Biochemical and Serological Comparisons of Australian Bunyaviruses Belonging to the Simbu Serogroup

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SUMMARY

Comparative analysis of the structural and possible non-structural proteins of seven Simbu serogroup bunyaviruses isolated in Australia revealed them all to be similar in size to those of Bunyamwera virus, the prototype of the Bunyavirus genus. The molecular weights of the structural proteins for these bunyaviruses (Akabane, Aino, Tinaroo, Douglas, Peaton, Facey’s Paddock and Thimiri viruses) were 193K to 205K (L), 103K to 125K (G1), 33K to 37K (G2) and 25K to 26K (N). Analysis of the virion RNA of three viruses (Akabane, Douglas and Facey’s Paddock) showed them all to be similar to Bunyamwera virus RNA, apparent Mr values being 2-6 x 10^6 (L), 1.4 x 10^6 to 1.9 x 10^6 (M) and 0.24 x 10^6 to 0.42 x 10^6 (S). Host cell protein synthesis was switched off late during infection, revealing four structural proteins L, G1, G2 and N. Comparative analysis of these protein profiles in infected Vero cells showed each virus, although similar, to be unique and easily identified; this method of comparison was efficient and rapid compared to the difficulty in obtaining adequate amounts of purified virus for analysis. Additionally, for all viruses except Douglas, two to four possible non-structural proteins were identified, with an Mr range from 12K to 30K. The viruses Akabane and Tinaroo, which have previously been shown to cross-react by plaque inhibition virus neutralization tests, were readily distinguished in migration of the G1 glycoprotein and by analysis of plaque reduction virus neutralization data using linear regression analysis of the dose–response curves. Using these same analyses, the differences between Aino and Douglas viruses, also related by plaque inhibition, were even greater. Application of the biochemical analysis of virus-specified proteins and some serological comparisons identified a mixed pool of different viruses in two unknown isolates grouped as Simbu serogroup viruses, and further identified a potential teratogenic strain in one of the two pools.

INTRODUCTION

The Bunyaviridae family of arthropod-borne viruses comprises four genera (Bunyavirus, Nairovirus, Phlebovirus, Uukuvirus), and possibly a fifth genus (Hantavirus), as well as several unassigned members (Bishop et al., 1980; Bishop, 1986). Viruses have been assigned to individual genera primarily on serological and morphological criteria; more recently these assignments have been substantiated by biochemical analyses. Of the 17 known and possible members of the Bunyaviridae isolated in Australia, protein analyses using PAGE of virion and virus-specified proteins for seven of them have shown all viruses to be bunyaviruses (supported by serological evidence) or bunyavirus-like (biochemically similar to viruses of the Bunyavirus genus) (McPhee & Della-Porta, 1981; McPhee & Westaway, 1981a; D. A. McPhee, unpublished data). All viruses possessed four structural proteins, similar in Mr, and relative amounts to Bunyamwera virus (the prototype of the Bunyavirus genus).

Of the seven Simbu serogroup viruses (Bunyavirus genus) isolated in Australia, Akabane (AKA) virus causes congenital deformities in cattle and sheep (Parsonson et al., 1981b); three

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others, Aino (AINO), Peaton (PEA), and Tinaroo (TIN), have been implicated as possible causal agents of deformities in cattle and/or sheep by serology (Parsonson et al., 1981b) or experimental inoculation of sheep (Parsonson et al., 1982). Nothing is known about the disease-causing capabilities of Australian bunyaviruses outside the Simbu serogroup. AINO, TIN and PEA viruses are serologically related to AKA virus, TIN being the most closely related and showing extensive cross-reactions using the plaque inhibition (PI) virus neutralization test; these cross-reactions may pose problems in identifying Simbu serogroup members (McPhee & Della-Porta, 1981). We now report on analyses of the proteins specified by all presently known Simbu serogroup viruses isolated in Australia, and detail serological relationships between cross-reacting members to aid in the identification of any new isolates, particularly any suspected of being teratogenic.

