

## IDENTIFICATION OF THE SEROTYPE-SPECIFIC AND GROUP-SPECIFIC ANTIGENS OF BLUETONGUE VIRUS

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### ABSTRACT

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The bluetongue virus (BTV) core particle contains 2 major polypeptides, P3 and P7, and is surrounded by an outer capsid layer that is composed of the 2 major polypeptides, P2 and P5. Analysis of the immune precipitates from soluble  $^{14}\text{C}$ -labelled BTV polypeptides and hyper-immune rabbit and guinea-pig sera indicated that polypeptide P2 precipitates only with homologous BTV sera. This would indicate that P2 is the main determinant of serotype specificity. It was also found that in sheep infected with BTV the P2-precipitating antibodies in the serum correlate with the neutralizing antibody titres, whereas the appearance and subsequent decline of P7-precipitating antibodies correspond well with those of the complement fixing antibodies. This suggests that BTV group specificity, as measured by a complement fixation test, is determined by the core protein P7. This result was supported by the observation that mouse ascitic fluid, which contains a high titre of BTV-specific complement fixing antibodies and a very low titre of neutralizing antibodies, contains almost exclusively antibodies that precipitate P7.

### Résumé

#### IDENTIFICATION D'ANTIGÈNES DU VIRUS DE LA FIÈVRE CATARRHALE DU MOUTON SPÉCIFIQUES DE SÉROTYPE ET DE GROUPE

La particule nucléaire du virus de la fièvre catarrhale du mouton (BTV) contient deux polypeptides majeurs, P3 et P7 et est entourée par une coque (capside) qui est composée de deux polypeptides majeurs, P2 et P5. L'analyse des précipités immunes de polypeptides solubles du BTV conjugués au  $^{14}\text{C}$  et de sera de lapin et de cobaye hyperimmunisés a indiqué que le polypeptide P2 se précipite seulement avec les sera BTV homologues. Ceci indiquerait que P2 est le déterminant principal de la spécificité de sérotype. Il a également été trouvé que chez le mouton infecté du BTV les anticorps précipitant P2 dans le serum sont en corrélation avec les titres d'anticorps neutralisant, tandis que l'apparence et le déclin subséquent des anticorps précipitant P7 correspondent bien avec ceux des anticorps fixant le complément. Ceci suggère que la spécificité de groupe du BTV, telle que mesurée par la fixation de complément, est déterminée par la protéine nucléaire P7. Ces résultats sont confirmés par l'observation que le liquide ascitique de souris, qui contient un titre élevé d'anticorps fixant le complément spécifiques du BTV et un titre très faible d'anticorps neutralisant, contient presque exclusivement des anticorps qui précipitent P7.

### INTRODUCTION

Bluetongue virus (BTV) is a member of the orbivirus subgroup in the Reoviridae family of double-stranded RNA viruses (Verwoerd, Huismans & Erasmus, 1979). The virion consists of a core particle composed of 32 capsomers arranged in icosahedral symmetry (Els & Verwoerd, 1969). The core is surrounded by a diffuse, structureless, outer capsid layer (Verwoerd, Els, De Villiers & Huismans, 1972) composed of 2 major polypeptides, P2 and P5, while the core contains 2 major and 3 minor capsid polypeptides (Martin & Zweerink, 1972; Verwoerd *et al.*, 1972).

The antigenic properties of BTV until recently have been studied mainly by means of various serological reactions. The BTV serogroup was found to consist of as many as 20 different serotypes which cross react in complement fixation (CF) tests but can be distinguished by neutralization tests (Howell, Kümm & Botha, 1970; Verwoerd *et al.*, 1979).

It is not known which of the capsid polypeptides of the virus are involved in the observed antigenic variation in the BTV serogroup, but several reports seem to indicate that most likely the variation is associated with the polypeptides in the outer capsid layer of the virus (Huismans & Howell, 1973; De Villiers, 1974).

An immune precipitation technique was used in the current investigation of the antigens involved in antigenic variation.

### MATERIALS AND METHODS

**Cells:** Baby hamster kidney (BHK) cells were obtained from the American Type Culture Collection, USA, and grown in modified BHK-medium, supplemented with 5% bovine serum.

**Virus:** The different BTV serotypes were all from the egg-adapted vaccine strains available at the Onderstepoort Veterinary Research Institute. After plaque purification they were propagated in cultures of BHK cells confluent with serum-free medium, the lowest possible tissue culture passage stock being used as inoculum. Virus was harvested 48 h after infection and titrated on L-strain mouse fibroblast cells (Howell, Verwoerd & Oellermann, 1967). The virus was purified as described by Huismans (1979). In the case of partially purified virus the sucrose gradient step in the purification was omitted.

The Alberta and New Jersey strains of epizootic haemorrhagic disease virus (EHDV) used were both obtained from the Arthropod-borne Animal Diseases Research Laboratory, USDA Denver, USA.

#### Preparation of BTV antibodies

**Rabbits:** Rabbits were injected with 1.5 mg of partially purified BTV of a specific serotype, together with complete Freund's adjuvant. After 2 weeks a booster was given with 750  $\mu\text{g}$  of the virus followed by another booster 1 week later. One week after the last booster the animals were bled and the serum collected. Serum was frozen and stored at  $-20^\circ\text{C}$ .

