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FACTORS AFFECTING THE BLUETONGUE VIRUS NEUTRALIZING ANTIBODY RESPONSE AND THE REACTION BETWEEN VIRUS AND ANTIBODY

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ABSTRACT

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A study was made of different aspects of the bluetongue virus neutralizing antibody response and the reaction between the virus and antibody. Optimum neutralization was obtained in a 2 mM Tris-HCl buffer, pH 9,0, at a temperature of 4 °C. The reaction of virus and antibody could be demonstrated by electron microscopy in the formation of clumps which were shown to be serotype specific. It was found that both IgM and IgG antibodies can neutralize the virus, but that IgM reached its maximum level sooner after infection than IgG.

Résumé

FACTEURS AFFECTANT LA RÉPONSE IMMUNITAIRE EN ANTICORPS NEUTRALISANT DU VIRUS DE LA FIÈVRE CATARRHALE DU MOUTON ET LA RÉACTION ENTRE VIRUS ET ANTICORPS

On a étudié divers aspects de la réponse immunitaire en anticorps neutralisant du virus de la fièvre catarrhale du mouton et de la réaction entre virus et anticorps. On a obtenu la meilleure neutralisation à la température de 4 °C et au pH 9,0 dans un tampon Tris-HCl à concentration 2 mM. Le microscope électronique a fourni une démonstration de la réaction entre virus et anticorps en mettant en évidence la formation d'amas dont on a pu montrer qu'ils sont spécifiques du sérotype. On a trouvé que le virus peut être neutralisé tant par l'anticorps IgM que par l'IgG; mais l'IgM atteint son niveau maximum plus rapidement après l'infection que l'IgG.

INTRODUCTION

Serum virus neutralization is not only the most commonly used serological test in the determination of bluetongue virus (BTV) antibodies (Howell & Verwoerd, 1971), but the typing of BTV strains is also based on this reaction (Howell, 1970).

Certain aspects of the neutralization reaction, which includes the effect of contact conditions on the reaction (Svehag, 1963a) and the kinetics of the neutralizing antibody response (Klontz, Svehag & Gorham 1962; Howell, 1969), have already been studied. These determinations, however, were carried out before the plaque count method was introduced for BTV (Howell, Verwoerd & Oellermann, 1967). The introduction of the plaque reduction neutralization technique for titrating BTV antibodies (Thomas & Trainer, 1970) led to the reinvestigation of certain aspects of the neutralization reaction which are reported in this paper. The morphology of the reaction was studied according to the method of Almeida & Waterson (1969). Factors that influence the yield of BTV neutralizing antibodies and the roles of IgM and IgG in the neutralizing antibody response to BTV were considered.

MATERIALS AND METHODS

Cells

Baby hamster kidney cells [BHK-21 (C-13)] and cells from the L strain of mouse fibroblasts (LF cells) were obtained from the American Type Culture Collection.* Cells were grown as monolayers in Roux flasks in about 60–70 mℓ of modified Eagle's medium (MacPherson & Stoker, 1962) as described by Howell *et al.* (1967).

Virus propagation, titration and purification

Attenuated type 10 bluetongue virus (BTV type 10A) was used in all the tests unless otherwise indicated. The virus was propagated by inoculating BHK monolayers with 10–15 plaque-forming units (PFU) per cell and harvesting the cultures after incubation for 72 hours at 37 °C. The virus titres were determined by the method of Howell *et al.* (1967) and expressed as PFU/mℓ. Virus was purified according to the method described by Verwoerd (1969).

Stability tests

Verwoerd (1969) showed that the virus was most stable in 2 mM Tris-HCl buffer (pH 10,0) at 4 °C and the conditions he described were therefore used as controls. While one of these factors was varied, the others were kept constant. An unpurified sample of BTV type 10A (titre 1×10^8 PFU/mℓ) was diluted to 100 PFU/0,1 mℓ in Tris-HCl buffer which had previously been adjusted to the molarity, pH and temperature values shown in Table 1. The molarity

TABLE 1 The stability of BTV after dilution and incubation in Tris-HCl buffer at various temperatures, pH's and molarities