**METHODS**

**Viruses and cells.** The sources of the Simbu serogroup viruses were as follows: AKA virus isolates CSIRO16 and CSIRO217, Douglas (DOU) isolate CSIRO150, PEA virus isolate CSIRO110, Thimiri (THI) virus isolate CSIRO1, TIN virus isolate CSIRO153 and the two unknown isolates CSIRO10 and CSIRO296 were all obtained from Dr T.G. St. George, CSIRO Division of Tropical Animal Science, Long Pocket Laboratories, Indooroopilly, Queensland, Australia; AKA virus isolates B8935 and R7949, AINO virus B7974 and Facey's Paddock (FP) virus isolate Ch16129 were provided by Dr R.L. Doherty, Queensland Institute of Medical Research, Herston, Queensland, Australia. Bunyamwera (BUN) virus was obtained from Dr I. Holmes, Department of Microbiology, University of Melbourne, Australia. Each of the known Simbu serogroup viruses from virus pools of infected suckling mouse brain was recloned three times by plaque assay and growth in Vero cells, the plaque from the last clone being used to grow stock pools. Growth and maintenance of Vero and BHK-21/13 cells have been described previously (McPhee & Westaway, 1981a).

**Growth, concentration and purification of virus.** The techniques used were essentially as described previously (McPhee & Westaway, 1981a). BHK-21/13 cell cultures in roller bottles (approx. 1 x 10^6 cells/bottle) were infected with cloned virus stocks at a low m.o.i. (0.001 to 0.1) for 30 min at 37 °C. After this period, maintenance medium was added. For labelling virus with [3H]leucine, [35S]methionine or [32P]orthophosphate the medium was deficient in leucine, methionine or phosphate respectively (10% of the normal concentration). The radioactive labels were added 6 h post-infection (p.i.) for all virus-infected cells. Cell culture fluids were removed and clarified (10000 g for 10 rain) 24 to 72 h p.i. when + 3 to +4 c.p.e. had developed. Virus particles from cell culture fluids were concentrated 50-fold using polyethylene glycol-sodium chloride precipitation as described previously (Obijski et al., 1976), or by ultrafiltration in an Amicon model 402 cell with an Amicon XM-50 membrane (M, cut-off 50000) which had been precoated by passing 200 ml of maintenance medium through the membrane.

After clarification, concentrated virus particles were purified by rate zonal sedimentation using a Beckman SW28 rotor at 24000 r.p.m. for 3 h at 4 °C in a linear 20% to 50% (w/v) sucrose gradient in TES buffer (0.01 M-Tris–HCl pH 7.6 at 4 °C, 0.001 M-EDTA, 0.1 M-NaCl) containing 0.2% bovine serum albumin (BSA). Virus particles were often purified a second time under the same conditions. Purified virus particles were diluted in TES buffer containing 0.2% BSA, pelleted at 24000 r.p.m. in the Beckman SW28.1 rotor for 2 h at 4 °C and resuspended in the appropriate buffer.

**Labelling of infected cells.** The technique for labelling virus-infected Vero cell monolayers with [35S]methionine was as used previously for BUN virus (McPhee & Westaway, 1981b). The cells were infected at an m.o.i. of 1 p.f.u./cell. The time of labelling infected cells was chosen such that extensive c.p.e. had developed by the end of the labelling period, generally between 12 and 72 h p.i. The cells were solubilized in 0.25 ml of 2% SDS and stored at -20 °C.

**Radiochemicals.** The radiochemicals used were L-[35S]methionine (1115 to 1500 Ci/mmol), L-[4,5-3H]leucine (150 to 197 Ci/mmole) and [32P]orthophosphate (10 mCi). The methionine and leucine were purchased from Amersham and the [32P]orthophosphate from New England Nuclear. M, values of virus proteins were estimated using [14C]methylated high (CFA. 646) and low (CFA. 645) molecular weight protein mixtures purchased from Amersham.

