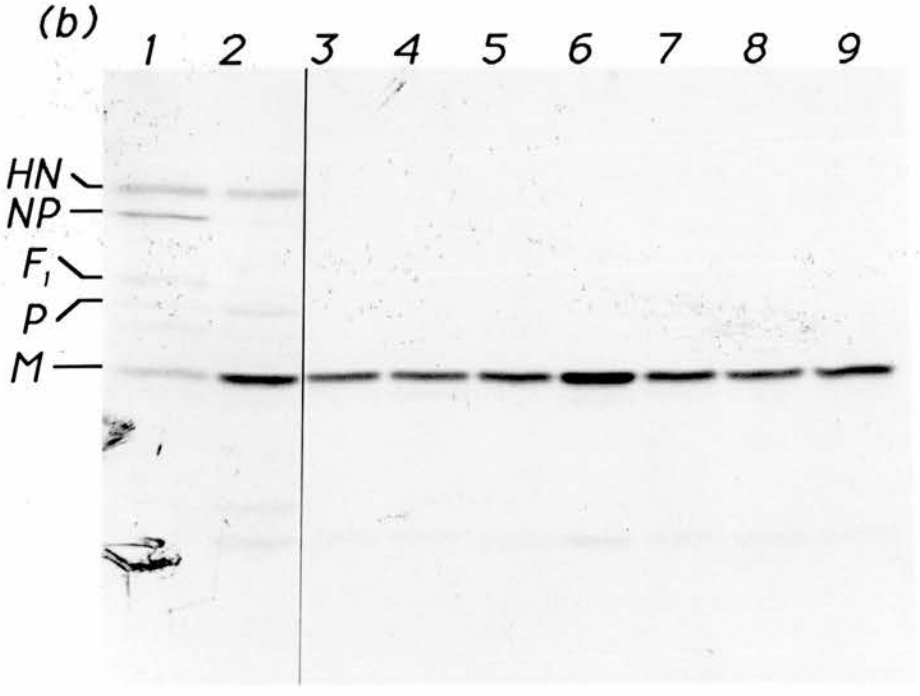
| Monoclonal antibody | Western blot activity | Binds Pr.A. | Neutralizes virus (titre[a]) | Reacts with SV5 isolated from: humans | dogs | monkeys | Reacts with PIV-2 |
|---|---|---|---|---|---|---|---|
| SV5-F-1a | - | + | + (1/8000) | + | - | + | - |
| SV5-HN-1a | - | + | + (1/1000) | + | + | + | - |
| HN-1b | - | + | + (1/4000) | + | + | + | - |
| HN-1c | - | + | + (1/200) | + | + | + | - |
| HN-2a | Not available | | | | | | |
| HN-3a | - | - | + (N.D.[b]) | + | + | - | - |
| HN-4a | - | + | + (1/128000) | + | + | + | - |
| HN-4b | - | + | + (1/2000) | + | - | + | - |
| HN-4c | - | w | + (1/64000) | + | + | + | - |
| HN-4d | - | + | + (N.D.) | + | + | + | - |
| HN-4e | - | + | + (1/8000) | + | + | + | - |
| HN-4f | - | + | + (1/128000) | + | + | + | - |
| HN-5a | - | + | + (1/12800)0) | + | + | + | - |
| HN-5b | - | w | + (1/64000) | + | + | + | - |
| HN-5c | - | + | + (1/16000) | + | + | + | - |
| HN-5d | - | + | + (1/16000) | + | + | + | - |
| HN-5e | - | + | + (1/16000) | + | + | + | - |
| HN-x | - | - | + (1/8000) | + | N.D. | N.D. | N.D. |
| HN-y | - | - | + (1/8000) | + | + | + | - |
| HN-z | - | + | +(1/1000) | + | - | + | - |
| SV5-M-a | - | - | N.D. | + | + | + | - |
| M-b | - | + | N.D. | + | + | + | - |
| M-c | - | + | N.D. | + | + | + | - |
| M-d | - | - | N.D. | + | + | + | - |
| M-e | - | - | N.D. | + | + | + | - |
| M-f | - | + | N.D. | + | + | + | - |
| M-g | - | - | N.D. | + | + | + | - |
| M-h | + | + | N.D. | + | + | + | - |
| M-i | - | + | N.D. | + | + | + | - |
| M-j | - | - | N.D. | + | + | + | - |
| M-k | - | - | N.D. | + | + | + | - |
| M-l | - | + | N.D. | + | + | + | - |
| M-m | - | + | N.D. | + | + | + | - |
| M-n | - | + | N.D. | + | + | + | - |
| M-o | - | + | N.D. | + | + | + | - |
| M-p | - | + | N.D. | + | + | + | - |
| M-q | - | - | N.D. | + | + | + | + |
| M-r | - | - | N.D. | + | + | + | - |
| M-s | - | w | N.D. | + | + | + | + |
| M-t | - | + | N.D. | + | + | + | - |
| M-u | - | w | N.D. | + | + | + | - |
| M-v | - | + | N.D. | + | + | + | - |
| SV5-NP-a | - | + | N.D. | + | + | + | - |
| NP-b | - | + | N.D. | + | + | + | - |
| NP-d | + | - | N.D. | +[c] | + | + | - |
| SV5-P-a | + | + | N.D. | + | + | - | - |
| P-b | + | + | N.D. | + | + | + | - |
| P-c | + | + | N.D. | + | + | + | - |
| P-d | + | + | N.D. | + | ± | ± | - |
| P-e | + | + | N.D. | + | + | + | + |
| P-f | + | + | N.D. | + | + | + | - |
| P-g | + | - | N.D. | + | + | + | - |
| P-h | + | + | N.D. | + | + | - | - |
| P-i | + | - | N.D. | + | + | + | - |
| P-j | + | + | N.D. | + | + | + | - |
| P-k | + | + | N.D. | + | +[d] | + | - |
| PIV-2-HN-1a | - | + | + (N.D.) | - | - | - | + |
| HN-2a | - | + | + (N.D.) | - | - | - | + |
| HN-3a | - | + | + (N.D.) | - | - | - | + |
| PIV-2-NP-a | - | + | N.D. | + | + | + | + |
| NP-b | - | - | N.D. | + | + | + | + |
| PIV-2-P-a | + | + | N.D. | - | - | - | + |

[a] Highest dilution of ascitic fluid that neutralizes virus infectivity.
[b] N.D. Not determined.
[c] Reacts with DN, LN and RQ but not with MT or NR.
[d] Reacts with M7 and CP1+ but not with CP1-.

immune precipitated complexes of the L, NP and P-proteins from standard soluble antigen extracts of infected cells (ie. in which 0·5% NP40 was the sole detergent). Addition of 0·1% SDS to these soluble antigen extracts resulted in the release of the NP-protein from such complexes (Fig. 3; compare lanes 5 & 7). A higher concentration of SDS (0·5%) was required to dissociate the P and L-proteins (data not shown). Anti-NP MAbs also precipitated complexes of L, NP and P-proteins in the absence of SDS although the level of contamination, by these complexes obtained using anti-NP MAbs, was lower than when using anti-P MAbs. Longer exposures of autoradiograms of immune precipitation tests showed the presence of numerous specific minor bands particularly in the case of immune precipitates using anti-P MAbs (eg. see Fig. 3 lanes 5 and 7). Immune precipitation tests using BHK and vero cell extracts demonstrated that soluble antigen extracts made from infected vero cells are more susceptible to proteolysis than those made from infected BHK cells (Fig. 4). However not all proteins showed the same degree of susceptibility to proteolysis, the HN and F-polypeptides showed very low levels of or no degradation in the same extracts that exhibit extensive degradation of for instance the M-polypeptide (Fig. 4a). In addition, the array of proteolytic fragments immune precipitated by

**Fig. 4** Analysis of $^{35}$S-methionine-labelled polypeptides present in immune precipitates formed by the reaction of mouse polyvalent anti-SV5 serum (lanes 1), a mixture of MAbs to the HN, NP, P and M-proteins of SV5 (lanes 2) or anti-SV5 MAbs M-f, h, i, k, p, c and m (lanes 3 to 9 respectively) with soluble antigen extracts of SV5 infected (a) vero or (b) BHK cells. The polypeptides were separated by electrophoresis through a 15% SDS-polyacrylamide slab gel and visualized by autoradiography. The positions of the SV5 polypeptides are indicated in the left hand column, positions with arrows are those of intact polypeptides where proteolysis has occurred.

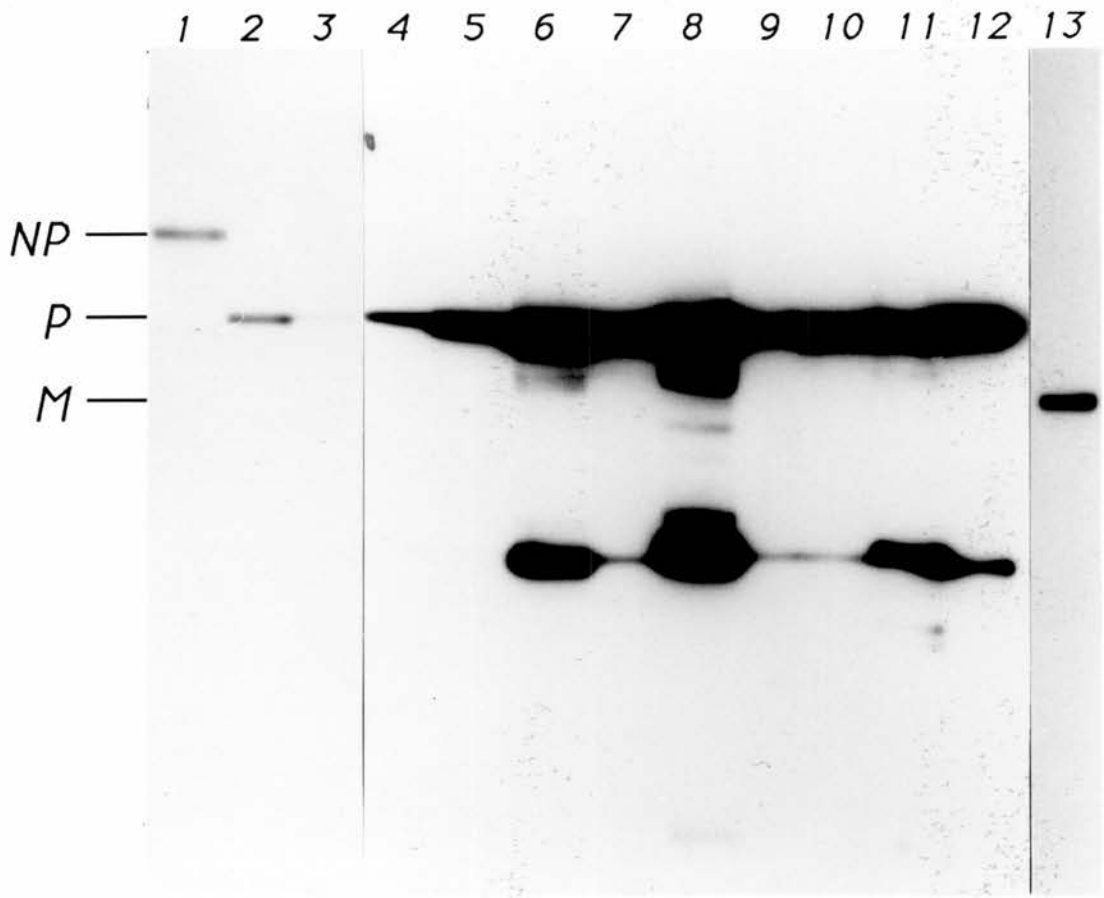

(a)
1 2 3 4 5 6 7 8 9
HN
NP
F1
P
M


(b)
1 2 3 4 5 6 7 8 9
HN
NP
F1
P
M

anti-M MAbs was dependent on the particular MAb being used (Fig. 4a; compare lanes 3 to 9). This presumably indicated the presence of different antigenic determinants on the M-protein residing on different proteolytic fragments. Other studies showed that fewer minor polypeptide bands existed in immune precipitates formed when using soluble antigen extracts of purified virus than when the extracts were made from infected cell lysates (data not shown). This was in the absence of noticeable proteolysis of other virus proteins.