**Guinea-pigs:** Highly specific neutralizing antisera were prepared in guinea-pigs by a series of 5-6 weekly



intraperitoneal inoculations of BTV grown in BHK cells and adsorbed onto freshly prepared aluminium phosphate, as described by Wallis & Melnick (1967).

**Sheep:** Three young Merino sheep fully susceptible to all BTV serotypes were used. Each sheep was injected intravenously with 0.5 ml of virulent BTV-10 containing sheep blood. The sheep were examined daily and serum samples were collected first at daily and later at weekly intervals. All 3 sheep developed marked febrile reactions, severe mouth lesions and mild coronitis, but recovered without further complications.

**Mouse ascitic fluid:** Young adult albino mice were immunized by intraperitoneal injection of BTV in Freund's adjuvant according to the schedule described by Sommerville (1967).

#### *Preparation of soluble extracts of BTV polypeptides*

BHK cells, grown as monolayer cultures in Roux flasks, were infected with BTV at a multiplicity of infection of between 20 and 40 plaque-forming units (pfu)/cell and the cells incubated at 31 °C. At 16 h after infection the cells were rinsed with amino acid-free Eagle's medium and each Roux flask ( $10^8$  cells) was incubated for 2 h at 37 °C with 5 ml of this medium containing 1  $\mu$ Ci/ml  $^{14}$ C-protein hydrolysate.\* At the end of the 2 h incubation period, cells were harvested by being scraped from the glass and collected by centrifugation for 15 min at 1 500 rpm. The cells were resuspended at a concentration of  $5 \times 10^7$  cells/ml in buffer containing 0.01 M NaCl, 0.005 M MgCl<sub>2</sub>, 0.01 M Tris, pH 7.4 and 0.5% Triton X100. After 5 min at 4 °C the nuclei from the disrupted cells were removed by centrifugation for 5 min at 3 000 rpm. The nuclei were rinsed once in half the original volume of the TX100-containing buffer. The 2 supernatant solutions were combined and centrifuged for 2 h at 40 000 rpm in a SW 50.1 Beckman rotor after addition of NaCl to a final concentration of 0.15 M NaCl. The supernatant, or S100 fraction, was used in immune precipitation experiments within 6 h after preparation.

#### *Immune precipitation*

Immune precipitations were carried out with either guinea-pig, sheep or rabbit sera. Routinely, 0.05 ml of the guinea-pig immune sera or 0.1 ml of the sheep or rabbit immune sera were mixed with 0.2 ml of a  $^{14}$ C-labelled S100 extract from BTV infected cells. After 16 h at 4 °C, 1 ml of cold 0.15 M STE (0.15 M NaCl, 0.01 M Tris-HCl pH 7.4, 1 mM EDTA) was added and immune precipitates were collected by centrifugation for 20 min at 3 000 rpm. The precipitates were rinsed with 2 ml of cold 0.15 M STE, centrifuged as before and stored at -20 °C until analysed by electrophoresis.

#### *Electrophoresis*

Freeze-dried samples or dried immune precipitates were resuspended in 20  $\mu$ l of a solution containing 0.8 M NaCl, 8 M urea, 1.5% SDS and 4% mercapto ethanol. The samples were layered on to 7.5% phosphate-buffered polyacrylamide slab gels in the presence of urea (Stone, Smith & Joklik, 1974). Electrophoresis was for 16 h at 55 V/gel.

Gels were stained in Coomassie Brilliant Blue G-250 (Anderson, Cawston & Cheeseman, 1974) and destained in 4% acetic acid.

#### *Autoradiography*

Gels were dried on filter paper under vacuum on a heated geldrying apparatus before being exposed in the dark to Kodak DF 96 X-ray film.

Where indicated, specific bands on the autoradiograms were scanned with a Vitatron densitometer coupled to a device for integrating the different peak areas.

#### *Plaque reduction neutralization test*

Multi-well, disposable plastic panels were used and a suspension of Earle's L929 was distributed in 1.0 ml volumes to each well. Twofold serial dilutions (usually 1:20-1:640) were made in Eagle's medium and then mixed with equal volumes of BTV. These had previously been titrated and diluted to contain 30-40 pfu per 0.2 ml. The mixtures were well agitated, incubated at 37 °C for 60 min and, after the growth medium had been decanted, 0.4 ml of each serum-virus mixture was transferred to 3 or 4 replicate wells. An equal volume (0.4 ml) of a 1% agarose suspension in Earle's balanced salt solution kept at about 43 °C was added to each well and allowed to solidify. The plates were incubated at 37 °C in an atmosphere of 5% CO<sub>2</sub> in air. On the 2nd or 3rd day 0.2 ml of a 0.5% agarose overlay containing neutral red (1:20 000) was added to each well. Plaques were counted 24-48 h after the addition of the stained overlay and antibody titres were expressed as the reciprocal of the serum dilution causing a 50% plaque reduction. An antibody titre of 20 or higher was regarded as significant.

#### *Complement fixation test*

The CF test was performed according to the method described by McIntosh (1958) for African horse sickness virus.