**PAGE.** Proteins were separated by electrophoresis in 10% or 10% to 20% gradient discontinuous SDS–polyacrylamide slab gels (Laemmli, 1970) as described by McPhee & Westaway (1981a) and Newton et al. (1981), respectively.

Virion RNA was phenol-extracted from purified virus particles according to the technique of Boulton & Westaway (1972) and, after ethanol precipitation, resuspended in 50 μl of electrophoresis buffer containing 1% SDS and analysed in 2% acrylamide-0.5% agarose slab gels (Boulton & Westaway, 1972). Autoradiographs were prepared and densitometer tracings were taken.

**Plaque inhibition and plaque reduction (PR) virus neutralization tests.** The PI method employed was that described by Della-Porta et al. (1981a), except that plates were stained on day 3 and read on days 4 and 5. The PR test was essentially that described by Della-Porta et al. (1981a) and by Westaway (1965a, b).
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Fig. 1. Electrophoresis in 10% to 20% gradient Laemmli slab gel of the structural proteins of Simbu serogroup viruses and BUN virus labelled with [3H]leucine (a) or [35S]methionine (b) and twice purified by rate zonal ultracentrifugation and pelleting. Mr values were determined using high Mr (A) and low Mr (B) markers.

Table 1. Molecular weights of proteins specified by Bunyamwera virus and by Australian Simbu serogroup viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>L</th>
<th>G1</th>
<th>G2</th>
<th>N</th>
<th>Possible non-structural proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN</td>
<td>187</td>
<td>113</td>
<td>35</td>
<td>27</td>
<td>16</td>
</tr>
<tr>
<td>AKA</td>
<td>193</td>
<td>124</td>
<td>36</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>AINO</td>
<td>195</td>
<td>103</td>
<td>35</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>TIN</td>
<td>193</td>
<td>112</td>
<td>36</td>
<td>26</td>
<td>30</td>
</tr>
<tr>
<td>PEA</td>
<td>193</td>
<td>120</td>
<td>37</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>DOU</td>
<td>200</td>
<td>105</td>
<td>36</td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td>FP</td>
<td>205</td>
<td>121</td>
<td>35</td>
<td>26</td>
<td>17</td>
</tr>
<tr>
<td>THI</td>
<td>205</td>
<td>125</td>
<td>33</td>
<td>26</td>
<td>22</td>
</tr>
</tbody>
</table>

*Mr are the means of two to six values obtained by comparison with migration of the following [14C]methylated standards in 10% to 20% gradient Laemmli slab gels: myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, cytochrome c and aprotinin.

RESULTS

Virus structural proteins and RNA species

Analysis of [3H]leucine- (Fig. 1 a) or [35S]methionine- (Fig. 1 b) labelled structural proteins of Australian Simbu serogroup members revealed them all to have typical bunyavirus profiles with the two external glycoproteins, G1 and G2, and the internal nucleoprotein, N, easily identified. The large L protein was only clearly recognized for AKA, AINO, TIN and PEA viruses (Fig. 1; McPhee & Della-Porta, 1981), while longer exposures (not shown) revealed the L protein for FP and DOU viruses. The Mr of the four virion proteins for the seven Simbu serogroup viruses studied were 193K to 205K (L), 103K to 125K (G1), 33K to 37K (G2) and 25K to 26K (N) (Table 1). The sizes and relative amounts were similar to those seen for BUN (McPhee & Della-Porta, 1981) and other bunyaviruses (Bishop & Shope, 1979; Ozden & Hannoun, 1980; Ushijima...
Fig. 2. (a to c) PAGE analyses of purified 32P-labelled virion RNA species of AKA CSIRO16 (a), DOU CSIRO150 (b) and FP Ch16129 (c). The RNA species are designated large (L), medium (M) and small (S) and $M_r$ values were estimated by reference to comigration of the virion RNA species of Bunyamwera virus in the same slab gel (positions of L, M and S indicated at top of figure). Absorbance measurements relate to the densitometer traces of autoradiographs.