All MAbs to the surface glycoproteins, HN and F, of SV5 showed neutralization of SV5 (data not shown), the titres of neutralization varied from 1/200 to 1/128,000. None of these antibodies reacted in Western blot tests. In contrast however, all 11 anti-P MAbs reacted in such tests (Fig. 5). In addition one each of the anti-NP (SV5-NP-d) and anti-M (SV5-M-h) MAbs reacted in Western blot tests (Fig. 5). Results of characterization tests are summarized in Table 3. Western blot tests using the anti-P MAbs also showed a series of minor polypeptide bands (Fig. 5 see also Fig. 11), several with apparent molecular weights higher than that of the P-polypeptide itself (see Fig. 11), this is consistent with immune precipitation

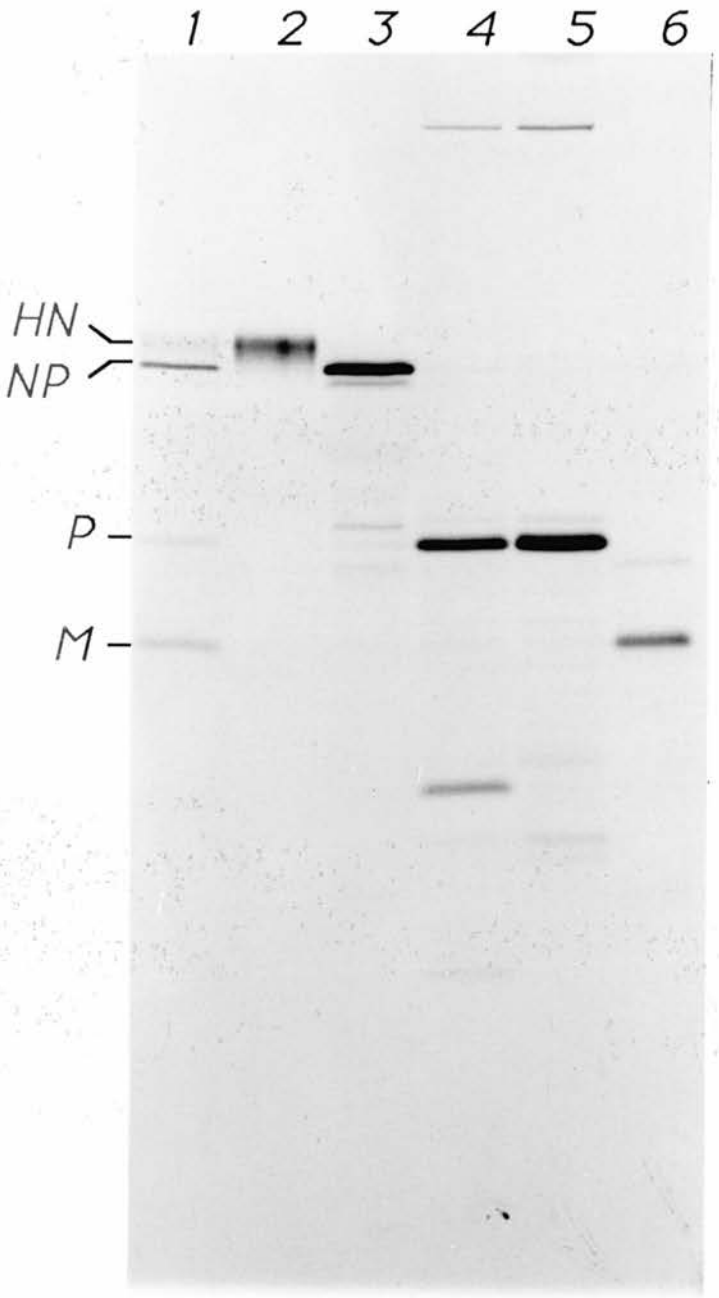
**Fig. 5** "Western blot" showing the reactivities of anti-SV5 monoclonal antibodies with SV5. SV5 infected cell polypeptides were separated by electrophoresis through a 15% SDS-polyacrylamide slab gel, electrophoretically transferred to a nitrocellulose membrane and reacted with monoclonal antibody NP-d (lane 1), all anti-P (lanes 2 to 12) or M-h (lane 13) monoclonal antibodies (all as 1/500 dilutions of ascitic fluid). Bound antibody was detected with $^{125}$I-labelled protein-A and visualized by autoradiography. The positions of the NP, P and M-polypeptides of SV5 are shown in the left hand column.


1 2 3 4 5 6 7 8 9 10 11 12 13
NP
P
M

studies. These other P-related polypeptides may appear as a consequence of translation of mRNAs of variable length arising from errors in transcription. It is known that full length P-mRNA transcription requires the insertion of non-templated ribonucleotide residues at a specific point in the genes coding for the P-proteins of SV5 (Thomas *et al.*, 1988) and PIV-2 (Southern *et al.*, 1990). It may be that similar but erroneous, insertions or deletions may occur at other points in the P-gene, resulting in variable length mRNAs.

The anti-PIV-2 MAbs were similarly characterized. Immune precipitation studies showed that the 6 anti-PIV-2 MAbs had specificities for the HN, NP and P-proteins of PIV-2 (Fig. 6). All 3 anti-HN MAbs neutralized PIV-2 infectivity (data not shown), and the anti-P MAb showed reactivity in a Western blot test (data not shown). Results of characterization studies are summarized in Table 3. The anti-PIV-2-P MAb also immune precipitated complexes of L, NP and P-proteins. Longer exposures of autoradiograms of immune precipitations using the anti-PIV-2-P MAb also showed the presence of minor polypeptide bands related to the P-polypeptide. Again soluble antigen extracts of PIV-2 infected vero cells showed greater susceptibility to proteolysis than those made using BHK cells (data

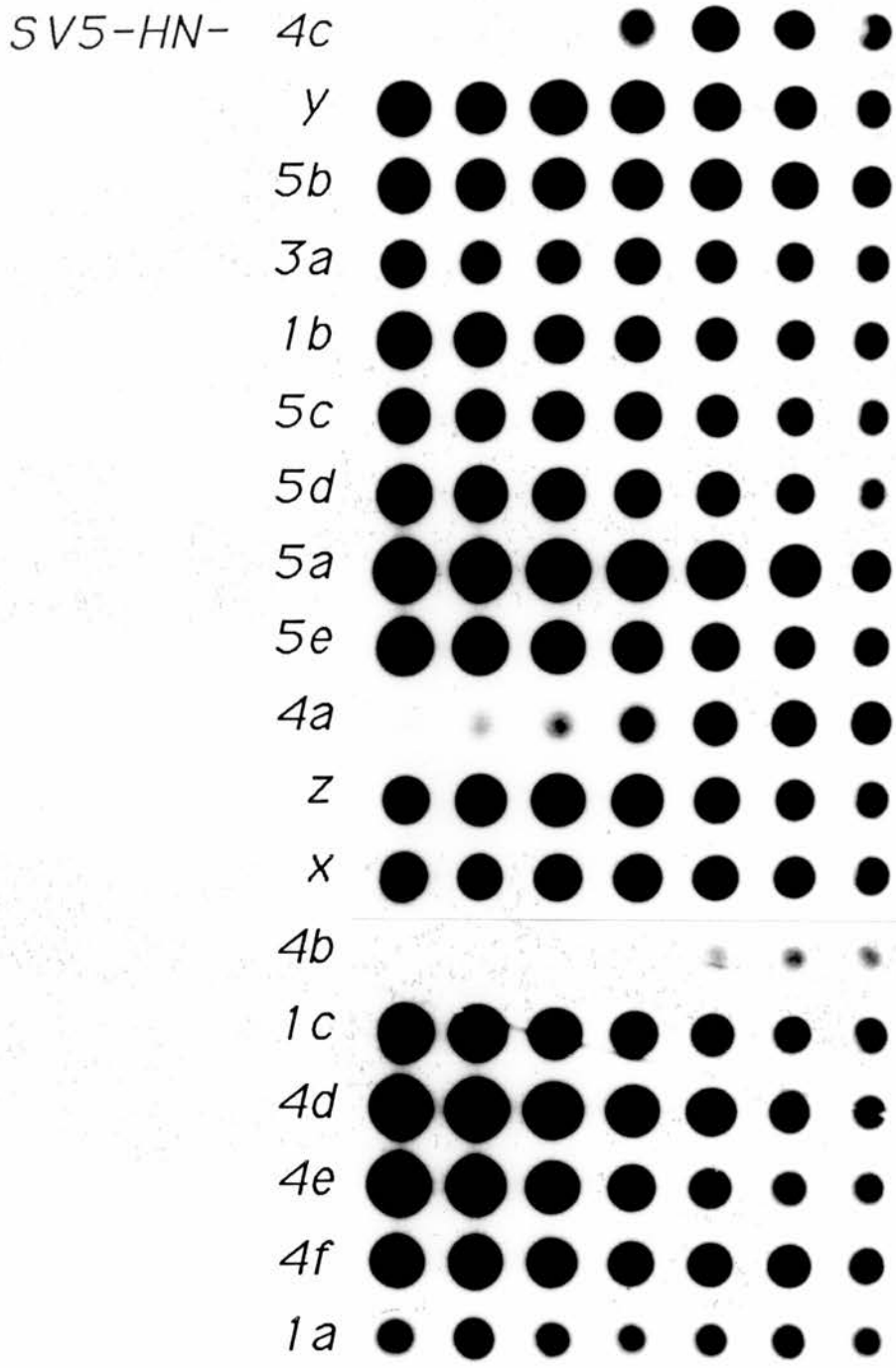
**Fig. 6** Analysis of $^{35}$S-methionine-labelled polypeptides, separated by electrophoresis through a 15% SDS-polyacrylamide slab gel, from immune precipitates formed by the reaction of soluble antigen extracts of PIV-2 infected BHK cells with, mouse polyvalent anti-PIV-2 serum (lane 1), monoclonal antibodies raised against PIV-2 with reactivities to the HN (lane 2), NP (lane 3) or P-proteins (lane 4), or with monoclonal antibodies raised against SV5 that cross-react with PIV-2 having specificities for the P (lane 5; SV5-Pq) or M-proteins (lane 6; SV5-Ms). The polypeptides were visualized by autoradiography.


1
2
3
4
5
6
HN
NP
P
M

not shown).

