J. gen. Virol. (1985), **66**, 1489–1500. *Printed in Great Britain*

Key words: *coronavirus/cows/antibody*

Chronic Shedding of Bovine Enteric Coronavirus Antigen–Antibody Complexes by Clinically Normal Cows

By C. F. CROUCH,[1] H. BIELEFELDT OHMANN,[1,2] T. C. WATTS[1] AND L. A. BABIUK[1,2]*

[1] *Veterinary Infectious Disease Organization and* [2] *Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada SN7 0W0*

(*Accepted 20 March 1985*)

SUMMARY

Using an ELISA for the detection of virus-specific immune complexes, ten cows were found to be shedding bovine enteric coronavirus. The shedding patterns from five of these animals were followed for a period of 12 weeks, and all were found to be chronically shedding virus. Despite the presence of both faecal and serum antibody the infection was not cleared; therefore, the role of cell-mediated immunity (CMI) was investigated by immunosuppressing the chronically shedding cows with dexamethasone. No major role for CMI in maintaining the chronic infection could be determined, although immunosuppression did result in a temporary reduction in the shedding of virus-specific immune complexes.

INTRODUCTION

Coronaviruses are associated with a wide variety of diseases in both humans and animals (Tyrrell *et al.*, 1978) and many members of the coronavirus family appear to be capable of establishing chronic infections in their hosts. These infections include chronic central nervous system disease in mice (Bailey *et al.*, 1949) and pigs (Mengeling & Cutlip, 1976), chronic nephritis in chickens (Alexander *et al.*, 1978) and chronic disease of the eye in rats (Lai *et al.*, 1976). The detection of coronaviruses associated with enteritis for several months after infection has also been reported in both humans (Moore *et al.*, 1977) and pigs (Underdahl *et al.*, 1975).

Bovine enteric coronavirus (BEC) has been shown to be a primary pathogen in neonatal calf diarrhoea (Mebus *et al.*, 1973). The incidence of BEC-associated enteritis in naturally occurring outbreaks of diarrhoea in young calves has been reported to vary from 15 to 70% (Crouch *et al.*, 1984; Langpap *et al.*, 1979; Marsolais *et al.*, 1978; Morin *et al.*, 1976) and serological evidence indicates that the virus may be widespread (Rodak *et al.*, 1982), at least in Western Canada. Little information is available concerning the infection of cows by BEC, and the possible role they may play in the epidemiology of infection. We have previously shown that the virus can be detected in the faeces from over 70% of clinically normal cows from a single herd (Crouch & Acres, 1984).

This report describes the shedding pattern and immune responses of clinically normal cows chronically shedding BEC in their faeces over a period of several months.

METHODS

Virus. The P.Q. isolate of BEC (originally obtained from S. Dea, Department of Pathology and Microbiology, Faculty of Veterinary Medicine, University of Montreal, St Hyacinthe, Quebec, Canada) grown in Madin–Darby bovine kidney (MDBK) cells as described previously (Crouch & Raybould, 1983) was used throughout this study.

Preparation of faecal samples. Faecal samples were collected and stored at 4 °C until diluted 1 in 3 (w/v) in 0·01 M-phosphate-buffered saline pH 7·2 (PBS) containing 0·05% Tween 20. Following low-speed centrifugation at 3000 r.p.m. (MSE Chilspin) for 20 min, the partially clarified supernatants were removed and stored at −20 °C until tested.

Specificity of immunoglobulin isotyping sera. The immunoglobulin isotypes associated with coronavirus-specific antibody and immune complexes were determined by using heavy chain-specific rabbit anti-bovine IgM, IgG_1, IgG_2 (Miles Laboratories) and guinea-pig anti-bovine IgA (a kind gift from Dr J. R. Duncan, ADRI, Ottawa, Canada) in the ELISAs described below. It was found necessary to purify the anti-bovine IgA serum by affinity chromatography. Bovine IgG (Miles Laboratories) was coupled to sodium metaperiodate-activated (Raybould & Chantler, 1979) Sepharose CL-4B (Pharmacia), at a concentration of 5 mg IgG per g of gel and used to form a column. The serum was passed through the column, and unbound fractions were collected and concentrated by vacuum dialysis to their original volume. The specificity of all antisera was confirmed by immunodiffusion against bovine serum. To further prove the specificity of the anti-bovine IgM and IgA sera, bovine IgG ELISA plates were reacted with each of the antisera in a standard ELISA assay. Only the anti-bovine IgG recognized the IgG-coated plates.