## RESULTS

#### *Immune precipitation with guinea-pig antisera*

Soluble  $^{14}$ C-labelled polypeptides of a number of different BTV serotypes were immune-precipitated with guinea-pig immune sera prepared against homologous and heterologous serotypes. The sera were those routinely used for serotyping BTV strains and show very little cross-reaction in neutralization tests. Precipitates were analysed by electrophoresis and autoradiography as described under Materials and Methods. A typical result obtained with a number of different serotypes is shown in Fig. 1.

From the homologous immune precipitates of serotypes 10, 3 and 1 (lanes b, k and t in Fig. 1) it can be seen that the sera contain predominantly antibodies that precipitate capsid polypeptides P2, P3 and P7. In the case of serotypes 10 and 3 there is also strong immune precipitation of P6A, a non-capsid polypeptide (Huisman, 1979). With heterologous sera the amount of P2 that is precipitated is reduced to just a fraction of that of the homologous control (lanes c to j) or hardly any precipitation is seen at all (lanes l to s). The precipitation of P3, P7 and P6A is not affected by the serotype of the serum used.

All the relevant immune precipitations with guinea-pig sera carried out are summarized in Table 1. The results reflect the ability of a specific serum to precipitate polypeptide P2 from a homologous or a heterologous BTV serotype. This ability is expressed either as a positive (+), indicating strong precipitation of P2

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such as illustrated in lanes b, k and t in Fig. 1, or a negative (-), indicating very weak precipitation when compared to that of a homologous control (such as shown in lanes c, d, l in Fig. 1). The few cases in which P2 was precipitated with intermediate efficiency (lane g in Fig. 1) are indicated by a  $\pm$ . The

precipitation of P3, P6A and P7, being unaffected by serotype specificity, is not reflected in Table 1.

It is evident that only the 8 homologous controls are positive. The heterologous immune precipitations are all negative except for 2 cases, both involving serotype 4, which are intermediate ( $\pm$ ).