Neutralization tests showed a wide range in the titres of neutralization between anti-HN MAbs, presumably because the MAbs recognize different antigenic sites on the HN-proteins both of SV5 and PIV-2. The MAbs that reacted with the HN-proteins of SV5 and PIV-2 were therefore examined specifically for their abilities to bind to different regions of the respective HN-proteins by use of a radio-immune competition assay. Fig. 7 shows an example of such a test. By this method the anti-HN MAbs were assigned to different groups, the criteria for assignation to a group being that any member of a group showed competitive binding with at least one other member of the same group but not with any members of another group. An antigenic site is defined here as the region of the HN-protein recognized by one group of MAbs. These antigenic sites can be divided into smaller overlapping immunogenic regions called epitopes (defined as the minimum region of the HN-protein required for the binding of an antibody). Thus MAbs that bind to the same antigenic site may not recognize the same epitope within that site. For example the anti-SV5 MAbs HN-4a and 4c competed poorly with the binding of $^{125}$I-labelled HN-4b in the same test that HN-4b was

**Fig. 7** Illustration of the radioimmune competition assay used to determine whether anti-HN MAbs bound to different antigenic sites on the HN-protein of SV5. Serial four-fold dilutions (starting at 1/20 dilution in the left-hand well of each row) of ascitic fluids of the anti-HN MAbs were made in an appropriate dilution of $^{125}$I-labelled HN-4b and then reacted with SV5-infected cell antigens bound to nitrocellulose membranes.


SV5-HN-
4c
y
5b
3a
1b
5c
5d
5a
5e
4a
z
x
4b
1c
4d
4e
4f
1a

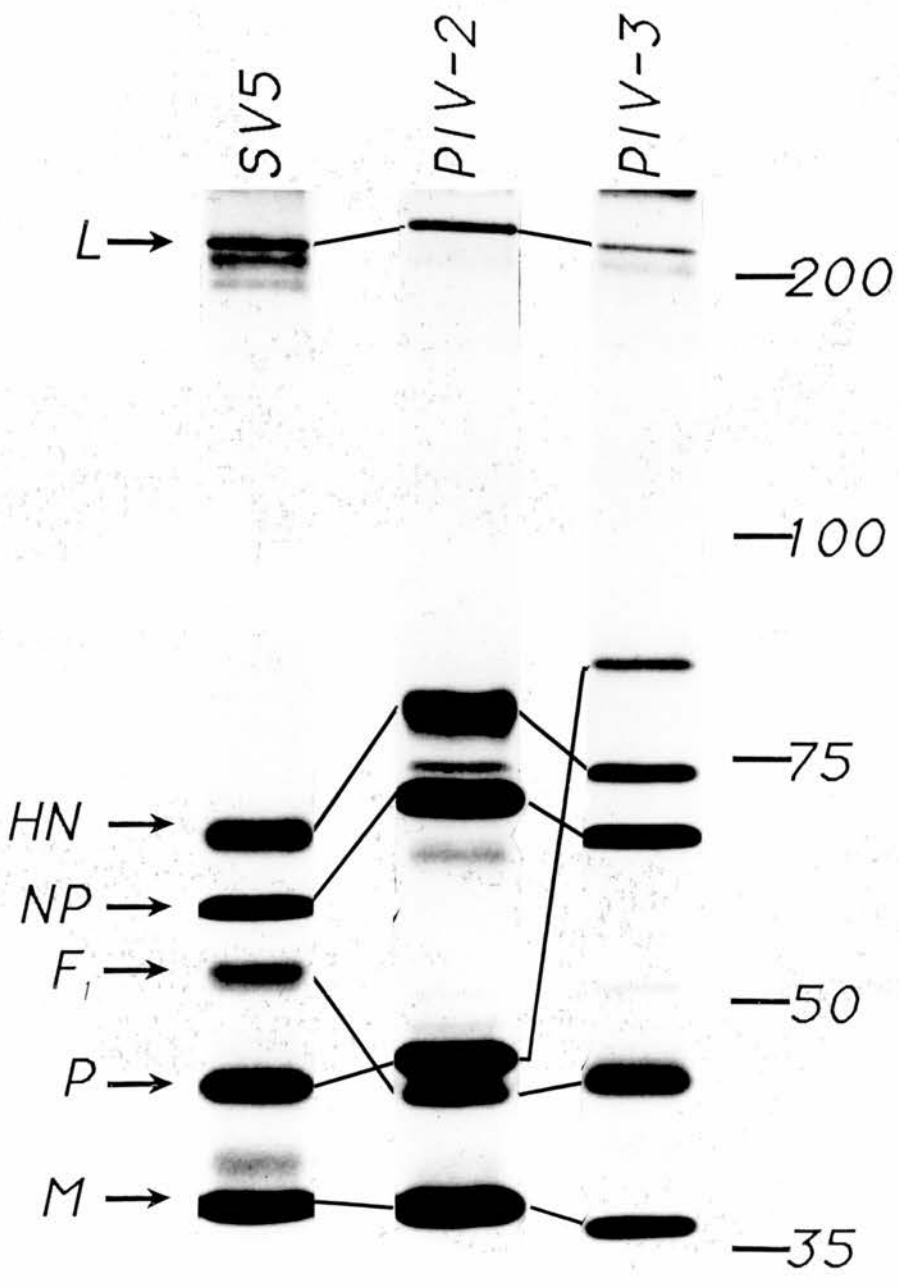
an efficient competitor (Fig. 7). In contrast, HN-4a was the most efficient competitor of $^{125}$I-labelled HN-4a while HN-4c and 4b showed moderately efficient and no competition respectively (data not shown). Furthermore HN-4d, 4e and 4f all successfully competed with the binding of $^{125}$I-labelled HN-4a (data not shown) but not with that of $^{125}$I-labelled HN-4b (Fig. 7). However, because HN-4a and 4b showed some competition with one another and were also both competed by HN-4c all these MAbs were placed in the same group. Not all the MAbs were purified and labelled with $^{125}$I for use in competition assays, if an unlabelled antibody competed with the binding of an $^{125}$I-labelled antibody then both antibodies were placed in the same group and a reciprocal analysis was not usually carried out. A summary of the grouping of the anti-HN MAbs is given in Table 3. It should be noted however that as well as competition between the binding of antibodies to the HN-protein it also appeared that binding of one antibody could enhance the binding of another. For instance compare the intensity of binding of $^{125}$I-labelled HN-4b in the presence of HN-5a to that in the presence of HN-3a (Fig. 7).

## COMPARISONS BETWEEN SV5 AND PIV-2

### SDS-PAGE analysis of virus proteins

SDS-PAGE analysis of polypeptides from purified preparations of L-[$^{35}$S]methionine labelled SV5, PIV-2 and PIV-3 virions demonstrated that although there was broad comparability between the three viruses the individual polypeptide patterns were clearly distinguishable (Fig. 8). The L-polypeptide of PIV-2 has a larger apparent molecular weight than the L-polypeptides of SV5 or PIV-3. The estimated molecular weight of the P-polypeptide of PIV-3 (91 kiloDaltons [kD]) is approximately twice that of the P-polypeptides of both SV5 and PIV-2 (46 and 47kD respectively). The estimated molecular weights of the HN and NP-polypeptides of PIV-2 (81 and 70kD respectively) are greater than those of PIV-3 (75 and 68kD respectively) which in turn are greater than those of SV5 (68 and 60kD respectively). The estimated molecular weight of the $F_1$-polypeptide of SV5 (54kD) is considerably greater than that of PIV-3 (45kD) which itself is just greater than that of PIV-2 (43kD). The M-polypeptide of PIV-3 is slightly smaller than those of SV5 and PIV-2.

**Fig. 8** Analysis of $^{35}S$-methionine-labelled polypeptide content of partially purified preparations of SV5, PIV-2 and PIV-3. The polypeptides were separated through a 10% SDS-polyacrylamide slab gel and were visualized by autoradiography and the mol. wts. of the virus polypeptides estimated by comparison with the electrophoretic mobilities of proteins with known molecular weights (Bethesda Research Laboratories protein mol.wt. standards; data not shown). The positions of the polypeptides of the structural proteins of SV5 are shown in the left hand column and those of PIV-2 and PIV-3 are indicated by lines drawn between the homologous polypeptides of the respective viruses.


SV5
PIV-2
PIV-3
L
200
100
HN
NP
F1
P
M
75
50
35

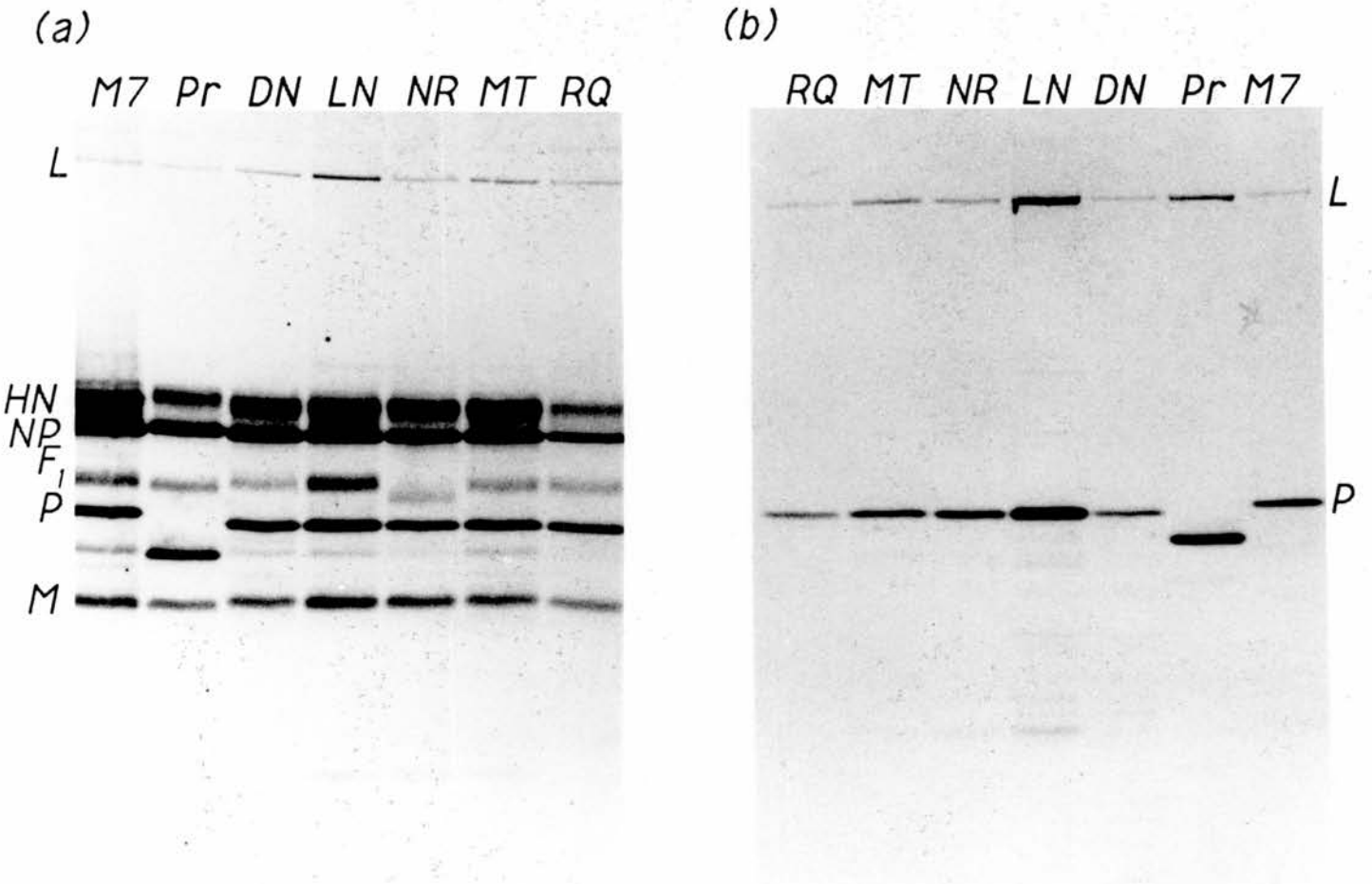
In contrast to the major differences between SV5 and PIV's 2 and 3, immune precipitation studies revealed only minor differences in the electrophoretic mobilities of virus proteins from different isolates of SV5. The P and $F_1$-polypeptides showed the most marked differences in mobility (Fig. 9). In particular the P-polypeptide of the canine isolate M7 migrated faster than the P-polypeptides of all the human isolates which also migrated faster than that of the prototype simian isolate Pr. There was less variation in the migration rates of the $F_1$-polypeptides between different isolates although that of the human isolate NR migrated fastest (Fig. 9).