Condition	Time after commencement of the test		
	½ h	2 h	30 h
Temp. °C:			
4	100	100	100
27	100	100	100
32	100	100	100
37	100	70	20
pH:			
7	10	10	10
8	90	90	90
9	100	100	100
10	100	100	100
11	10	10	10
Molarity of Tris-HCl $\times 10^{-3}$*:*			
2	100	100	100
50	100	100	100
100	100	100	100
150	100	100	100
200	30	30	30
600	30	30	30
1 000	10	10	10

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varied from 2–1 000 mM and the pH from 8–10. The diluted suspensions were then incubated at the required temperature for periods ranging from 15 min–30 hours. The stability of the virus is indicated by the number of PFU's retained in 0,1 mℓ of the test suspension after different time periods.

Determination of optimum conditions for the neutralization reaction and the titration of neutralizing antibodies

Titrations of serum neutralizing antibodies were carried out using the plaque reduction neutralization (PN) test described by Thomas & Trainer (1970).

For the controls, conditions were chosen where the virus is known to be optimally stable, i.e. in 2 mM Tris-HCl buffer (pH 9,0) at 4 °C (see above). To determine the optimum conditions for neutralization, one of these parameters was varied while the others were kept constant. The test serum and virus were titrated in Tris-HCl buffer which had previously been adjusted to the different pH's, molar concentrations and temperatures shown in Table 2. The pH varied between 7,0 and 10,0, the molarity between 50 and 150 and the temperature at which the test was incubated was either 4 °C or 28 °C.

TABLE 2 The influence of pH, temperature and molarities of Tris-HCl buffer on the neutralization of BTV by antibody as indicated by the plaque reduction neutralization titre of an antiserum measured under different conditions

Condition		Titre of antiserum (log_2)
pH	8,0	12
	9,0	12
	10,0	9
Molarity of Tris-HCl buffer × 10^{-3}	50	12
	100	13
	150	14
Temperature °C	4	11
	28	12

Immune electron microscopy

The method of Almeida & Waterson (1969) was basically followed. The serum sample to be used in the test was inactivated by being heated for 30 min at 56 °C. It was then centrifuged for 1 hour at 100 000 *g* to remove any clumped protein that might be present. A 0,1 mℓ sample of this serum (PN titre 1/32) was then mixed with 0,1 mℓ of a purified BTV suspension (titre 5×10^9 PFU/mℓ) and 1,8 mℓ of 2 mM Tris-HCl buffer, pH 9,0. In the case of the controls, the serum sample was replaced by buffer or negative serum. The mixture was incubated for 15 min at 28 °C and then centrifuged at 8 500 *g* for 15 min at 4 °C.

The supernatant was discarded and the pellet resuspended in 0,1 mℓ of Tris-HCl buffer. An equal volume of 3% phosphotungstic acid (PTA) pH 6,0 was added to this buffer. A drop of this mixture was then placed on a 200 mesh carbon-formvar-coated grid, the excess fluid withdrawn with filter paper, and the grid immediately placed in the electron microscope. The 2 criteria that were used to identify a positive reaction were: (1) the obscuring of the virus, and (2) clumping of the virus.

Stimulation of a BTV-neutralizing antibody response in rabbits

An indication of the antigenicity of BTV is given by the level of the neutralizing antibody response following the injection of the virus into rabbits.

To study the influence of various factors on the antigenicity of BTV, the virus was administered to rabbits either in a purified or an unpurified state, in varying concentrations and different routes of injection (Table 3). For this purpose 7 groups consisting of 3 rabbits each were used. All injections were repeated weekly for 3 weeks and the first bleeding took place a week after the last injection. The level of neutralizing antibodies in the following immune response were determined as described above.