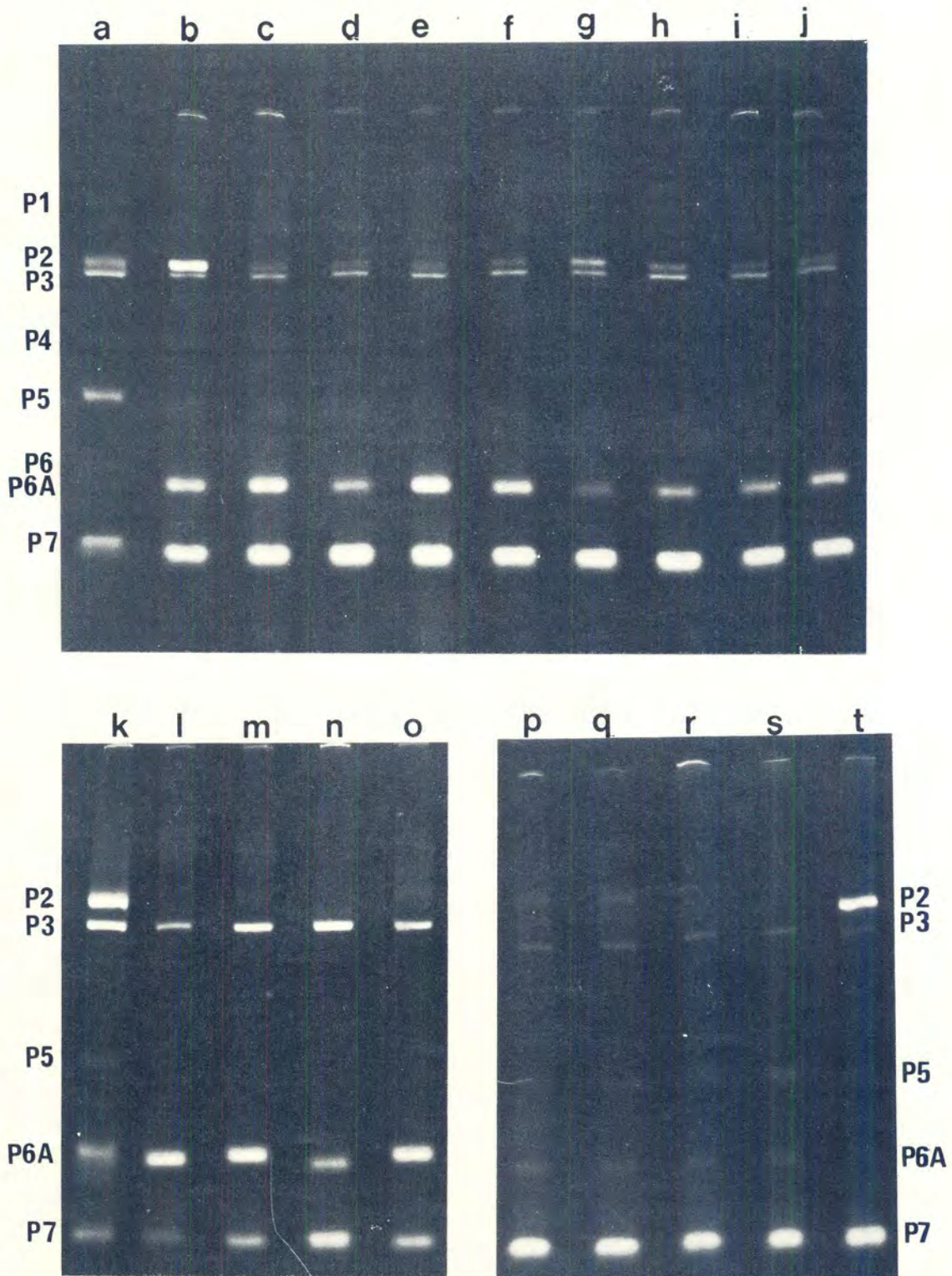


FIG. 1. Autoradiograms of the electrophoretic fractionation of the immune precipitates obtained in the reaction of  $^{14}\text{C}$ -labelled polypeptides of 3 different BTV serotypes with homologous and heterologous guinea-pig immune sera. BTV-10 polypeptides with sera against (b) BTV-10, (c) BTV-9, (d) BTV-8, (e) BTV-7, (f) BTV-6, (g) BTV-4, (h) and (n) BTV-3, (i) BTV-2 and (j) BTV-1. BTV-3 polypeptides with sera against (k) BTV-3, (l) BTV-1, (m) BT-4 and (o) BTV-10. BTV-1 polypeptides with G.P. sera against (p) BTV-15, (q) BTV-10, (r) BTV-14, (s) BTV-4, (t) BTV-1. (a) Control showing the polypeptides of BTV-10



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TABLE 1 Cross-immune precipitation of BTV polypeptide P2 in a reaction between <sup>14</sup>C-labelled polypeptides of a number of different BTV serotypes and GP sera from heterologous and homologous serotypes

Polypeptides from BTV type	GP immune sera against BTV serotype														
	1	2	3	4	6	7	8	9	10	11	12	13	14	15	
1.....	+	N	N	-	N	N	-	N	-	-	-	-	-	-	
3.....	-	N	N	-	N	N	-	N	-	-	-	-	-	-	
4.....	-	N	N	+	-	N	-	N	-	±	-	N	-	-	
6.....	-	N	-	-	+	N	N	N	-	N	-	N	N	N	
10.....	-	N	-	±	-	N	N	N	+	-	-	N	-	-	
11.....	-	N	-	-	N	N	N	N	-	+	-	N	-	N	
14.....	-	N	N	-	N	N	N	N	-	-	N	N	+	-	
15.....	-	N	N	N	N	N	N	N	-	-	N	N	-	+	

N indicates immune precipitations not carried out  
 - indicates very little or no immune precipitation of P2  
 + indicates strong precipitation of P2  
 ± indicates an intermediate result

The result is not restricted to BTV. An experiment was carried out in which <sup>14</sup>C-labelled polypeptides of the New Jersey (NJ) strain of epizootic haemorrhagic disease virus (EHDV) were precipitated with homologous GP antiserum and antiserum from a heterologous EHDV strain (Alberta). Immune precipitates are shown in Fig. 2.

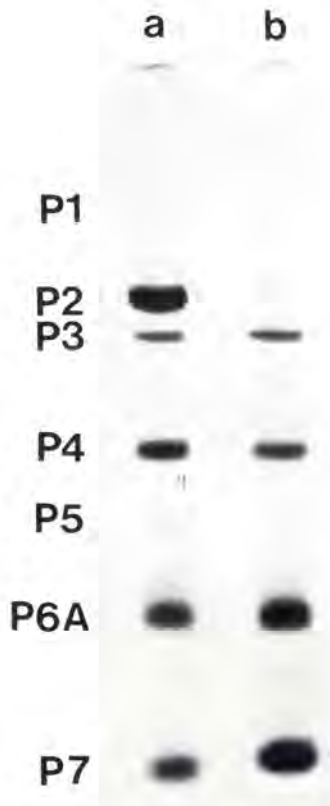


FIG. 2 Autoradiogram of the electrophoretic fractionation of immune precipitates obtained in the reaction of <sup>14</sup>C-labelled polypeptides from cells infected with EHDV (New Jersey strain) with guinea-pig sera prepared against (a) EHDV (NJ) and (b) EHDV (Alberta strain)

Polypeptide P2 is again only precipitated with homologous serum.

*Immune precipitation with rabbit antisera*

Since GP sera do not contain P5-precipitating antibodies, it was necessary to repeat the immune precipitations with rabbit antisera against the different BTV serotypes. The P5 immune response in rabbits appears to be much better than in guinea-pigs (Huisman, 1979). A typical result is shown in Fig. 3.

It is evident that, whereas P2 is not (or at best weakly) precipitated with heterologous sera, this is not the case with the precipitation of P5.

In some instances P5 is precipitated strongly with heterologous sera (lanes e and b, Fig. 1). In others P5 is either precipitated in relatively small amounts or not at all (lanes f and g, Fig. 1). These results, together with all the other immune precipitations carried out with rabbit sera, are summarized in Table 2.

Table 2 classifies the ability of a serum to precipitate P5 as either positive (+), negative (-) or intermediate (±). Almost half (21) of a total of 46 heterologous immune precipitations are considered positive, whereas 15 are negative and 10 intermediate.