Analysis of different field isolates of PIV-2 was hampered by their poor growth properties in tissue culture cells. However the HN-polypeptide of the prototype isolate of PIV-2 migrated slower than that of either of the two field isolates (see Fig. 14).

## Antigenic comparison between SV5, PIV-2 and other paramyxoviruses

The radioimmune assay used to show reactivities of the bank of MAbs to various paramyxoviruses demonstrated that the vast majority of MAbs were specific for the virus against which they

**Fig. 9** Analysis of $^{35}S$-methionine-labelled polypeptides present in immune precipitates formed by the reaction of various soluble antigen extracts with (a) mouse polyclonal anti-SV5 serum or, (b) an anti-SV5-P MAb. The polypeptides were separated by electrophoresis through a 15% SDS-polyacrylamide slab gel and visualized by autoradiography. The soluble antigen extracts were made from BHK cells infected with canine (M7), simian (Pr) or human (DN, LN, NR, MT and RQ) isolates of SV5.


(a)
M7 Pr DN LN NR MT RQ
L
HN
NP
F1
P
M
(b)
RQ MT NR LN DN Pr M7
L
P

were raised, an example of such a test is shown in Fig. 10. A few (5 out of 60) MAbs did show cross-reaction between PIV-2 and SV5, these MAbs had specificities for the M, NP and P-proteins. One MAb (SV5-P-e) also cross-reacted with mumps virus (Fig. 10, rows 2 wells 6). None of the MAbs reacted with PIV-3 or measles virus. Confirmation of cross-reaction was obtained from immune precipitation tests (data not shown) or Western blot analysis (eg. Fig. 11). The results of this analysis are summarized in Table 3.

In contrast the same type of assay showed that the the vast majority of anti-SV5 MAbs reacted with all isolates of SV5 tested. However a few MAbs did reveal antigenic differences between different isolates. Fig. 12 shows an example of such a test using three isolates of SV5; LN (human; against which the anti-SV5 MAbs were raised), Pr (simian) and M7 (canine). Not surprisingly all MAbs reacted with the human isolate LN (Fig. 12a). However four MAbs failed to react or reacted very weakly with the simian isolate Pr (eg. Fig. 12b row 3, wells 1, 4 and 6). Four MAbs (Fig. 12c; row 1, wells 7 and 8; row 3, well 4; row 4 well 4) failed to react or reacted very weakly with the canine isolates. In addition one anti-P MAb (SV5-P-k) was shown to distinguish between the two closely

**Fig. 10** Illustration of the radioimmune assay used to screen for cross-reactivities of monoclonal antibodies to SV5 and PIV-2 with various paramyxoviruses. In the test shown, monolayers of vero cells were infected with (a) SV5, (b) PIV-2 or (c) mumps virus and reacted with monoclonal antibodies that bound protein-A directly; bound antibodies were thus detected with $^{125}$I-labelled protein-A and autoradiography. Rows 1, wells 1 to 5 show the reactivity of anti-PIV-2 (well 1, anti-P; well 2, anti-NP; wells 3 to 5, anti-HN) antibodies; rows 2, 3 and 4 show the reactivity of anti-SV5 (rows 2, anti-P; rows 3, anti-M and rows 4, anti-HN) antibodies. In this illustration the anti-SV5 antibody that shows cross-reactivity to PIV-2 and mumps is a monoclonal antibody to the P-protein termed SV5-Pe. Wells are numbered, starting from the left hand side.

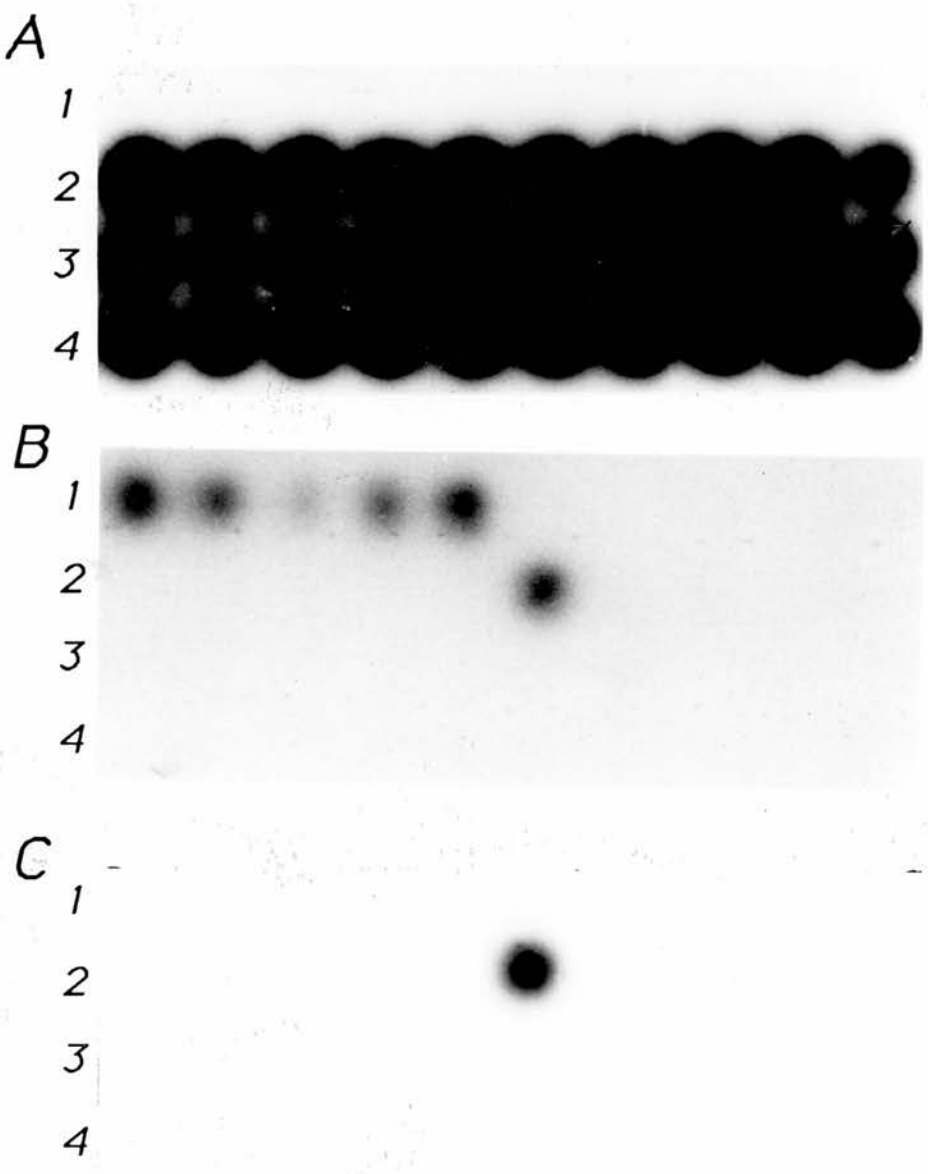

A
1
2
3
4
B
1
2
3
4
C
1
2
3
4

**Fig.11** "Western blot" showing the reactivity of monoclonal antibody SV5-Pe with uninfected vero cells and with vero cells infected with SV5, PIV-2, mumps, PIV-3 or measles viruses. Infected cell polypeptides were separated by electrophoresis through a 15% SDS-polyacrylamide slab gel, electrophoretically transferred to a nitrocellulose membrane and reacted with monoclonal antibody SV5-Pe (1/500 dilution of ascitic fluid). Bound antibody was detected with $^{125}$I-labelled protein-A and visualized by autoradiography. The position of the SV5 P-polypeptide is indicated in the left hand column.