Comparison of virion protein profiles for AKA isolates B8935, CSIRO16, R7949 and CSIRO217 (results for isolates B8935 and CSIRO16 shown in Fig. 1a; others not shown), revealed them all to be identical when compared or co-electrophoresed in 10% to 20% gradient Laemmli slab gels.

In addition to the four above-mentioned structural proteins, a protein of $M_r$ 43K was detected in a number of purified virus preparations (Fig. 1a, b). This protein comigrated with a prominent host cell protein, probably actin ($M_r$ 43K), in extracts of infected cells (see Fig. 3 and 4). Actin is thought to be incorporated adventitiously during maturation of a number of enveloped viruses (Wang et al., 1976). For THI virus, purification of satisfactory amounts of labelled virus was very difficult because of low yields and the fact that host cell protein contaminants could not be eliminated (Fig. 1b).

The virion RNAs for the Simbu serogroup viruses AKA, DOU and FP (Fig. 2) were compared with those of BUN virus (not shown) by PAGE and were all found to be similar. Each contained three RNA species of apparent $M_r$ 2.6 × 10^6 (L), 1.4 × 10^6 to 1.9 × 10^6 (M) and 0.24 × 10^6 to 0.42 × 10^6 (S). These apparent $M_r$ values, determined under non-denaturing conditions, may not be indicative of their true values, as is the case for other Bunyaviridae members (Obijeski & Murphy, 1977; Bishop & Shope, 1979). AKA and DOU virus RNA species were very similar in migration, whereas for FP virus, the M and S species appeared slightly larger, especially the latter (0.42 × 10^6 for S of FP virus compared to 0.24 × 10^6 for S of AKA virus).
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Virus-specified protein synthesis

The kinetics of protein synthesis were compared in Vero cells infected with AKA CSIRO16 and BUN viruses (Fig. 3). By using 10% to 20% gradient gels we could detect proteins in the \( M_r \) range \( 6.5 \times 10^3 \) to \( 200 \times 10^3 \) (see markers in Fig. 1a). As shown previously for BUN (McPhee & Westaway, 1981b), in addition to the four structural proteins found in infected cells, two other proteins were detected late in infection (Fig. 3, lane 9). Using a discontinuous gradient gel (Laemmli, 1970), the estimated \( M_r \) values were 16K and 13K, slightly higher values than those previously determined (13K and 8K respectively) using a continuous SDS–phosphate gel system (McPhee & Westaway, 1981b). In AKA CSIRO16 virus-infected cells, four structural proteins, L, G1, G2 and N, and two possible non-structural proteins (Table 1) similar in \( M_r \) and relative amounts to the corresponding proteins were detected in cells infected with BUN virus. Thus, in the late stages of infection, all structural proteins and two possible non-structural proteins were identified relative to the host cell proteins. Similar profiles were observed using BHK-21/13 cells except that host cell switch-off was not as effective (results not shown). We therefore exploited this rapid technique to identify the structural (and possible non-structural) proteins of other Simbu serogroup viruses because virus yields were often too low for easy analysis of virion proteins.

Analysis of the proteins specified late in infection by FP, THI, AKA, TIN, DOU, AINO and PEA viruses on a 10% to 20% gradient slab gel (Fig. 4) easily identified the structural proteins L, G1, G2 and N for most viruses. The resultant profiles were unique for each virus serotype making all of them easily identifiable. The G2 structural protein of THI, DOU and AINO was not as readily identified as those of the other viruses studied. In addition to the four structural proteins, at least two possible virus-infected proteins smaller than G2 were identified in infected cells for most viruses studied (Fig. 4, open arrowheads). These were of variable size except for the smaller possible non-structural protein, designated p22, observed in cells infected with THI, AKA, TIN and PEA viruses. The strongly labelled band migrating as a doublet with N of AINO and PEA may represent an additional intracellular form of N (Fig. 4).