Cross-neutralization by the different rabbit sera was tested with the standard neutralization test described under Materials and Methods. There was no evidence of a higher than usual level of cross-neutralization in the cases in which cross-immune precipitation of P5 was observed. No evidence that P5 is implicated in serotype specificity or that antibodies against P5 are involved in neutralization of the virus was therefore obtained.

*Immune precipitation and the levels of CF and neutralizing antibodies in sheep sera*

In this investigation no attempt was made precisely to quantify the immune precipitating antibody levels in sheep sera. It was assumed, however, that a large decline in CF titre should be accompanied by a decline in the amount of CF antigen that could be precipitated by the sheep sera. The immune precipitation conditions in the experiment were selected to avoid a large excess of antibodies over the available pool of soluble BTV polypeptides.

Three sheep were injected with BTV type 10, as described under Materials and Methods and serum was collected first at daily, later at weekly and then



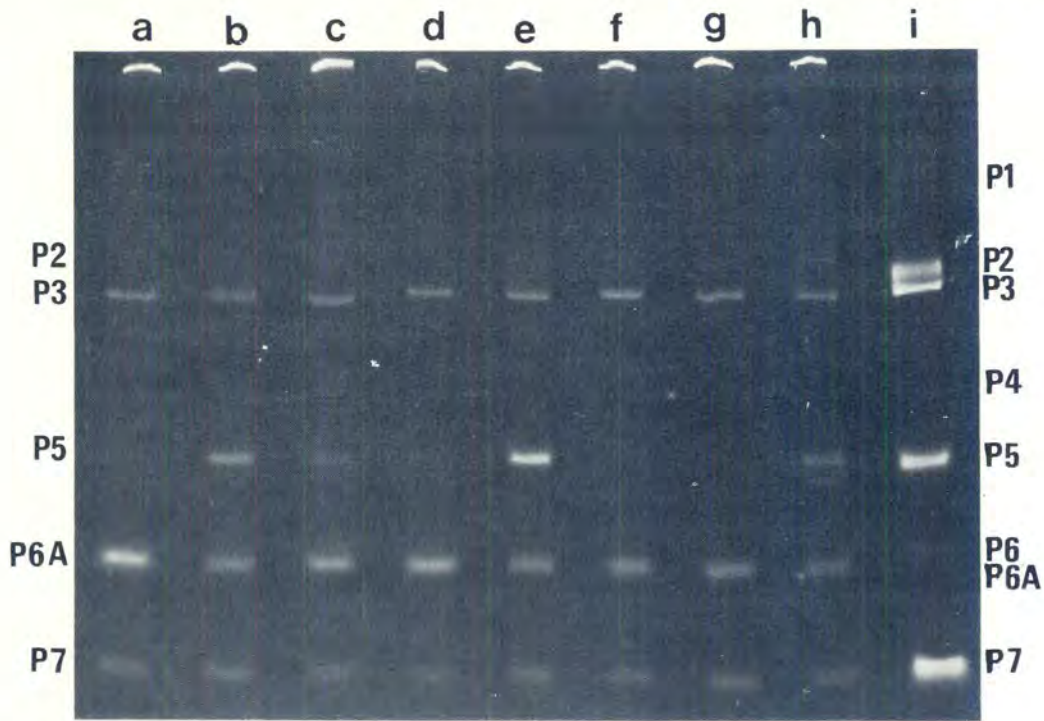


FIG. 3 Autoradiogram of the electrophoretic fractionation of immune precipitates obtained in the reaction between  $^{14}\text{C}$ -labelled polypeptides from cells infected with BTV-3 and rabbit immune sera against (a) BTV-1, (b) BTV-8, (c) BTV-10, (d) BTV-11, (e) BTV-13, (f) BTV-14, (g) BTV-15 and (h) BTV-16. (i) Control showing the polypeptides present in BTV-10

TABLE 2 Cross-immune precipitation of BTV polypeptide P5 in a reaction between  $^{14}\text{C}$ -labelled polypeptides of a number of different BTV serotypes and rabbit sera of homologous and heterologous serotypes

Polypeptides from BTV type	Rabbit immune sera against BTV serotype								
	1	4	8	10	11	13	14	15	16
1.....	+	-	+	+	-	+	-	-	N
3.....	-	+	+	+	-	+	-	-	+
4.....	+	+	N	+	±	N	±	N	N
10.....	-	+	N	+	±	N	±	+	N
11.....	±	+	+	+	±	+	±	-	+
14.....	-	+	+	±	-	+	+	±	+
15.....	-	±	N	±	-	N	-	+	N

N indicates immune precipitations not carried out  
- indicates very little or no precipitation of P5

+ indicates strong precipitation of P5  
± indicates an intermediate result

at longer intervals after injection. CF tests were carried out on all the sera and the neutralizing antibody levels measured by a plaque inhibition test. Each serum was then tested for its ability to precipitate BTV soluble polypeptides by immune precipitation. The precipitates were analysed by electrophoresis and autoradiography. The results obtained with a few of the sera of one of the sheep are given in Fig. 4.