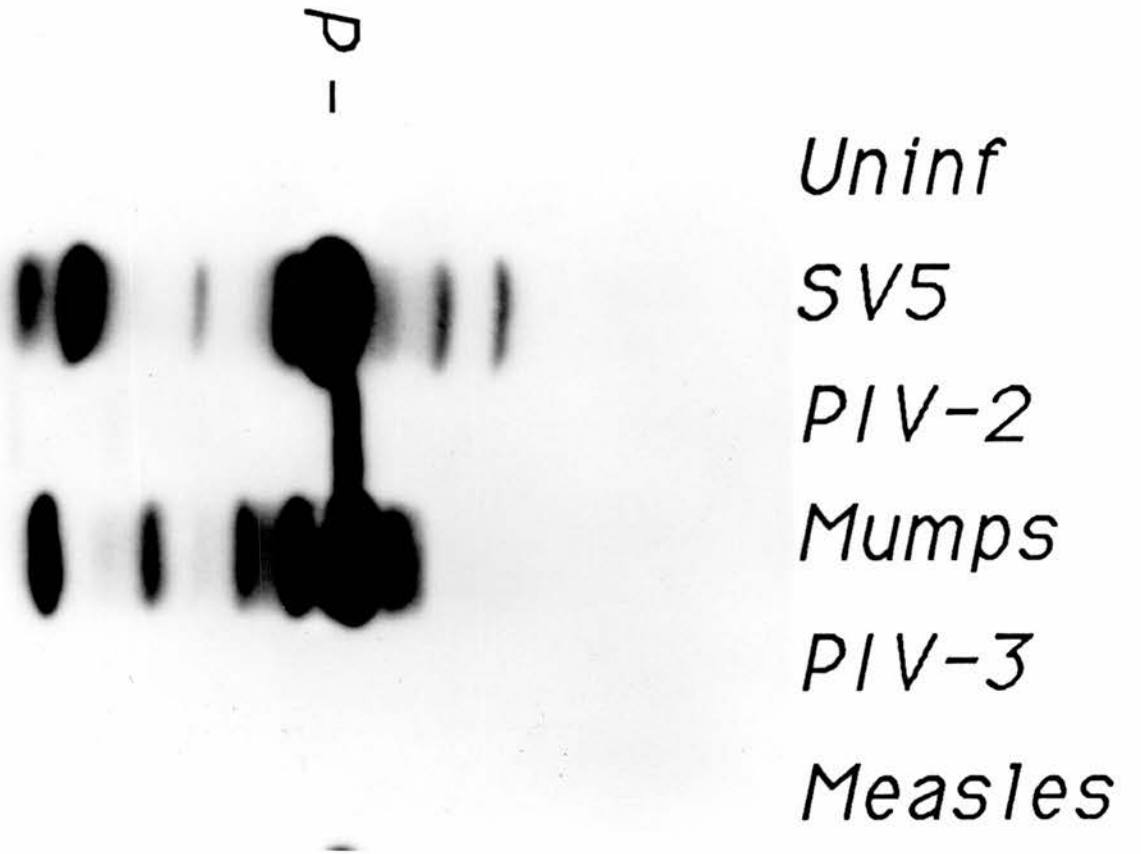
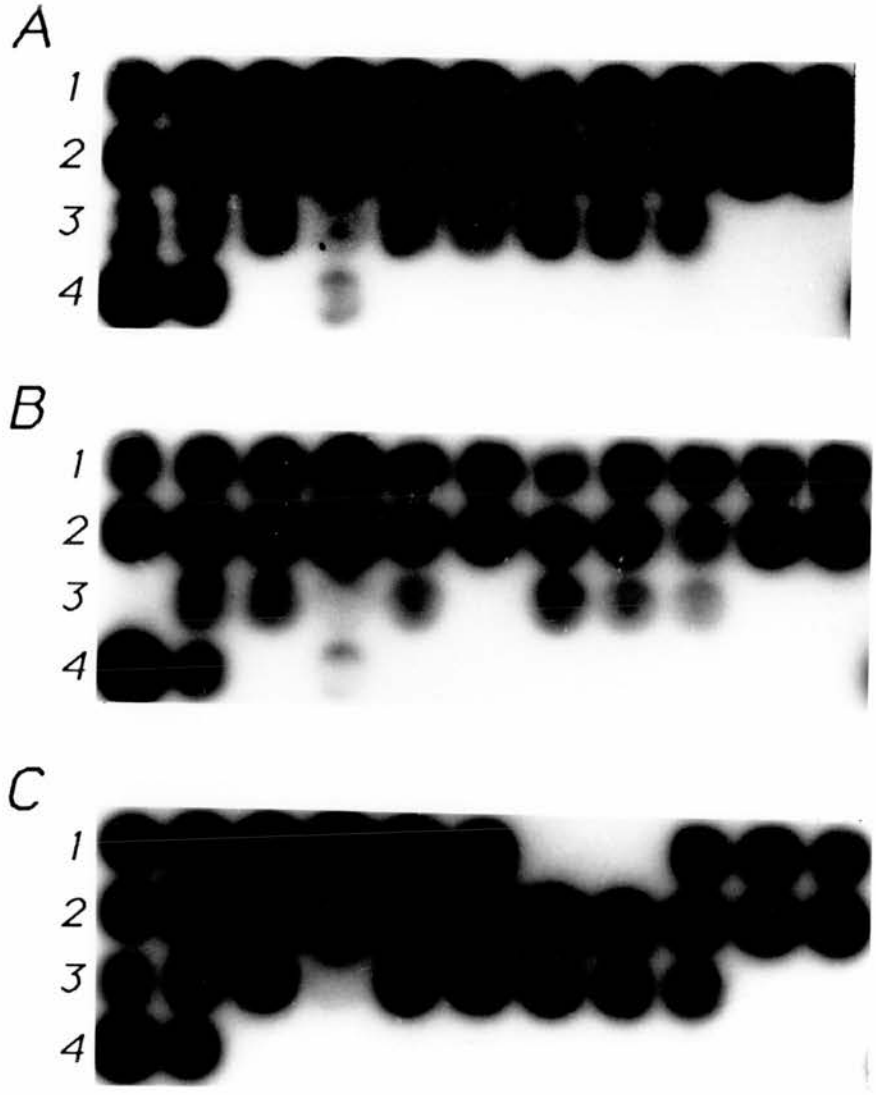

P-
Uninf
SV5
PIV-2
Mumps
PIV-3
Measles

**Fig. 12** Illustration of the assay used to screen for antigenic differences between different isolates of SV5. In the test shown, monolayers of vero cells in 96-well microtitre plates were infected with human (LN; panel a), simian Pr; panel b) or canine (M7; panel c) isolates of SV5, fixed and permeabilized. The plates were then reacted with protein-A binding MAbs, bound antibodies thus being detected with $^{125}$I-labelled protein-A and autoradiography. The illustration shows the reactivities of anti-HN (Rows 1, wells 1 to 11), anti-M (rows 2, wells 1 to 11), anti-P (rows 3, wells 1 to 9) anti-NP (rows 4, wells 1 and 2) and anti-F (rows 4, well 4) MAbs. The antibodies used in this experiment that did not react with all the isolates shown are HN-4b and HN-z (rows 1, wells 7 and 8 respectively), Pa, Pd and Ph (rows 3, wells 1, 4 and 6 respectively) and F-1a (rows 4, well 4). A summary of all the results is given in Table 3. Wells are numbered, starting from the left hand side.


A
1
2
3
4
B
1
2
3
4
C
1
2
3
4

related canine isolates CP1+ and CP1- (data not shown), also one anti-NP MAb (SV5-NP-d) was shown to react with three (DN, LN and RQ) of the human isolates but not with the remaining two (NR and MT; data not shown). Confirmation of the inability of these MAbs to react with certain isolates was obtained from immune precipitation studies (eg. Fig. 13) and the results of these tests are summarized in Table 3. Similar tests showed that only one of the anti-PIV-2 MAbs revealed differences between isolates of PIV-2. PIV-2-HN-1a reacted with the prototype isolate of PIV-2 but failed to react with either of the two field isolates (Fig. 14).

**Further studies on the antigenicity of the HN-proteins of SV5 and PIV-2**

The immunization scheme used to produce MAbs and also the methods used to screen hybridoma cells for secretion of MAbs to SV5 and PIV-2 may result in a biased selection of antibodies with high affinities for their respective proteins. Consequently the bank of MAbs produced may not reflect the broad spectrum of low and high affinity antibodies produced during a polyclonal immune response. Therefore to further examine the antigenic similarities and differences between the HN-proteins of SV5 and PIV-2 a series of

**Fig. 13** Analysis of $^{35}S$-methionine-labelled polypeptides present in immune precipitates formed by the reaction of the anti-F monoclonal antibody SV5-F-1a, with soluble antigen extracts of BHK cells infected with canine (M7), human (RQ, MT, NR, LN and DN) or simian (Pr) isolates of SV5. The polypeptides were separated by electrophoresis through a 15% SDS-polyacrylamide slab gel and visualized by autoradiography.

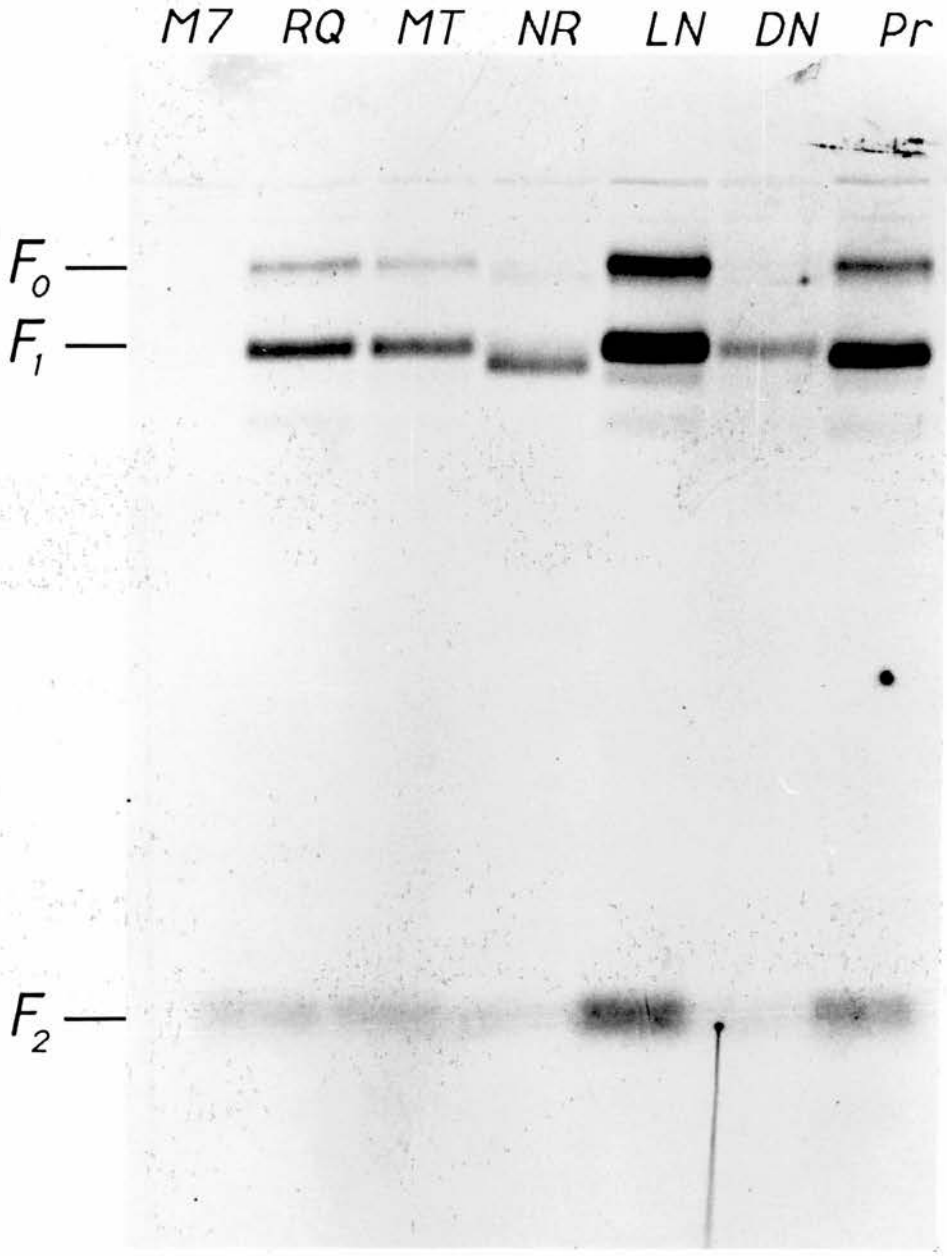
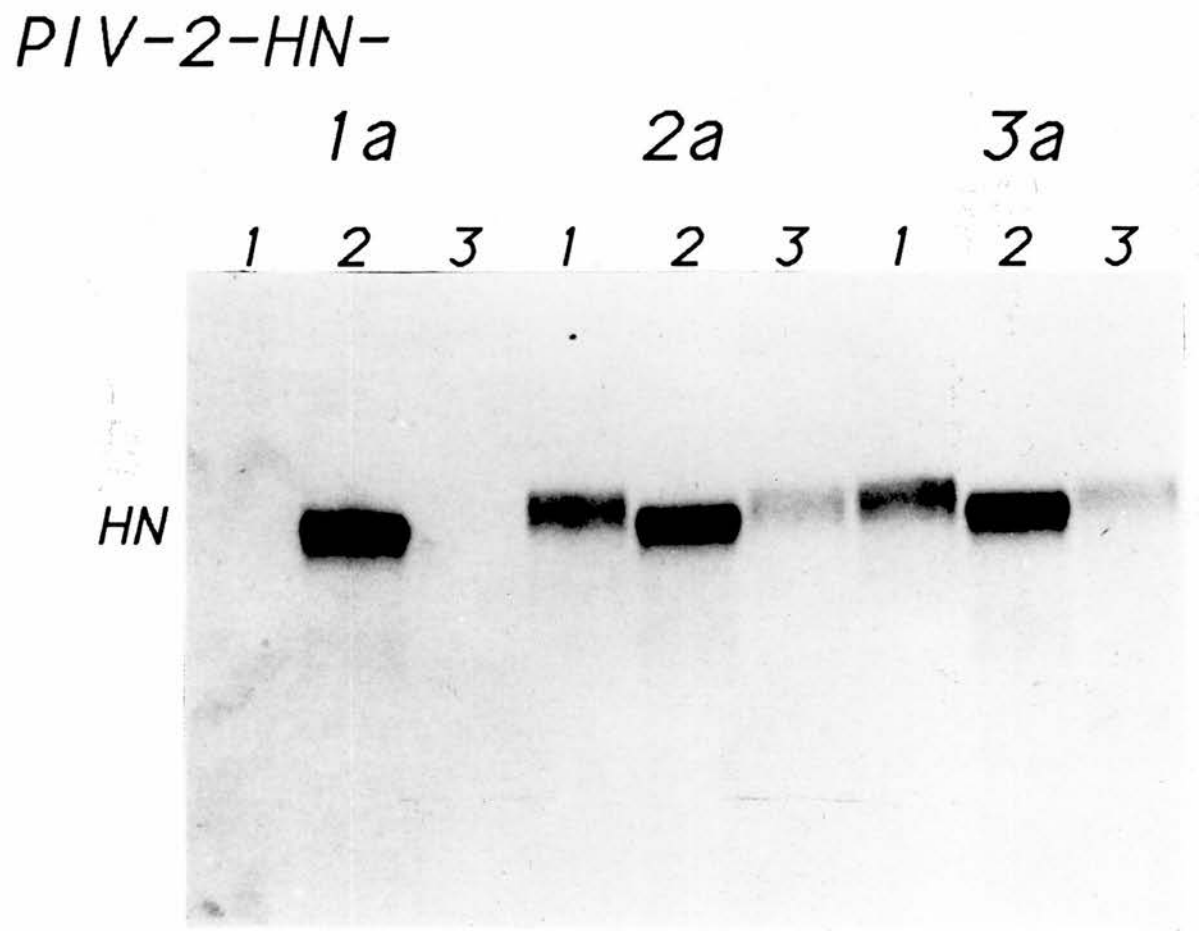