**Fig. 3.** Kinetics of protein synthesis of mock- (lanes 1 to 3), AKA CSIRO16- (lanes 4 to 6) and BUN- (lanes 7 to 9) infected Vero cells. Cells were labelled with [\(^{35}\)S]methionine (20 \( \mu \)Ci/ml) at 6 to 10 (lanes 1, 4 and 7), 14 to 18 (lanes 2, 5 and 8) and 22 to 26 (lanes 3, 6 and 9) h p.i. After labelling, cells were disrupted in 2% SDS and the treated samples were electrophoresed in a 10% to 20% gradient Laemmli slab gel. Virus structural proteins (\( \blacktriangleright \)) are designated L, G1, G2 and N and the possible non-structural proteins (\( \blacktriangleleft \)) are designated NS. \( M_r \) markers were run alongside (lane 10).
Fig. 4. Electrophoresis in a 10% to 20% gradient Laemmli slab gel of [35S]methionine-labelled proteins synthesized in mock- (lane 2), BUN- (lane 1), FP- (lane 3), THI- (lane 4), AKA B8935- (lane 5), TIN- (lane 6), DOU- (lane 7), AINO- (lane 8) and PEA- (lane 9) infected cells. Cells were labelled for a 4 h period between 12 and 72 h p.i. depending on when c.p.e. was evident and host cell protein synthesis was most depressed. Structural and possible non-structural proteins are designated as for those in Fig. 3. Mr markers were run alongside (lane 10).

Table 2. Analysis of dose–response relationships in plaque reduction virus neutralization assay comparisons of AKA and TIN viruses

<table>
<thead>
<tr>
<th>Antiserum in test</th>
<th>AKA</th>
<th>TIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A*</td>
<td>B*</td>
</tr>
<tr>
<td>AKA</td>
<td>3.81</td>
<td>-1.33</td>
</tr>
<tr>
<td>TIN</td>
<td>2.67</td>
<td>-0.84</td>
</tr>
</tbody>
</table>

* A and B are values from the regression line equation \( Y = A + BX \), where \( Y \) represents \( -\log_{10} \) of the surviving fraction of virus after neutralization and \( X \) represents \( -\log_{10} \) of the antiserum dilution that produced the neutralization. After Westaway (1965a, b) and Della-Porta et al. (1981a).

Cross-reactivity between Australian Simbu serogroup strains in virus neutralization tests

Previous studies utilizing the PI virus neutralization assay showed extensive serological cross-reactions between AKA and TIN viruses, and minor cross-reactions between AINO and DOU viruses (McPhee & Della-Porta, 1981). Limited reactions of AINO, PEA, DOU and FP antisera with TIN virus were also observed. In order to define more clearly the relationships between these viruses, dose–response relationships were defined using the PR virus neutralization assay (Della-Porta et al., 1981a; Westaway, 1965a, b). The results from these assays are shown as linear regression curves of the dose–response relationships (Tables 2 and 3). They show that AKA and TIN could be easily distinguished, although the TIN antiserum did react strongly with AKA virus. Both the \( y \) axis intercepts (A) differed significantly (2.67 for AKA and 4.42 for TIN,..
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Table 3. Analysis of dose–response relationships in plaque reduction virus neutralization assay comparisons of AINO and DOU viruses

<table>
<thead>
<tr>
<th>Antiserum in test</th>
<th>AINO</th>
<th>DOU</th>
</tr>
</thead>
<tbody>
<tr>
<td>AINO</td>
<td>3.77</td>
<td>0.20</td>
</tr>
<tr>
<td>DOU</td>
<td>0.87</td>
<td>3.85</td>
</tr>
</tbody>
</table>

* See Table 2.