Fig. 4 demonstrates the rapid appearance of antibodies that precipitate polypeptides P2 and P7 in the period 10 and 11 days after injection. The ability to precipitate P7 is the highest in this early period. However, there is a noticeable decline in P7 precipitation with sera collected later than 72 days post-injection. There is also a steady decline in the ability, to precipitate P2, but much less so than that observed in the case of P7. This is illustrated by the fact that shortly after injection (period 6-20 days) the relative amount of P7 in the precipitate appears to be much more than that of P2, whereas in the period 136-184 days it is much less.

The P2 and P7 bands on autoradiograms such as the one shown in Fig. 4 were quantified by scanning and the integration values expressed as a percentage of the maximum for either P2 or P7. In Fig. 5A the relative amounts of P7 precipitated by the different sera are plotted against days after injection together with a plot of the CF titres (also expressed as a percentage of the maximum). In Fig. 5B the relative amount of precipitated P2 with the time after injection is shown together with neutralization titres of the sera expressed as a percentage of the maximum.

CF tests are used to indicate group specificity. The close agreement between the decline in CF titres of a sheep serum and the decline in its ability to precipitate P7 (Fig. 5A) would therefore indicate that P7 is the group specific or CF antigen. Neutralization tests, on the other hand, are used to distinguish between different BTV serotypes. The correlation between neutralization titres and the ability to precipitate P2 (Fig. 1B) suggests that P2 is the neutralization specific or serotype specific antigen.



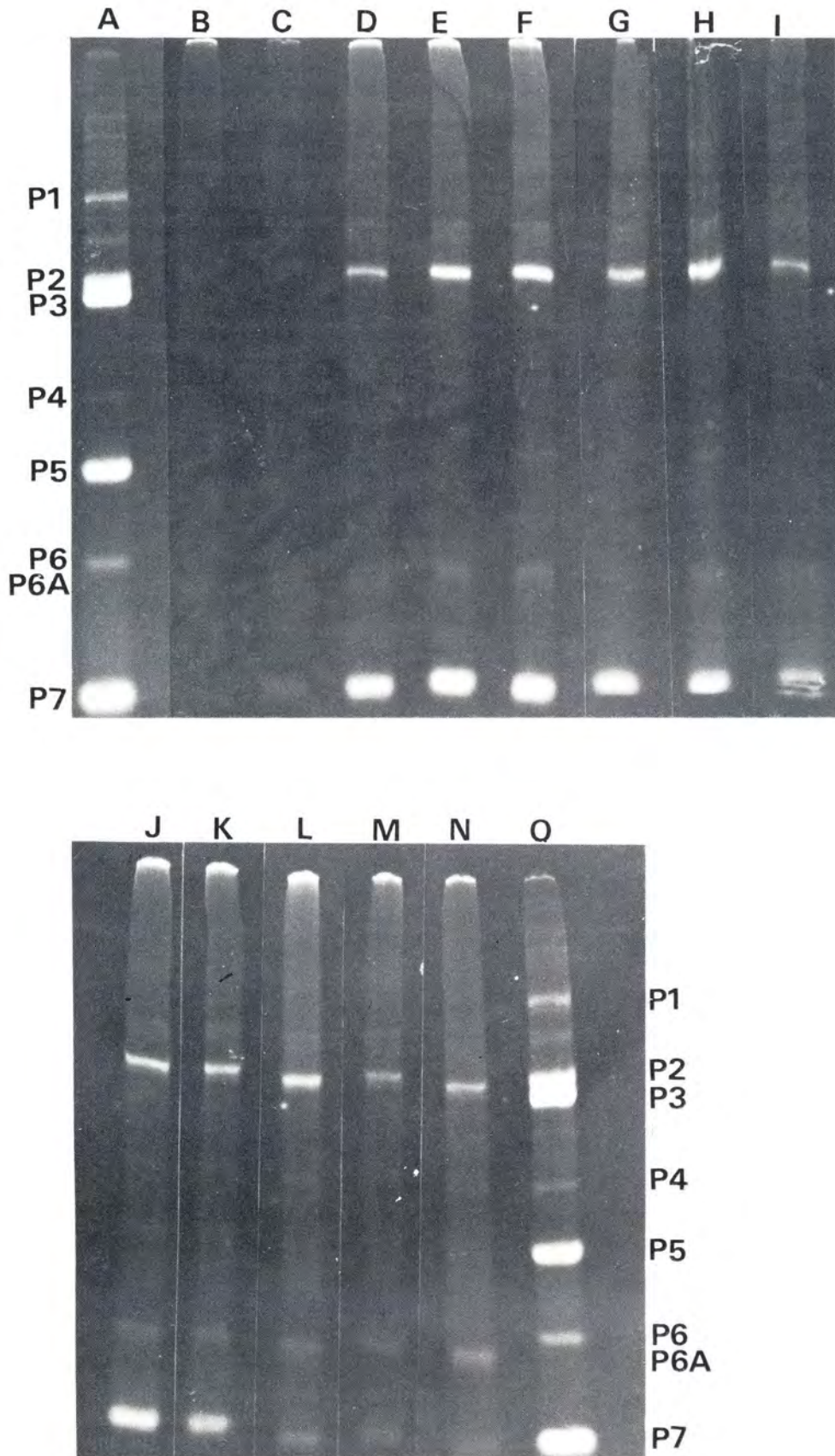


FIG. 4. Autoradiogram of the electrophoretic fractionation of immune precipitates obtained in the reaction of  $^{14}\text{C}$ -labelled polypeptides from cells infected with BTV-10 and sheep sera collected on the following days after injection with BTV-10: (B) 9, (C) 10, (D) 11, (E) 12, (F) 13, (G) 23, (H) 37, (I) 62, (J) 72, (K) 93, (L) 136, (M) 156, (N) 184. Control (A) represents the BTV-10 polypeptides and it was run together with samples (B) to (I) on one gel. Control (O) also represents BTV-10 polypeptides and was run together with samples (J) to (N) on another gel