TABLE 3 The influence of various factors on the antigenicity of BTV

	Virus	Route	Titre/ml	Additions	Average titre over 12 weeks
A	Purified...	i.m.	1×10^{10}	—	7,9
B	Purified...	i.m.	1×10^{5}	—	6,5
C	Purified...	i.m.	2×10^{10}	Incomplete Freund's adjuvant	9,1
D	Purified...	i.v.	1×10^{8}	—	9,6
E	Purified...	s.c.	1×10^{8}	—	5,7
F	Unpurified	i.m.	1×10^{8}	—	8,5
G	Unpurified	i.m. and i.v.	1×10^{8}	—	7,9

i.m.—intramuscular
i.v.—intravenous
s.c.—subcutaneous
A–G—different groups of 3 rabbits each. All injections were repeated weekly for 3 weeks. The first bleeding took place a week after the last injection. Antiserum titres are expressed as log_2 of the dilution at which 50% neutralization takes place

To identify the immunoglobulins involved in the neutralization of BTV, a rabbit was given a single 2 mℓ intramuscular injection containing 3×10^8 PFU/mℓ and then bled at various time intervals, as shown in Table 4. The serum of each sample was separated by centrifugation after the blood was allowed to clot at room temperature for 2 hours.

TABLE 4 Identification of the immunoglobulin classes involved in the antibody reaction to BTV

Days after injection	Percentage of BTV neutralized by purified Ig fractions	
	IgM	IgG
0	0	0
2	0	0
6	100	64
13	100	68
22	96	72
30	68	100
40	45	100

Separation of IgM and IgG serum fractions and determination of their neutralizing capacity

Immunoglobulins of each serum sample were separated into IgM and IgG fractions by sucrose density gradient centrifugation, as described by Forghani, Schmidt & Lennette (1973). The gradients

were made in SW-50 tubes with a Beckman gradient former, using starting solutions of 4% and 40% sucrose in phosphate buffered saline (Dulbecco & Vogt, 1954). Centrifugation was done at 4 °C in a Beckman SW-50 rotor at 38 000 rpm for 18 hours. Fractions were collected dropwise from the bottom of the tube. The first 0,5 mℓ was taken as the IgM fraction, the next 0,5 mℓ was discarded and the rest of the gradient was taken as the IgG fraction. The purity of the gradient fractions was determined by radial immunodiffusion with antisera, specific for the heavy chains of either IgM or IgG, obtained from Cappel Laboratories.*

To determine the neutralizing capacity of the different fractions, 100 PFU of BTV were added to 0,5 mℓ of both the IgM and IgG fractions and titrated on a monolayer of L cells in 60 mm petri dishes. The percentage of PFU's neutralized was taken as an indication of the neutralizing capacity of each fraction.

Results

Stability of BTV type 10A

The stability, i.e. conservation of infectivity, of the virus, was investigated to exclude the posibility of confusing neutralization of virus with inactivation.

The stability of the virus under the various conditions investigated is shown in Table 1. At temperatures of 4–32 °C the virus was stable, but at 37 °C a marked reduction in infectivity occurred.

The virus was stable between pH 8 and 10 but very unstable between pH 7,0 and 11,0. By varying the molar concentration of the buffer, it was established that the virus is stable between 10 mM and 150 mM. Above 200 mM there was a marked reduction in the number of PFU's within 15 min but thereafter no further reduction was observed. The subsequent neutralization experiments were therefore carried out within the ranges in which the virus appeared to be stable.

Optimal conditions for the neutralization reaction

The effect of various conditions on the neutralization reaction is shown in Table 2. The highest titres were obtained at pH 8,0 and pH 9,0, but at pH 10,0 a slight loss of infectivity occurred. The variations in temperature and molar concentration of the buffer did not appear to affect the results significantly. Subsequent PN tests were carried out using 2 mM Tris-HCl buffer (pH 9,0) at 28 °C as diluent, since the virus was stable under these conditions and the PN test showed optimum sensitivity.

Electron microscopic demonstration of the BTV-antibody reaction

The reaction of BTV type 10A and type 4A in the PN test was investigated by electron microscopy. As controls, BTV type 10A was incubated with either buffer alone or with a known negative serum. According to the results demonstrated in Fig. 1, it is evident that the reaction of BTV type 10A with the homologous antiserum is characterized by clumping of the virus (Fig. 1C). No indication of clumping was seen where BTV type 4A was incubated with the heterologous type 10A antiserum (Fig. 1D), nor was there any indication of a reaction where BTV type 10A was incubated with buffer (Fig. 1A) or a negative serum (Fig. 1B), in the case of the 2 controls (Fig. 1A and B).