Table 4. Facey's Paddock virus: cross comparison by plaque inhibition virus neutralization testing of various cloned isolates

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plaque inhibition zone (mm) of antiserum against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FP Ch16129*</td>
</tr>
<tr>
<td>FP Ch16129</td>
<td>21.5</td>
</tr>
<tr>
<td>CSIRO503‡</td>
<td>0</td>
</tr>
<tr>
<td>CSIRO504§</td>
<td>12</td>
</tr>
</tbody>
</table>

* Hyperimmune mouse ascitic tumour fluids prepared against FP Ch16129 or CSIRO504.
† Rabbit antiserum prepared against CSIRO10.
‡ Plaque-purified clone from CSIRO10, neutralized by rabbit anti-CSIRO10 serum.
§ Plaque-purified clone from CSIRO10, picked in the presence of rabbit anti-CSIRO10 serum.

the homologous system) and the slope of the curve for the homologous reaction was slightly steeper (B = -1.03) than that for the heterologous system (-0.84). However, the similarity of the slopes of the curves was such as to suggest that the avidity or binding efficiency of the TIN antiserum was similar for both viruses. In contrast, the slopes and y axis intercepts for the dose–response curves for AINO and DOU (Table 3) were clearly very different.

AINO, PEA, DOU, and FP antisera did not neutralize TIN virus in the PR assay. However, it was observed that both the AINO and FP antisera increased the number of TIN plaques counted and that this enhancement was dependent on the dilution of the antiserum in the assay. The AINO antiserum gave maximum enhancement ratio of 1.7 at a dilution of 1 in 100 and the FP antiserum a maximum enhancement ratio of 1.5 at a dilution of 1 in 10.

Identification of mixed virus isolates from field specimens

Two viruses isolated during epidemiological studies in Northern Australia were thought to be Simbu serogroup viruses from preliminary serological studies (H. A. Standfast, T. D. St. George & D. H. Cybinski, personal communication). CSIRO10 was isolated from a mixed Culicoides pool collected near Darwin in November 1974. (H. A. Standfast, personal communication) and CSIRO296 was isolated from bull blood collected at Peachester in December 1979 (D. H. Cybinski, personal communication). Several unusual findings in the preliminary investigations prompted more detailed analyses.

For CSIRO10, during routine PI testing, large plaques were observed growing in the presence of reference antiserum (prepared in rabbits against CSIRO10). These plaques were picked and grown in the presence of the same antiserum and the stock was designated CSIRO504. Additional plaques were picked from the CSIRO10 stocks grown under normal conditions and designated CSIRO503. The latter virus was not affected by antiserum against FP Ch16129 (Table 4), and in further tests was not related to any Australian Simbu serogroup virus by group- or type-specific serological tests. Analyses of the virus-specified proteins (Fig. 5a) revealed an N protein very different to that of FP Ch16129 and indeed to those of all Simbu serogroup viruses.
Fig. 5. Comparative electrophoretic profiles of various clones from field isolates of FP isolate CSIRO10 (a) and CSIRO296 (b) viruses. Clones of FP virus (CSIRO503, lane 5; CSIRO504, lanes 4 and 7) were compared with reference isolate Ch16129 (lanes 2, 3 and 6) along with mock-infected cells (lanes 1 and 8). CSIRO296 virus clones (CSIRO505, lane 3; CSIRO506, lane 2; CSIRO507, lane 6) were compared with AKA CSIRO16 (lane 1), AINO B7974 (lane 5), DOU CSIRO150 (lane 8) and an isolate from a pregnant sheep (SG949, lanes 4 and 7) along with mock-infected cells (lane 9). Samples were electrophoresed in 10% to 20% gradient gels.