ELISA procedure. ELISA tests were performed using the microplate modification (Voller & Bidwell, 1975) of the method of Engvall & Perlmann (1972). A standard washing procedure of six washes in distilled water was adopted (Crouch & Raybould, 1983) and incubations were performed at room temperature (20 °C) for 1 h unless otherwise stated. Polystyrene 96-well plates (Immulon 2, Dynatech) were sensitized for each of the assay systems used as follows.

(i) For the detection of free coronavirus antigen, plates were indirectly sensitized with a mixture of monoclonal antibodies against bovine enteric coronavirus as previously described (Crouch *et al.*, 1984). After sensitization, the plates were washed and test samples serially diluted twofold in PBS containing 0·05% Tween 20 to give a final volume of 100 µl per well. Positive and negative control samples (from experimental infections of specific pathogen-free calves) were included in each test. Plates were incubated, washed, then further incubated following the addition of an optimal dilution of the rabbit anti-coronavirus peroxidase conjugate previously described (Crouch *et al.*, 1984). Finally the plates were again washed and bound conjugate reacted with Chromagen and enzyme substrate [100 µl/well of recrystallized 5-aminosalicylic acid diluted to 1 mg/ml in 0·01 M-phosphate buffer containing 0·01 M-sodium EDTA pH 5·95 to which 0·005% hydrogen peroxide was added immediately before use (Gielkens & Ellens, 1981)]. After 30 min at room temperature, the absorbance (A_{450}) of each well was determined using a micro-ELISA reader (MR580, Dynatech). This assay can detect both free virus and virus–antibody complexes which have some free viral epitopes exposed.

(ii) For the detection of coronavirus-specific immune complexes a modification of the method of Scherrer *et al.* (1981) was used. An optimal dilution (previously determined by titration) of rabbit anti-bovine IgG (H + L) serum (Zymed Laboratories, Burlingame, Ca., U.S.A.) was passively adsorbed to the plates at pH 9·6 (0·05 M-carbonate–bicarbonate buffer) overnight at 4 °C. After sensitization, the plates were washed and test samples serially diluted fourfold (30 µl in 90 µl) in PBS containing 0·05% Tween 20 from an initial dilution of 1 in 4. Positive and negative control samples (from experimental infections of specific pathogen-free calves) were included in each test. Plates were incubated, washed, then 100 µl of an optimal dilution (previously determined by titration) of rabbit anti-coronavirus peroxidase conjugate was added per well. After one further incubation, plates were washed and bound conjugate was detected as described above. For the determination of immunoglobulin isotypes associated with immune complexes, the anti-bovine IgG serum used to sensitize the plates was replaced with optimal dilutions of immunoglobulin isotyping sera (see above).

(iii) For the detection of coronavirus-specific antibody, plates were sensitized with purified whole coronavirus preparations as described previously (Crouch & Raybould, 1983). After sensitization, the plates were washed and test samples serially diluted twofold in PBS containing 0·05% Tween 20, to give a final volume of 100 µl per well. Faecal samples were titrated starting with an undiluted sample and sera diluted from an initial dilution of 1 in 100 or 1 in 10. Sera, positive and negative for coronavirus-specific antibody (from experimental infections of specific pathogen-free calves) were included in each test. Plates were incubated, washed, then 100 µl of an optimal dilution (previously determined by titration) of affinity-purified, peroxidase-conjugated rabbit anti-bovine IgG (H + L) (Zymed Laboratories) was added per well. After one further incubation, plates were washed and bound conjugate was detected as described above. For the determination of anti-coronavirus immunoglobulin isotypes the peroxidase-conjugated rabbit anti-bovine IgG (H + L) was replaced by an indirect detection system consisting of optimal dilutions of immunoglobulin isotyping sera (see above) followed after incubation and washing by the addition of 100 µl per well of an optimal dilution of either peroxidase-conjugated goat anti-rabbit immunoglobulin (H + L) (Cappel Laboratories) (IgM, IgG_1, and IgG_2) or peroxidase-conjugated rabbit anti-guinea-pig IgG (H + L) (Miles Laboratories) (IgA). Bound conjugate was detected as described above.