M7
RQ
MT
NR
LN
DN
Pr
$F_0$
$F_1$
$F_2$

**Fig. 14** Analysis of $^{35}S$-methionine-labelled polypeptides present in immune precipitates formed by the reaction of three monoclonal antibodies (PIV-2-HN-1a, HN-2a and HN-3a) to the HN-protein of PIV-2 with soluble antigen extracts of vero cells infected with either the prototype strain (lanes 2) or with 2 recent field isolates (lanes 1 and 3) of PIV-2. The polypeptides were separated by electrophoresis through a 10% SDS-polyacrylamide slab gel and visualized by autoradiography.


PIV-2-HN-
1a
2a
3a
1 2 3 1 2 3 1 2 3
HN

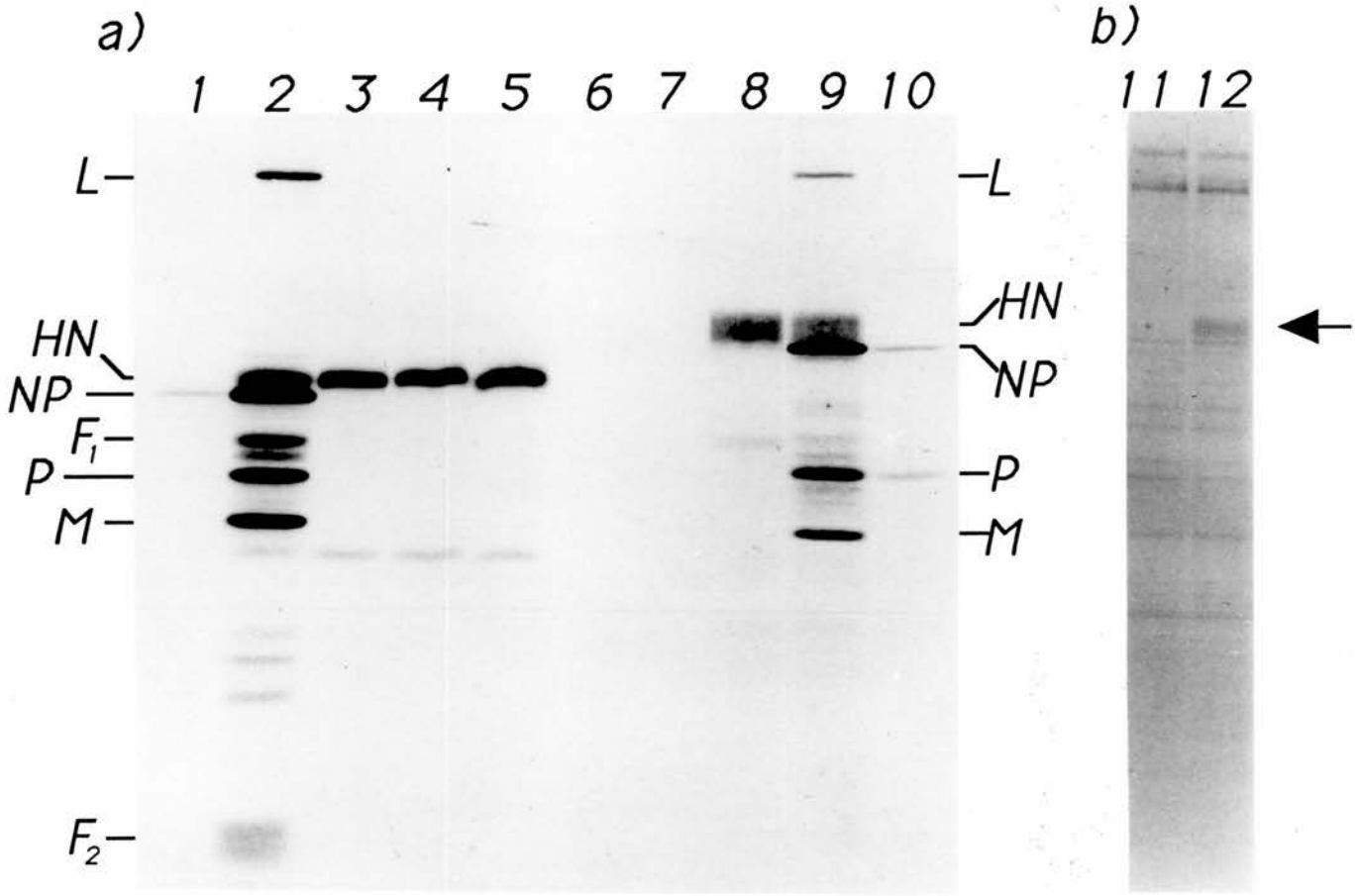
polyclonal antisera were raised in mice by immunization with purified preparations of the HN-proteins of SV5 and PIV-2. The sera collected from these animals were analysed for their ability to neutralize infectivity of SV5 and PIV-2 (Table 4). Tests showed that these sera were dominated by antibodies specific for the HN-protein of the virus against which they were raised. Cross-neutralizing antibodies were detected although the titre of neutralization against the homologous virus was always at least ten-fold greater than against the heterologous virus. For instance serum raised against SV5-HN from one mouse neutralized SV5 and PIV-2 at titres of 1/1600 and 1/160 respectively. However sera from other mice immunized with the same preparation of SV5-HN showed the same neutralizing titre against SV5 but failed to show neutralization of PIV-2 (Table 4). These same polyclonal anti-HN sera were also tested for their ability to immune precipitate the HN-proteins of both SV5 and PIV-2. Fig. 15 shows the result of a test using the anti-SV5-HN sera to immune precipitate the HN-proteins of SV5 (lanes 4-5) or PIV-2 (lanes 6-7 also lanes 11-12). These assays thus demonstrate limited antigenic relationship between SV5 and PIV-2 with respect to their HN-proteins. The variation in immune response of inbred mice to the HN-proteins of both SV5 and PIV-2 is likely to

**Table 4.** Neutralization of SV5 and PF-2 with polyclonal sera raised against purified preparations of the HN-proteins of SV5 or PF-2.

| | | Neutralization titre* against: | |
|---|---|---|---|
| | | SV5 | PIV-2 |
| Anti-SV5-HN serum: | 1 | 3200 | 40 |
| | 2 | 6400 | <20 |
| | 3 | 400 | <20 |
| | 4 | 400 | <20 |
| | 5 | 3200 | <20 |
| | 6 | 800 | 20 |
| | 7 | 1600 | <20 |
| | 8 | 1600 | 20 |
| | 9 | 1600 | 80 |
| | 10 | 400 | 40 |
| | 11 | 400 | 20 |
| | 12 | 100 | <20 |
| | 13 | 1600 | 160 |
| | 14 | 1600 | 40 |
| | 15 | 1600 | <20 |
| | 16 | 6400 | 20 |
| | 17 | 800 | 20 |
| | 18 | 1600 | 40 |
| | 19 | 1600 | 40 |
| Anti-PIV-2-HN serum: | 1 | <20 | 800 |
| | 2 | <20 | 3200 |
| | 3 | <20 | 1600 |
| Monoclonal antibody: | | | |
| SV5-HN-4a | | $>2.10^6$ | <20 |
| PIV-2-HN-3a | | <20 | 3200 |

* Highest dilution of serum that significantly neutralizes virus infectivity.

**Fig. 15** Analysis of $^{35}$S-methionine-labelled polypeptides, separated by electrophoresis through a 15% SDS-polyacrylamide slab gel, from immune precipitates formed by the reaction of various antisera with soluble antigen extracts of SV5 (lanes 1 to 5) or PIV-2 (lanes 6 to 12) infected BHK cells. The antisera used for the immune precipitation reactions were; mouse polyvalent sera raised against purified preparations of PIV-2 (lanes 1 and 9) or SV5 (lanes 2 and 10) virions; four different mouse polyclonal antisera raised against purified preparations of the HN-protein of SV5 (serum 1, lanes 4 and 6; serum 2, lanes 5 and 7; serum 3, lane 11 and serum 4 lane 12); or monoclonal antibodies to the HN-protein of SV5 (lane 3) or PIV-2 (lane 8). The autoradiogram shown in panel (a) was exposed for two weeks, while that shown in panel (b) was exposed for ten weeks. The positions of the SV5 and PIV-2 polypeptides are shown in the left and right hand columns respectively of panel (a). The position of the HN-polypeptide of PIV-2 is arrowed in panel (b).


a)
1 2 3 4 5 6 7 8 9 10
L
HN
NP
F1
P
M
F2
L
HN
NP
P
M
b)
11 12

be much greater in the outbred human population. This heterogeneity will also presumably be amplified as a result of sequential or persistent infections with different paramyxoviruses with varying degrees of antigenic relatedness. Studies with human sera containing antibodies to both SV5 and PIV-2 showed that in general the titre of neutralization was higher against PIV-2 than against SV5. Preadsorption of these sera with preparations of SV5 or PIV-2 antigens resulted in a subsequent decrease in the neutralization titre of the sera to the homologous virus (Fig. 16). Overall, preadsorption of the sera with PIV-2 antigens resulted in a decrease of approximately four-fold in the titre of neutralization against PIV-2 whilst the titre against SV5 remained virtually unchanged. However preadsorption of the same sera with SV5 antigens resulted in a smaller (approximately 1·6-fold) but consistent decrease in the neutralization titre against SV5 while the titre against PIV-2 remained almost the same.