Table 5. CSIRO296: cross-comparison by plaque inhibition virus neutralization testing of the various cloned isolates

<table>
<thead>
<tr>
<th>Virus</th>
<th>AKA B8935</th>
<th>AINO B7974</th>
<th>CSIRO296 (uncloned)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKA B8935</td>
<td>15</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>AINO B7974</td>
<td>0</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>CSIRO505*</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CSIRO506*</td>
<td>21</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>CSIRO507*</td>
<td>0</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

* Plaque-purified clones from mixed pool of CSIRO296.

tested (not shown). In contrast, the isolate that formed plaques in the presence of rabbit anti-CSIRO10 serum (CSIRO504) had a protein profile identical to that of FP Ch16129 (Fig. 5a), except for the G1 protein which migrated more slowly. PI tests also showed strong cross-reactions between FP Ch16129 and CSIRO504 (Table 4). Thus CSIRO10 yielded two virus isolates both distinct from FP Ch16129, although CSIRO504 was closely related to FP Ch16129 in both virus neutralization tests and virus-specified protein analyses.

Initial characterization of isolate CSIRO296 revealed that antiserum raised against the virus reacted to both AKA and AINO viruses by PI testing (Table 5). For this reason it was thought that perhaps this isolate was a mixture of AKA- and AINO-like viruses. In an attempt to isolate possible distinct viruses CSIRO296 was cloned in the presence of AKA antiserum; a 1000-fold drop in p.f.u. titre was observed. Three plaques of very different morphology were then cloned as for prototype stocks (see Methods). These virus clones, designated CSIRO505, 506 and 507, all proved distinct in their serological patterns on PI testing with AKA, AINO and antiserum raised against the original CSIRO296 stocks (Table 5). CSIRO505 reacted only with AKA antiserum whereas CSIRO506 reacted with both AKA and CSIRO296 antisera. The third clone (CSIRO507) reacted strongly with both AINO and CSIRO296 antisera. Virus-specified protein
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profiles (Fig. 5b) showed CSIRO505 and CSIRO506 to be identical to AKA virus and CSIRO507 to be similar to, yet distinct from, AINO virus. These analyses suggest that there were at least three different viruses within the original CSIRO296 virus pool, two AKA-like and one AINO-like, the latter being distinct from both AKA-like isolates. Interestingly, the original uncloned virus isolate CSIRO296 has been inoculated into pregnant sheep to test for virus teratogenicity because of the serological relationship to AKA (Parsonson et al., 1982). The virus infected all the pregnant sheep but in only one instance was virus isolated from foetal tissues. The virus-specified proteins of this isolate were compared to those of all the cloned isolates (Fig. 5b) and revealed that this virus, which was able to cross the placental junction and infect the foetus, was identical in G1 and N profiles to the AKA-like isolates and AKA virus.

**DISCUSSION**

All of the Australian Simbu serogroup viruses that have been studied appear to be distinct by comparative analysis of the virus structural and possible non-structural proteins by PAGE (Table 1). Thus, the tentative classification of a new isolate could be made by comparison of its virus-specified proteins with known serotypes and confirmed by serology and analysis of the RNA. The protein and migration patterns were similar to those of BUN virus, the prototype of the *Bunyavirus* genus, and support serological data for the inclusion of the Simbu serogroup in this genus, rather than one of the other four genera (Bishop et al., 1980; Bishop, 1986).

Of the virus structural proteins, the migrations of the N and L proteins were the most conserved (Table 1; Fig. 1). Occasionally an apparent doublet of N was observed in cells infected with AINO and PEA virus (see Fig. 4): the reason for this is not known and this observation has not been reported for other bunyaviruses. Greater variation was observed in the migration of the two surface glycoproteins G1 and G2 (Table 1; Fig. 1). All viruses showed differences in the migration of G1, within the *M* range 103K to 125K, except for AINO and DOU. The migration of G2 showed less variation, *M* 35K to 37K, with THI, at 33K, showing more variation. THI virus is serologically the most distinct of the Australian Simbu serogroup viruses and has a different ecological niche, being associated with avian hosts (McPhee & Della-Porta, 1981). 'A wide range of AKA isolates have been examined and all have an indistinguishable protein profile (Fig. 1a; Della-Porta et al., 1981b).