In all ELISAs, the titres were expressed as the reciprocal of the highest sample dilution that gave an A_{450} of greater than 3 standard deviations over the mean background absorbance obtained with twenty known negative samples (from uninfected specific pathogen-free calves).

Blocking assay. Specificity testing for the coronavirus-specific immune complex ELISA was performed using a blocking assay in which the reaction between the test sample and the peroxidase conjugate was blocked in one of a pair of duplicate sample wells using a 1:10 dilution of bovine anti-coronavirus serum and, in the second well, using a 1:10 dilution of a bovine serum negative for coronavirus antibody as previously described (Crouch *et al.*, 1984).

Following washing, and the addition of peroxidase conjugate, the standard capture-ELISA procedure was then continued as outlined above. A positive reaction was considered specific if the absorbance value was reduced by >50% in the well treated with bovine anti-coronavirus serum.

Virus neutralization (VN) test. A microneutralization assay was used in which equal volumes (125 µl) of heat-inactivated (56 °C for 30 min) serum, in a fourfold dilution series was mixed with a coronavirus suspension containing 200 $TCID_{50}$ and incubated at 37 °C for 1 h. Aliquots (100 µl) of this incubation mixture were inoculated in duplicate onto confluent MDBK cell monolayers in flat-bottomed 96-well plates. These plates were incubated for 5 days at 37 °C, the cells then fixed using 10% formaldehyde in isotonic saline and stained with 0·1% (w/v) crystal violet in 20% methanol. The titres were expressed as the reciprocal of the highest serum dilution giving a 50% reduction in cytopathic effect.

Plaque assay. Virus samples were serially diluted tenfold in minimal essential medium (MEM). Growth medium was removed from confluent MDBK cell monolayers in six-well tissue culture dishes. The cell monolayers were washed with 0·01 M-PBS and 500 µl of each virus dilution was added to separate well. After adsorption at 37 °C for 1 h, the inoculum was removed and each well was overlaid with 3 ml of MEM containing 1·5% Noble agar, 2% foetal bovine serum (FBS) and 100 µg/ml DEAE-dextran. The infected monolayers were incubated at 37 °C in 5% CO_2 for 7 days at which time they were stained using 0·02% neutral red, and the plaques counted.

Haemagglutination assay (HA). A 20% suspension of mouse red blood cells (MRBC) in Alserver's solution was stored at 4 °C. A working concentration of 0·4% MRBC was made up in PBS containing 0·2% gelatin (HA buffer) as required. MRBC stored for longer than 5 days were not used in the assay.

Serial twofold dilutions of virus suspensions in HA buffer (50 µl in 50 µl) were prepared, in duplicate, in 96-well U-bottomed microtitre plates (Cooke) and an equal volume of a 0·4% suspension of MRBC was added to each well. After gentle agitation, the plates were covered and allowed to stand at room temperature for 90 min before endpoint agglutination titres were recorded.

Haematological studies. Standard methods were used for determination of total and differential counts of peripheral blood leukocytes in heparin-stabilized blood samples.

Preparation of mononuclear leukocytes and mitogenic assay. Venous blood was drawn into syringes containing citrate/dextrose and the peripheral blood leukocytes (PBL) were isolated as described (Bielefeldt Ohmann *et al.*, 1983) by centrifugation on Ficoll-Hypaque (Pharmacia). The blastogenic response of PBL was measured by incubating triplicate cultures of 1×10^5 cells in RPMI 1640 containing 10% FBS with 50 µg/ml phytohaemagglutinin (PHA; Miles) for 72 h at 37 °C in a humidified 5% CO_2 atmosphere. During the last 18 h the cultures were pulse-labelled with 0·2 µCi tritiated thymidine ([^{3}H]TdR, Amersham). The plates were harvested with the aid of a multiple sample harvester (Skatron, Flow Laboratories) and incorporation of [^{3}H]TdR was quantified in a liquid scintillation counter (TriCarb 450 CD, Packard). The results are expressed as mean c.p.m. per culture. Standard deviations were consistently less than 5%.