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Factors influencing the antigenicity of BTV

It was found that the mode of administering BTV has only a slight influence on its antigenicity when measured by the production of neutralizing antibodies. The best responses were obtained when either purified virus suspended in incomplete Freund's adjuvant injected intramuscularly (Group C), purified virus injected intravenously (Group D) or unpurified virus injected intramuscularly (Group F) was used. The amount of virus injected did not seem to affect its antigenicity.

Identification of the immunoglobulins involved in the neutralization of BTV at different stages of the antibody response

The results of this investigation are shown in Table 4. Both IgM and IgG antibodies were found to neutralize the virus for the whole duration of the experiment. The neutralizing activity in the IgM fractions reached an optimum in the early stages of the reaction, i.e. 6–13 days after injection. The neutralizing activity in the IgG fractions, however, increased more slowly than that in the IgM fractions, but remained high until 40 days after injection.

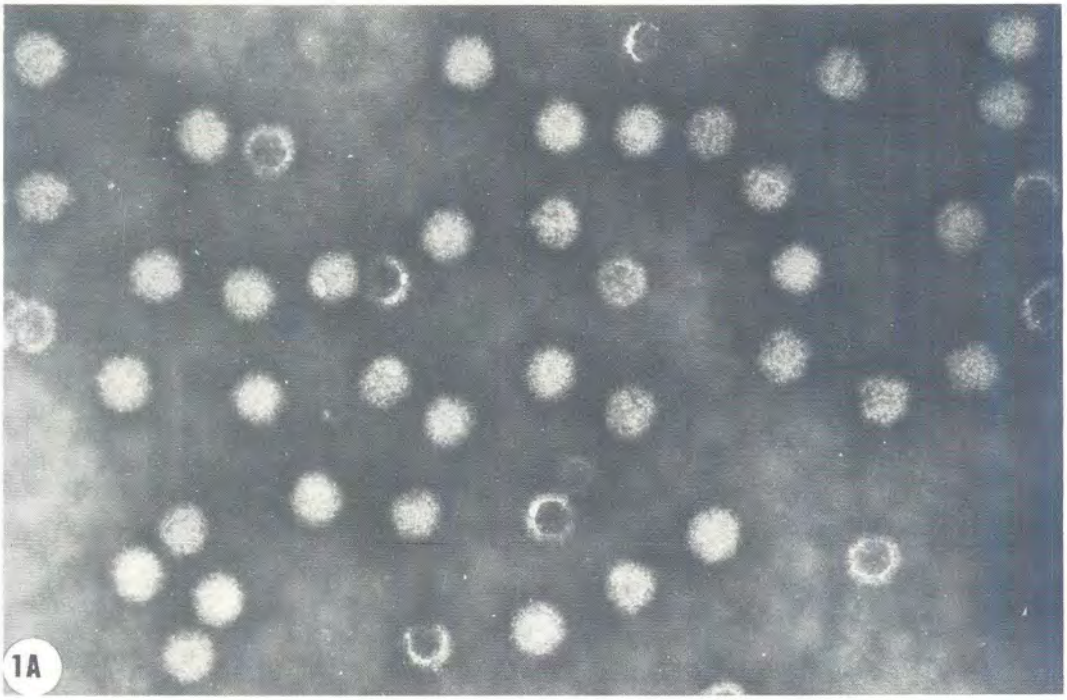
Discussion

Several aspects of the antibody response to BTV have already been studied. Klontz *et al.* (1962) compared the time of onset and duration of the precipitating and neutralizing antibody responses in sheep. The role of complement fixing antibodies in the response to BTV was reviewed by Howell & Verwoerd (1971). The importance of neutralizing antibodies in the response to BTV was stressed by correlating them to the level of resistance of sheep against the disease (Howell, 1969). The present paper reports on several aspects of the neutralizing antibody response to BTV.

The stability tests that were carried out to exclude the possibility of confusing neutralization with virus inactivation showed that in spite of differences in the test procedures the optimum stability of unpurified BTV type 10A appeared to be the same as that of a purified preparation (Verwoerd, 1969). Variations in the stability of BTV as previously reported by other workers (Alexander, 1947; Neitz, 1948; Svehag, 1963b; Owen, 1964; Howell *et al.*, 1967; Svehag, Leendersten & Gorham, 1966), underlined the importance of defining the serotype of the virus, the purity of the virus preparation, the presence of stabilizing factors in the test suspension and the duration of the test.