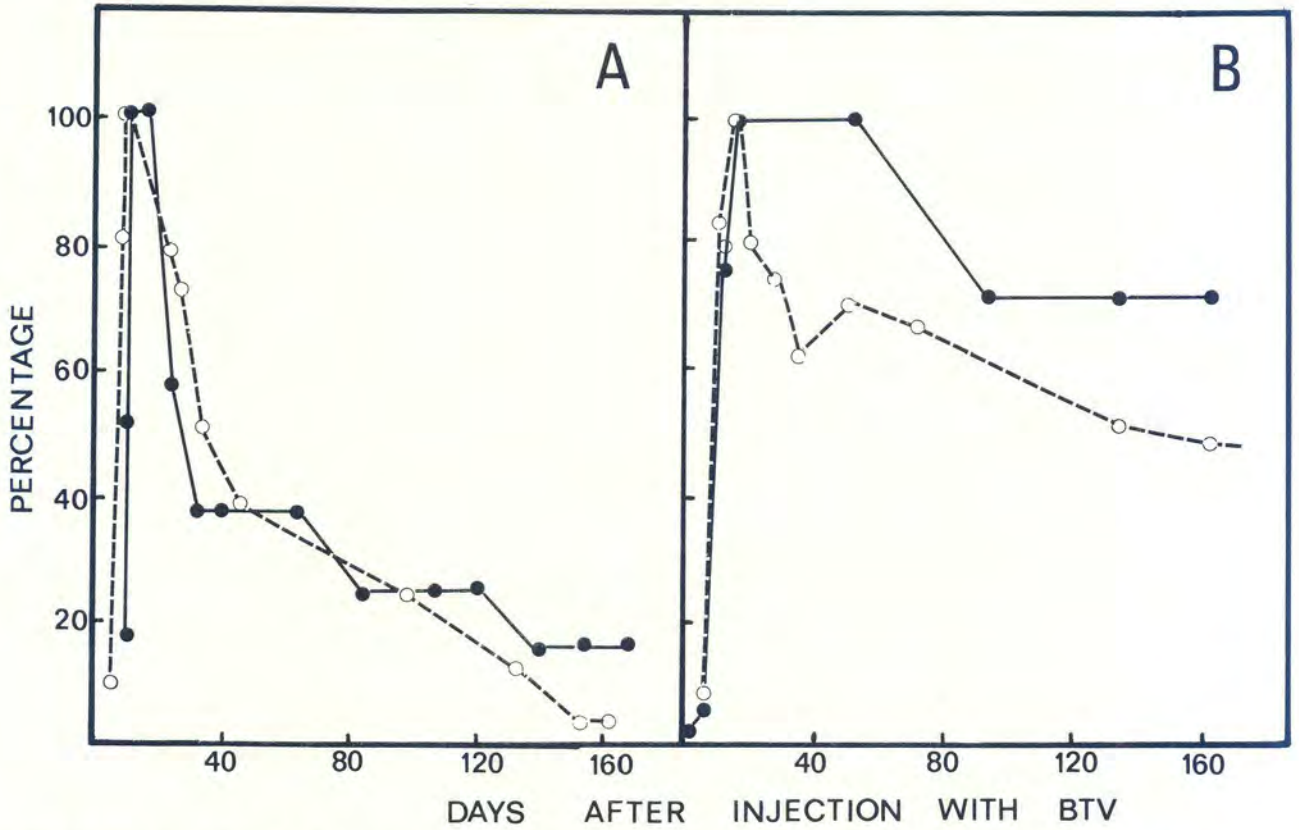


FIG. 5A ---○---○--- The relative amount of P7 precipitated by sera collected at different times after injection with BTV  
 —●—●— The relative CF titres in the sera

FIG. 5B ---○---○--- The relative amount of P2 precipitated by sera collected at different times after injection with BTV  
 —●—●— The relative neutralization titres in the sera