Isolation of polymorphonuclear leukocytes (PMN), measurement of migration activity and O_2^- production. PMN (usually more than 95% neutrophilic granulocytes) were isolated and their migratory activity, spontaneous and chemotactic, was measured by an agarose plate assay as described previously (Bielefeldt Ohmann & Babiuk, 1984*a*). Superoxide anion release was measured by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C on cells in suspension as described previously (Bielefeldt Ohmann & Babiuk, 1984*b*). Differential counts were performed on cytosmears stained with Wright's stain.

Effect of dexamethasone on coronavirus replication in vitro. Confluent Georgia bovine kidney (GBK) cell cultures were infected at a multiplicity of infection of 1 or 0·01 p.f.u./cell in the usual manner. Following adsorption for 1 h, the unadsorbed virus was removed by washing twice with Hanks' balanced salt solution. Cultures were incubated for 60 h in the presence or absence of various concentrations of dexamethasone phosphate (Dexagen-5 Rogar/STB BTI Products Inc., London, Ontario, Canada) after which the media were removed, clarified by centrifugation to remove infected cells and assayed for the presence of cell-free virus. The cells (99%) which remained attached to the plates were removed with a rubber policeman and sonicated briefly to release cell-associated virus. The virus in both cases was quantified by haemagglutination and plaque assay.

RESULTS

Specificity and reproducibility of the virus-specific immune complex ELISA

Virus-specific immune complexes produced by incubating mixtures of tissue culture-derived coronavirus and bovine anti-coronavirus serum overnight at 4 °C were tested by ELISA for the presence of both free and complexed virus. At high concentrations of antiserum, neither virus-specific immune complexes nor free virus could be detected. At intermediate serum dilutions, both free virus and immune complexes could be detected, whilst at high serum dilutions only free virus could be detected. In an attempt to demonstrate the specificity of the assay, bovine

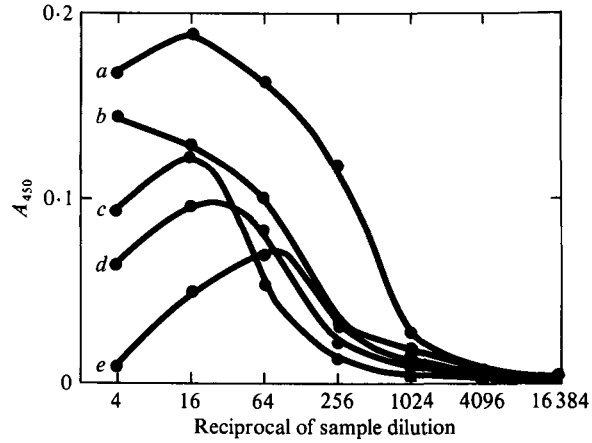

Fig. 1. Detection of virus-specific immune complexes by ELISA. Each curve (*a* to *e*) represents a different faecal sample from different cows.