The sizes of RNA segments of AKA and DOU viruses were indistinguishable. The M and S RNA segments of FP virus migrated less rapidly than those of AKA and DOU viruses. Although the S RNA of FP is apparently larger, the N protein (coded for by the S RNA) is virtually identical in *M* to that of other Simbu serogroup viruses. However, the RNA species were compared using non-denaturing conditions which may not reflect their true *M*; additionally, the S RNA gene codes for more than one virus protein (Fuller & Bishop, 1982; Elliott, 1985).

Non-structural proteins specified by several viruses of the *Bunyavirus* genus have recently been identified, most of which migrate faster than N (Ushijima et al., 1981; Fuller & Bishop, 1982; Short et al., 1982; Elliott, 1985). Two of these correspond to proteins of *M*, 16K and 13K noted in this report and detected previously for BUN virus (McPhee & Westaway, 1981b). It would appear that of the possible non-structural proteins migrating faster than N for Simbu serogroup viruses (Fig. 3 and 4), at least two may be virus-coded because they were unique for each virus and were not detected in mock-infected cells.

Previous serological studies using hyperimmune mouse ascitic fluids had shown strong virus-neutralizing antibody cross-reactions between AKA and TIN viruses and moderate cross-reactions between AINO and PEA viruses using the PI virus neutralization test (McPhee & Della-Porta, 1981). We investigated these cross-reactions further using a PR virus neutralization test (Della-Porta et al., 1981a) in which we determined the linear regression curves for the homologous and heterologous antibody reactions with each pair of viruses (Table 2 and 3). Each of the viruses could be distinguished easily but the results indicate that care should be taken in interpreting low antibody titres, especially from field sera, when testing animals for previous infections associated with AKA and/or AINO viruses. Further studies are under way to define the importance of these cross-reactions in sheep and cattle; the work reported in this study...
involved hyperimmune mouse ascitic fluids. The similarity of the slopes of the dose–response curves (Table 2) for TIN antiserum with AKA and TIN suggests that the TIN antiserum contained antibodies that were able to recognize the same antigenic determinant(s) on both viruses, hence explaining the similar avidities observed with each virus.

An interesting observation was the enhancement of TIN virus infectivity by AINO and FP antisera. Such enhancement has been observed for flaviviruses (Hawkes & Lafferty, 1967; Della-Porta & Westaway, 1977; Peiris & Porterfield, 1981) and for alphaviruses and bunyaviruses (Peiris & Porterfield, 1981). The observation would suggest that there are a small number of antigenic determinants on the surface of TIN virus to which antibodies to AINO and FP can bind without neutralizing virus infectivity (Della-Porta & Westaway, 1977). The use of Vero cells in this assay may have resulted in lower levels of enhancement when compared to the Fc receptor-containing macrophage line used by Peiris & Porterfield (1981), although enhancement has been observed in non-macrophage cell lines and its mechanism is unknown (Halstead, 1982). Whether immune enhancement plays a role in Bunyavirus infection or disease is unclear, but it is certainly unnecessary for the production of teratogenic defects by AKA virus (Parsonson et al., 1981a, c).

The usefulness of the serological and biochemical methods described in this paper was demonstrated by the identification and analysis of mixtures of virus strains in two field isolates: one from insects (CSIRO10; Table 4; Fig. 5a) and one from the blood of a bull (CSIRO296; Table 5; Fig. 5b). The virus identified in a mixed pool (SG949) that was able to cross the ovine placenta and infect the foetus was clearly similar to AKA virus in its protein profile. Thus, any new isolate within the Simbu serogroup that has a similar profile to AKA could be teratogenic. These results emphasize the need for caution in the use of field isolates which have not been plaque-cloned as prototype strains of viruses. Thus, serology and protein PAGE profiles proved to be valuable in defining virus strains within virus pools and may be useful in identifying any future virus isolates thought to be members of the Simbu serogroup, particularly those suspected of being teratogenic.

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REFERENCES


Comparisons of Simbu serogroup bunyaviruses


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