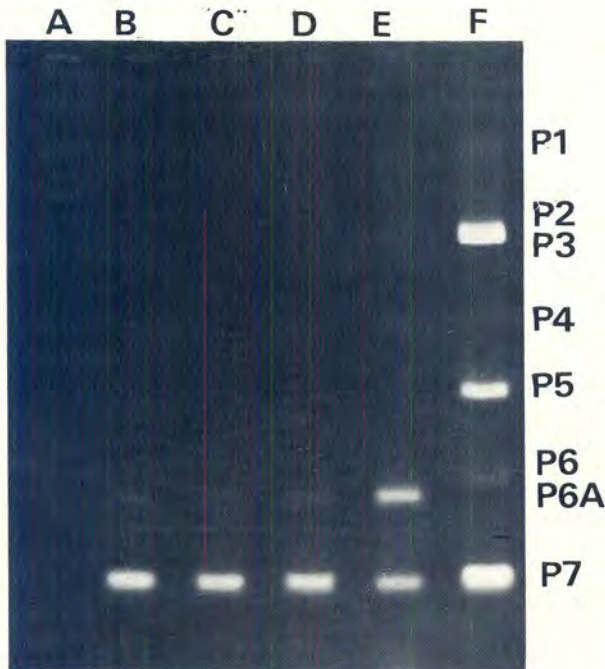


FIG. 6 Autoradiogram of the electrophoretic fractionation of the immune precipitates obtained from the reaction between <sup>14</sup>C-labelled polypeptides in 0.2 ml of an S100 fraction from cells infected with BTV-10 and amounts of mouse ascitic fluid: (A) 0.01 ml; (B) 0.02 ml; (C) 0.05 ml; (D) 0.1 ml (E) 0.2 ml. The control (F) contained polypeptides from BTV-10

These conclusions were substantiated by the results obtained with the other sheep. In one of the sheep the result was almost identical with that shown in Fig. 5 A and B. In the other the response was different in that the appearance of CF antibodies was delayed and could not be detected until 21 days after injection. After just more than 1 month the CF titre declined rapidly. This particular pattern again corresponded to the ability of the sera to precipitate polypeptide P7 (result not shown).

*Immune precipitation with mouse ascitic fluid*

An immune precipitation was also carried out with mouse ascitic fluid from BTV-infected mice. Since ascitic fluid from BTV-infected mice is characterized by comparatively high CF antibodies but very low neutralizing antibody titres, it is often used in CF tests. Fig. 6 shows the immune precipitates obtained in the reaction of BTV-10 polypeptides with increasing amounts of ascitic fluid.

Polypeptide P7 is the only capsid polypeptide precipitated from the pool of soluble BTV antigens. Precipitation of P6A (a non-capsid polypeptide) is also seen when a large excess of ascitic fluid is used.

DISCUSSION

The results obtained indicate that the outer capsid polypeptide, P2 of BTV, is the main serotype-determining antigen, whereas core polypeptide P7 is probably the major determinant of group specificity.



Several reports have also indicated serotype-related variation in polypeptide P2. Huismans & Howell (1973) could demonstrate by hybridization experiments that the largest variation in the nucleic acid of the different BTV serotypes was found to be associated with genome segment 2, the segment that presumably codes for polypeptide P2 (Verwoerd *et al.*, 1972). This result was substantiated by comparative electrophoresis of the polypeptides of the different BTV serotypes which indicated that the largest variation in polypeptide size amongst the different serotypes was found in polypeptide P2 (De Villiers, 1974). The loosely structured outer capsid protein layer of BTV can apparently accommodate such P2 size variations. In the case of reovirus the outer capsid layer is more structured (Luftig, Kilham, Hay, Zweerink & Joklik, 1972) and serotype specificity is associated with a minor polypeptide component of this outer layer (Weiner & Fields, 1977).

Even though at least some of the antigenic determinants on polypeptide P2 appear to be serotype specific, it is by no means certain that all the P2 precipitating antibodies should necessarily be serotype specific. It was, in fact, rather surprising that so few cases of P2 cross-immune precipitations between different serotypes were found. In Table 1 only 2 cases of intermediate ( $\pm$ ) P2 immune precipitations were found. A few more such cases are reported in an accompanying paper (Huismans & Bremer, 1981). They all involve either type BTV-4 or the Australian BTV isolate, BTV-20. In most of these cases, however, some evidence for cross-neutralization has also been found.

The other outer capsid polypeptide, P5, also shows a considerable size variation amongst different serotypes (De Villiers, 1974). However, no evidence was obtained by immune precipitation that P5 contributes to serotype specificity. This does not rule out the possibility that P5 antibodies can still be involved in virus neutralization and it could be rewarding to investigate the role of P5 by a more direct method. Van Dijk & Huismans (1980) reported that polypeptide P2 can be removed selectively from the BTV particle without affecting P5. The immune response against such subviral particles under conditions where the virus does not replicate should provide further interesting information on the role of P5 and P2 in neutralization and serotype specificity.

The group-specific antigen of BTV, polypeptide P7, is part of the virus core particle. According to Martin, Pett & Zweerink (1973), polypeptide P7 is on the inside of the core and is less exposed to the core surface than the other major core polypeptide, P3. Recent investigations (Huismans, unpublished results) indicate, however, that the characteristic capsomeres on the surface of the BTV cores are composed of polypeptide P7. Large amounts of polypeptide P7 are present in the soluble fraction of infected cells (Huismans, 1979) and P7 could be the group-specific, soluble BTV antigen that reacts in immuno-diffusion tests (Wang, Lueker & Chow, 1972). All BTV strains investigated so far show cross-immune precipitation of P7. This also applies to other viruses such as EHDV that show CF cross-reaction with BTV (Huismans, Bremer & Barber, 1979).

It is impossible, at this stage, however, to exclude a contributory role for other virus polypeptides such

as P3 or the minor polypeptide components in the group specific response. A more direct approach, for example, investigating the immune response against single proteins or BTV sub-core particles, could provide answers to some of these questions.

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