## Studies on intracellular localization of SV5 and PIV-2 proteins using immunofluorescence

Immunofluorescence studies revealed differences in the patterns of intracellular distributions of SV5 and PIV-2 proteins (Fig. 17). In

**Fig. 16** Illustration of the effects of preadsorption with SV5 or PIV-2 antigen on the subsequent neutralization titres of various human (1-5) or mouse (anti-SV5-F, 6-7 or; anti-PIV-2-HN, 8-10) sera against SV5 or PIV-2. Replication of virus was detected by incubating the fixed cells with either anti-SV5 or anti-PIV-2 monoclonal antibodies, bound antibodies were then detected with $^{125}$I-labelled protein-A and visualized by autoradiography. Rows a, unadsorbed; rows b, preadsorbed with PIV-2 antigen and rows c, preadsorbed with SV5 antigen. Human sera were preadsorbed by incubation with monolayers of SV5 or PIV-2 infected vero cells which had been fixed and permeabilized as for immunofluorescence. Two-fold serial dilutions of all sera were made starting at 1/10 (human sera) or 1/20 (mouse sera) dilution from the left hand side.

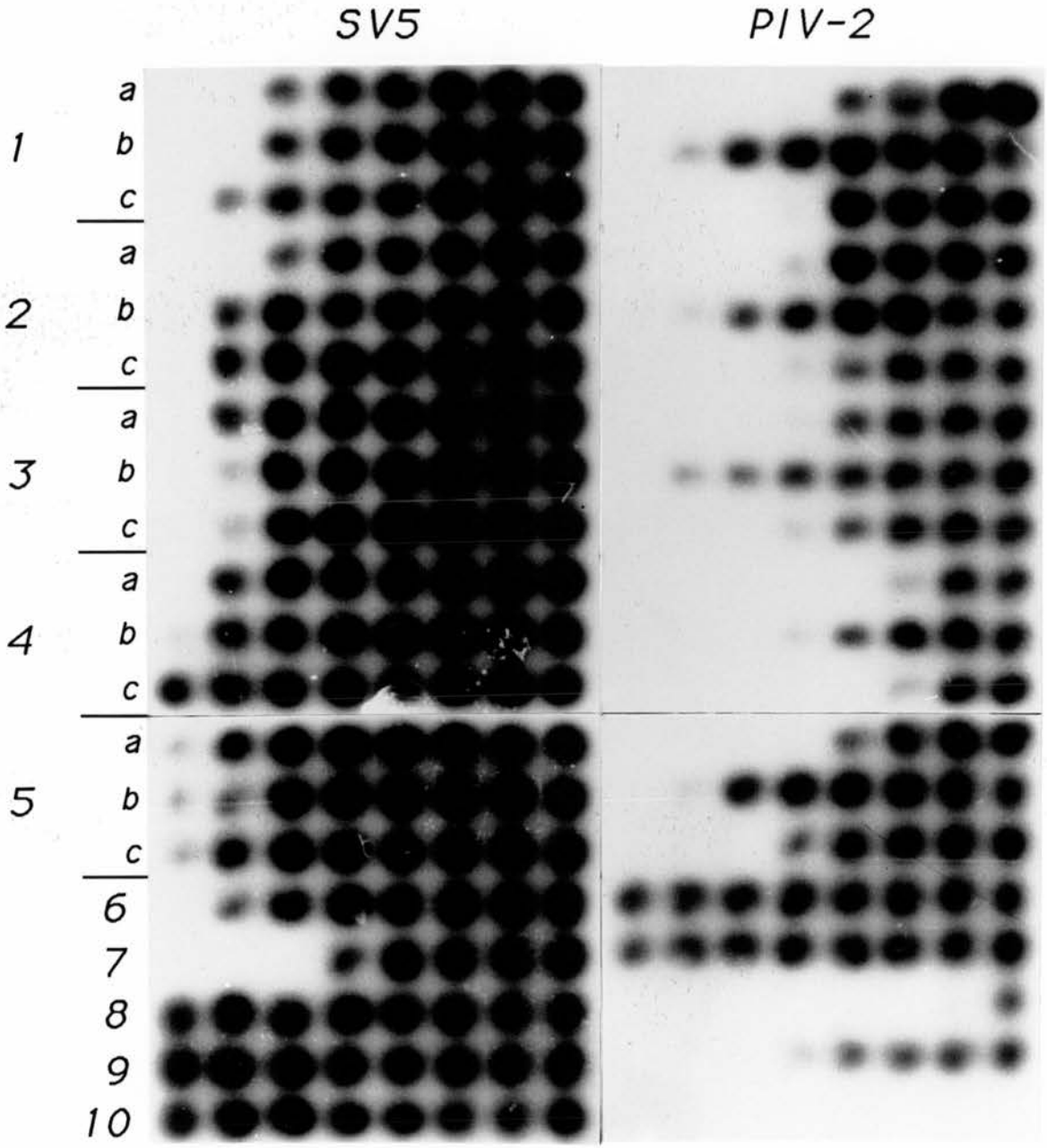
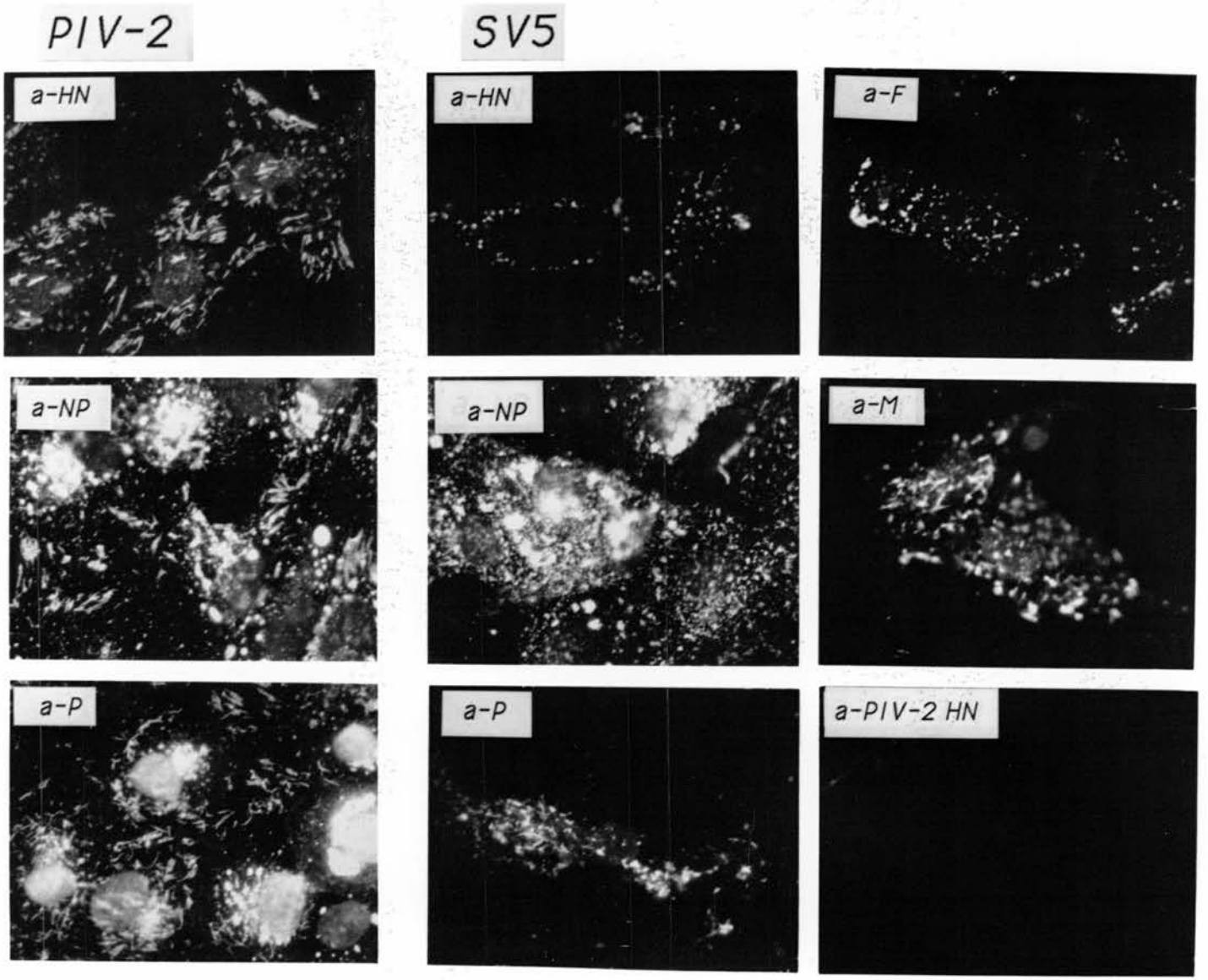

SV5
PIV-2
1
a
b
c
2
a
b
c
3
a
b
c
4
a
b
c
5
a
b
c
6
7
8
9
10

**Fig.17** Photomicrographs showing the intracellular localizations of the HN, NP and P-proteins in PIV-2-infected vero cells and the HN, F, NP, M and P-proteins in SV5-infected vero cells. Cells were fixed with formaldehyde 24hrs post-infection, permeabilized with a non-ionic detergent and stained with specific antibodies using indirect immunofluorescence.


PIV-2
SV5
a-HN
a-HN
a-F
a-NP
a-NP
a-M
a-P
a-P
a-PIV-2 HN

PIV-2 infected cells the HN-protein was found almost exclusively in filament-like structures and, whilst the NP and P-proteins were also located in such structures they were more abundant in large granular cytoplasmic foci (Fig. 17). In contrast far fewer filament-like structures were seen in SV5 infected cells and all the proteins occurred in greater abundance in more granular cytoplasmic foci. Differences in the abundance of the various SV5 proteins could easily be visualized, the NP-protein being the most abundant and located predominantly in large foci whereas the HN and F-proteins were seen in much smaller, restricted locations within infected cells (Fig. 17).