The importance of the effect of physiological conditions on the neutralizing reaction was stressed by Neurath & Rubin (1971). The present study made it possible to define the conditions where the virus is stable and the PN test is optimally sensitive. All subsequent PN tests were therefore carried out in 2 mM Tris-HCl buffer (pH 9,0) at 28 °C.

Immune electron microscopy was used to demonstrate a serotype specific BTV-antibody reaction. As it is an *in vitro* technique and very practical, it may also be of use in studies of the immune reaction and to demonstrate low levels of antibody (Almeida & Waterson, 1969). Although the reaction could be identified by a definite clumping of the virus, no specific obscuring of the fine structure could be detected. This finding is probably because the fine structure of the complete BTV particle is blurred by the outer protein layer of the virus (Verwoerd, Els, De Villiers & Huismans, 1972).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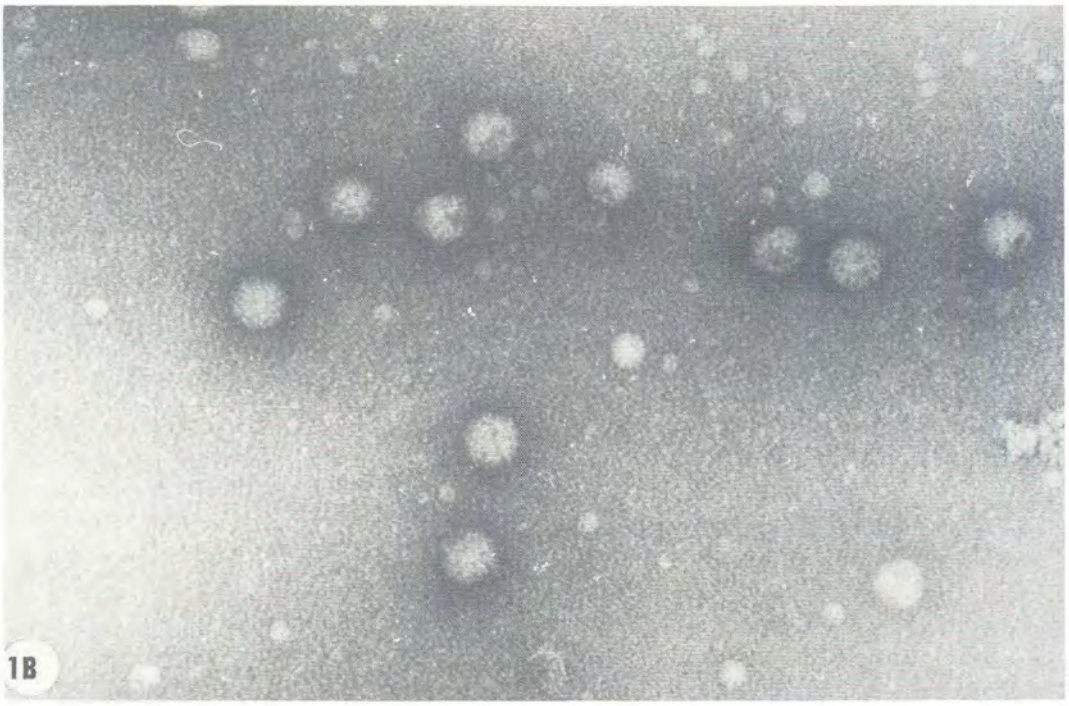
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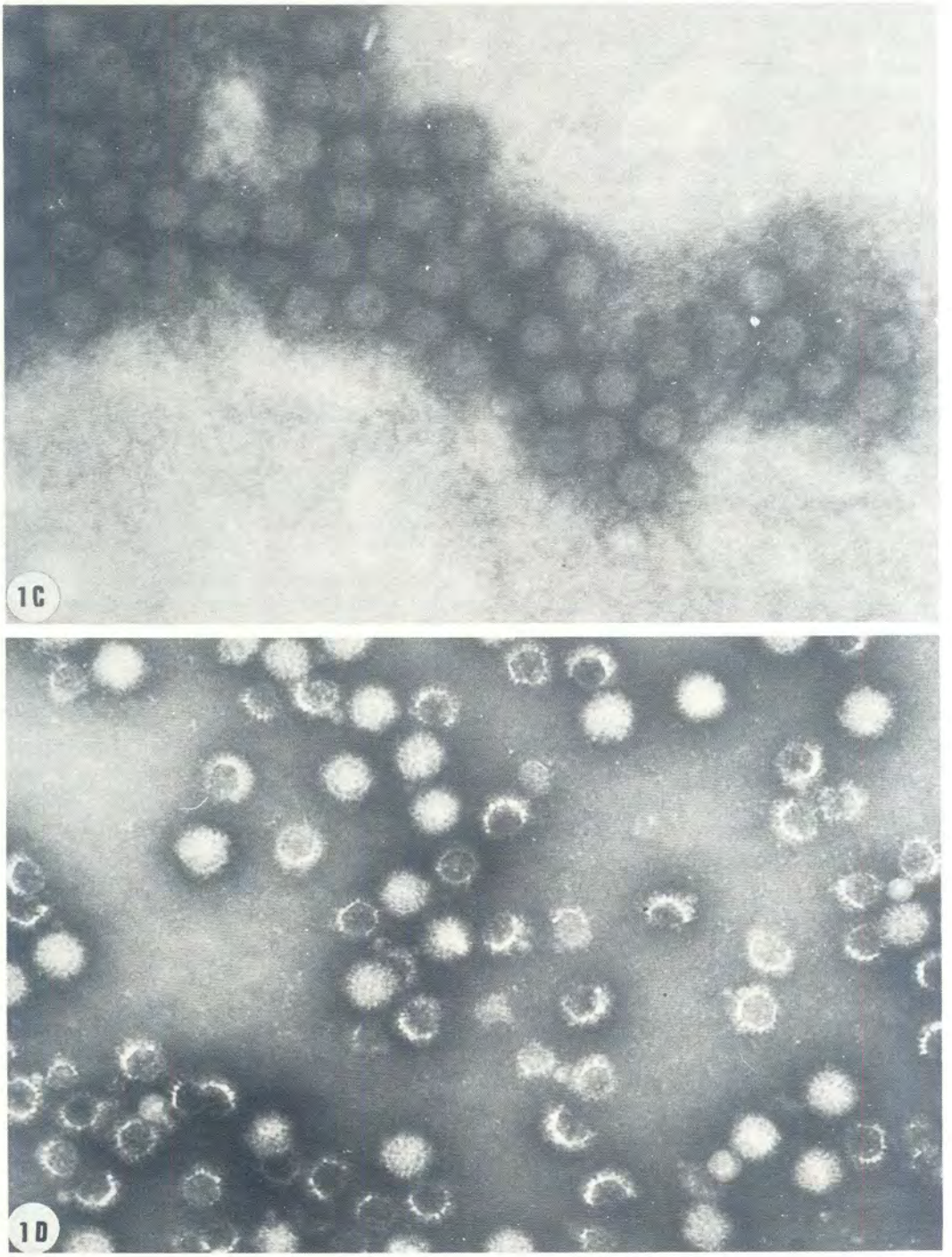

FIG. 1 The morphology of BTV before and after treatment with serum. Fig. 1A shows BTV type 10A suspended in 2 mM-Tris-HC buffer. The virus is also shown after incubation with a negative control serum (Fig. 1B) and a positive antiserum (Fig. 1C) In Fig. ID BTV type 4A is shown after incubation with antiserum against BTV type 10A. Magnification: ×100 000

As it is known that the antigenicity of many antigens is determined either by their physical and chemical properties, the use of adjuvants or the dosage, the influence of these factors on the antigenicity of BTV was investigated. The slight effect which was observed is probably because BTV is a good antigen, as it is a particulate protein of considerable size. The amount of virus injected appears not to be critical, probably because the virus multiplies in the rabbit, as it does in sheep (Howell, 1969).

Identification of the classes of immunoglobulin that neutralizes the virus could be important for the serodiagnosis of recent infections (Forghani *et al.*, 1973). It is also part of the characterization of the antibody response to BTV, but much remains to be done in this regard (Macario & De Macario, 1975).

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