# DISCUSSION

SDS-PAGE analysis of purified preparations of SV5, PIV-2 and PIV-3 showed that while the three viruses gave similar arrays of polypeptides the individual polypeptide patterns were readily distinguishable from one another (Fig. 8). The P-polypeptides showed greatest variability, that of PIV-3 having an estimated molecular weight approximately twice that of either the SV5 or PIV-2 P-polypeptide. The M-polypeptides demonstrated least variation in electrophoretic mobility between the three viruses. In contrast SDS-PAGE analysis of canine, human and simian isolates of SV5 revealed marked similarities between the different isolates, the polypeptides showing only minor differences in electrophoretic mobility. The P and $F_1$-polypeptides demonstrated greatest variation. The differences in migration rates of the P-polypeptides appeared to be a consequence of the species from which the isolate originated. The $F_1$-polypeptides however showed variations independent of the species of origin. Since there are only minor strain variations in the migration rates of SV5 polypeptides but major characteristic

differences between those of SV5 and PIV-2 it is unlikely that these two viruses are as closely related antigenically as has previously been suggested (Goswami and Russell, 1982).

To examine the extent of the antigenic relationships between SV5 and PIV-2 a bank of monoclonal antibodies (MAbs) was produced. The 54 raised against SV5 had specificities for the HN, F, M, NP and P-proteins and the 6 raised against PIV-2 were specific for the HN, NP and P-proteins. All the MAbs to the surface glycoproteins, HN and F, showed neutralizing activity but only to the virus against which they were raised. The titres of neutralization varied from 1/200 to 1/128,000 depending on the MAb used, this wide range indicated the likelihood of the existence of different antigenic sites on the HN-proteins of SV5 and PIV-2. Direct evidence for the presence of distinct antigenic sites came from competition assays. The anti-HN MAbs defined a minimum of 4 and 3 antigenic sites on the HN-proteins of SV5 and PIV-2 respectively. Within these antigenic sites the MAbs showed the presence of smaller overlapping immunogenic regions or epitopes. It was apparent that not all epitopes within an antigenic site overlapped with all other epitopes within the same site. Thus although some MAbs showed competitive binding with all other members of the same group, other MAbs in the

same group showed competitive binding with some but not all other members of the same group. There may be several possible explanations for these observations. As examples, binding of an antibody to one epitope could inhibit the binding of a second antibody by partly overlapping a neighbouring epitope; binding of an antibody to one epitope at a constriction on the HN-protein could block the access of an antibody to a second epitope beyond that constriction; binding of an antibody to one epitope could cause a conformational change in a region adjacent to a second epitope such that a constriction is formed which blocks the access of an antibody to the second epitope; binding of one antibody induces a conformational change at a second epitope thereby abolishing or reducing the efficiency of binding of an antibody to the second epitope. The converse to the last scenario may also be true for it appears that binding of some antibodies enhances the binding of others. For instance compare the binding of $^{125}$I-labelled HN-4b in the presence of HN-5a to that in the presence of HN-3a (Fig. 7). It is possible that such cooperative binding is an important factor in the interactions between antibodies and antigens in polyclonal sera. It is of interest to note that HN-4a defines an epitope common to all isolates of SV5 whereas HN-4b defines an epitope present on all but the canine

isolates of SV5.

Although the vast majority of anti-SV5 MAbs reacted with all isolates of SV5 tested, a few MAbs did reveal antigenic differences between canine, simian and human isolates of SV5. Four MAbs showed weak or no reactivity towards all three canine isolates, whilst one of these four and a further three MAbs showed weak or no reactivity towards the prototype simian isolate. Thus it would appear that isolates of SV5 may be grouped together according to their species of origin. Two MAbs revealed differences between isolates from the same species, one of these (SV5-NP-d) reacted with three of the human isolates (DN, LN and RQ) but not with the remaining two (MT and NR). The other of these MAbs (SV5-P-k) reacted with the canine isolates M7 and CP1+ but failed to react with CP1-, a variant isolated from a gnotobiotic dog intracerebrally infected with CP1+. The five human isolates of SV5 were all isolated from the same geographical region at about the same time and therefore may not be a representative sample of human strains of SV5. However, the canine isolates M7 and CP1+ were isolated in Britain and Germany respectively yet they show exactly the same pattern of reactivity with the 54 MAbs to SV5. It has been suggested that SV5 will infect humans, monkeys, dogs and more recently

hamsters and mice. With regard to the levels of antigenic differences detected here between SV5 isolates from different species it will be of interest to know whether such differences are sufficient to prevent SV5 crossing species barriers or whether the virus moves freely between species.

In marked contrast to the small number of differences observed between different isolates of SV5, major differences were apparent between SV5 and PIV-2 in that the vast majority of MAbs (55 out of 60) were specific for the virus against which they were raised. Thus the results presented here provide evidence that SV5 and PIV-2 are distinct species of virus and are not as closely related antigenically as has been previously thought. Indeed, more recent studies have shown that the HN (Kawano *et al.*, 1990, Precious *et al.*, 1990) and P (Ohgimoto *et al.*, 1990, Southern *et al.*, 1990) proteins share approximately 43% and 44% homology respectively between PIV-2 and SV5.

The 5 MAbs that did show cross-reaction between SV5 and PIV-2 were specific for the M, NP and P-proteins (see Table 3). The same anti-P MAb that cross-reacted between SV5 and PIV-2 also showed cross-reactivity with mumps virus thereby defining an important determinant that has been evolutionarily conserved. No

cross-reaction of the MAbs was observed with measles, canine distemper or PIV-3 viruses. Studies here using SDS-PAGE and MAbs indicate that the M-protein appears overall to be the most highly conserved protein, the M-polypeptide showing least variation in apparent molecular weight between SV5, PIV-2 and PIV-3. Furthermore all 22 anti-M MAbs reacted with all isolates of SV5 and 2 of these MAbs showing cross-reaction between SV5 and PIV-2. These findings are in agreement with sequence analysis which demonstrated that the M-protein shows the broadest homology between Paramyxoviridae (Galinski *et al*, 1987). However, in the studies carried out here, immune precipitations of SV5 and PIV-2 antigens by polyvalent antisera failed to show cross-reaction between the M-proteins of SV5 and PIV-2 (eg. Fig. 15). The proteins that showed the strongest cross-reaction between SV5 and PIV-2 were the NP and P-proteins (Fig. 15). Ito *et al.* (1987) using a series of polyvalent antisera concluded that the NP-protein showed broadest cross-reactivity between paramyxoviruses and that the M-protein exhibited greatest individuality amongst the same viruses. Goswami and Russell (1982) also noted strong cross-reaction between the NP-proteins of SV5 and PIV-2. Thus the NP and P-proteins are somewhat paradoxical for while they show

cross-reaction between SV5 and PIV-2 with certain MAbs (Table 3; also Fig. 10) and polyvalent sera (Fig. 15; lanes 1 and 10, Ito *et al.*, 1987, Goswami & Russell, 1982), other MAbs reveal significant antigenic differences. The functions of these proteins and analysis of their sequences may explain this paradox. It has been suggested that the role of the NP-protein is three-fold; firstly, to confer helical symmetry on the ribonucleoprotein core (Sánchez *et al.*, 1986); secondly, to interact with the positively charged M-protein (Morgan *et al.*, 1984) and; thirdly, to act in the process of switching from transcription to replication of the viral genome. Thus it may be for instance that the domain involved in conferring helical symmetry on the ribonucleoprotein core requires conservation, resulting in antigenic epitopes that are conserved between species whereas, the domain which interacts with the M-protein may only require maintenance of charge (this could be achieved by non-conservative substitutions) which, with evolutionary drift, may result in species specific epitopes. Sequence analysis has indeed shown that domains of high and low homology do exist within the NP-proteins of Paramyxoviridae. For example, although the overall homology between the NP-proteins of PIV-3 and Sendai virus is 59%, the central half of the protein shows 80% homology but the C-terminal

quarter shows only 15% homology (Sakai *et al.*, 1987). Similar situations are seen with the HN and F-proteins (Suzu *et al.*, 1987). The conserved and non-conserved domains of the P-protein are less well defined although the C-terminal half of the protein is more homologous (Spriggs & Collins, 1986, Southern *et al.*, 1990). The discrepancies between monoclonal and polyvalent analyses of the M-protein may in part be due to different techniques used in the production and screening of the the different sera. Sequence analysis of the genes encoding the M-proteins of PIV-2 and SV5 should resolve this paradox.

Although SV5 has never been associated with any acute disease in humans, studies have shown that 20 to 50 percent of human sera show neutralizing activity towards SV5 at 1/20 to 1/160 dilutions of sera (Hsuing, 1972; Goswami *et al.*, 1984). Interpretation of such data in terms of the epidemiology of SV5 infections in humans has been hampered by the antigenic relatedness of SV5 and PIV-2. However data presented here demonstrates that although some antigenic identity exists between the HN-proteins of SV5 and PIV-2 the major antigenic determinants are specific to each virus. In particular, none of the 21 MAbs to the HN-proteins of either SV5 or PIV-2 showed any cross-reaction with the heterologous virus.

However polyclonal antisera raised against purified preparations of the HN-proteins of SV5 and PIV-2, although dominated by antibodies specific for the homologous virusl, did show low levels of cross-reactivity with the heterologous virus. The titre of neutralization against the heterologous virus was always at least ten-fold lower than that against the homologous virus. Interestingly the data showed variation in the immune response of animals with the same genetic background immunized with the same preparations of antigen. Thus certain sera showed low levels of cross-reacting antibody while other sera with similar levels of neutralization against the homologous virus showed no detectable cross-neutralizing activity. This variation in the response of inbred mice with respect to the levels of cross-reacting SV5/PIV-2 anti-HN antibody induced, is likely to be far greater in the outbred human population. Furthermore persistent or sequential infection by different paramyxoviruses with varying degrees of antigenic relatedness would presumably lead to still more heterogeneity in the spectrum of antibodies in individual sera. In addition cross-neutralizing antibody may also be directed at the F-proteins of SV5 and PIV-2. However although two polyclonal sera, raised against the F-protein of SV5, showed neutralization of SV5 at 1/80 and 1/160

dilutions, they failed to show any neutralizing activity against PIV-2 (data not shown). Generally the level of neutralizing activity in human sera is higher against PIV-2 than against SV5 although some sera have similar titres of neutralizing activity against both viruses (data not shown). On the basis of data presented here it is unlikely that cross-reacting antibody alone could account for all the neutralizing activity. Accurate estimations of the precise incidence of SV5 in the human population will therefore depend on the use of sensitive and specific competition assays using MAbs of defined specificities (eg. Goswami & Russell, 1984).

Immunofluorescence studies on the location of SV5 and PIV-2 proteins in infected cells demonstrated that whilst the HN-protein of PIV-2 was located mainly in filamentous structures, that of SV5 had a more amorphous distribution. Similar short filamentous structures have been reported in SV5 infected cells although they were limited to 10% of all virus particles produced (Baumgärtner *et al.*, 1981). In addition, long filamentous structures have been noted in RSV infected cells and it has been suggested that the presence of such structures may be a contributing factor in the pathogenesis of RSV infections (Pringle, 1987). Thus it is of interest that whilst PIV-2 causes an acute respiratory disease in humans SV5 has no

such association (although SV5 infection in man presumably occurs via the respiratory tract). SV5 is however regarded as an important cause of respiratory disease in dogs (Rosenberg *et al.*, 1971). Whether the number and length of filamentous viral structures is dependent on the type of cell infected or the strain of virus involved remains to be established, as does the possible pathogenic role these structures play in paramyxovirus infections.

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