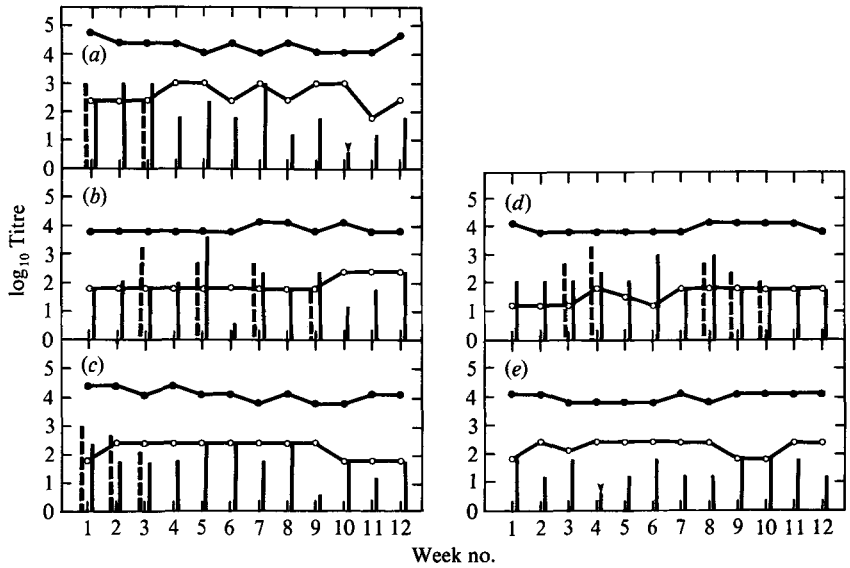

Fig. 2. Virus excretion and serum antibody titres in five cows chronically shedding BEC. Samples from animals (*a*) 83-72, (*b*) 83-73, (*c*) 83-76, (*d*) 83-80 and (*e*) 83-99. Vertical bars show shedding of free virus (¦) and virus-specific immune complexes (|). Arrows indicate titres below levels of detection. Horizontal lines denote serum antibody titre as determined by ELISA (●) and virus neutralization (○).

rotavirus antigen–antibody complexes were prepared as described for the coronavirus antigen–antibody complexes. In each instance the rotavirus antigen–antibody complex gave negative results in the coronavirus assay. The specificity was further confirmed by testing 50 ELISA-positive faecal samples in the blocking assay. No false positive reactions were detected. The reproducibility of the assay system was evaluated by testing five faecal samples in a twofold dilution series a minimum of five times on separate occasions. The range of titres obtained did not exceed one twofold dilution for any of the samples tested (data not shown).

Standardization of virus-specific immune complex ELISA

When different faecal samples containing virus-specific immune complexes were tested by fourfold serial dilutions in the ELISA, the curves shown in Fig. 1 were obtained. Although the slopes of the linear portions of the curves were parallel, the curves possess two unusual features;

firstly, a marked prozone effect was noticeable with all samples tested, and secondly even with samples containing similar titres of virus-specific immune complexes (samples *b*, *d* and *e*) significant differences occurred in the maximum absorbance values obtained. In an attempt to determine whether faecal material could alter the reactivity in ELISA and help explain the observed prozone effect at low dilutions we added various concentrations of a negative faecal sample to a known positive sample. Low dilutions (1/20) of normal faecal material did reduce the level of reactivity of a positive sample (data not shown). At higher dilutions, this effect was much less pronounced, indicating that faecal material can alter the reactivity and be responsible for the prozone effect observed at low dilutions.

Chronic shedding of BEC by cows

To determine the dynamics of coronavirus shedding in cattle, the faeces from ten cows were screened for the presence of BEC in either a free form or complexed with immunoglobulin. Free virus could be detected in only five of these animals, but virus-specific immune complexes could be detected in all ten. Five of these cows were selected for further studies. Virus excretion, serum and faecal antibody titres were monitored weekly for a period of 12 weeks (Fig. 2). Varying amounts of virus-specific immune complexes could be detected in the faeces of all five animals throughout the period of study. On only two occasions, each in different animals, could no virus-specific immune complexes be found (Fig. 2*a*, *e*). In contrast, free virus was apparently shed only intermittently, with titres ($\log_{10}$) varying from 1·8 to 3·3. In the case of cow 83-99 no free virus was detected during the entire sampling period (Fig. 2*e*). Significant levels of BEC coproantibody capable of reacting in the ELISA were not found in any of the cows examined, although cows 83-73 and 83-99 showed occasional trace reactivity by ELISA. In all animals, serum antibody measured either by ELISA or VN showed no significant variation in titre during the period of study.

Analysis of the immunoglobulin isotypes associated with virus-specific immune complexes indicated that IgG_1, IgG_2, IgM and IgA were all represented and that the relative proportions of these immunoglobulin subclasses did not change significantly during the period of study (Table 1). Similarly, coronavirus-specific serum antibody was also found to be associated with all immunoglobulin isotypes (Table 2). The relative proportions of coronavirus-specific reactivity associated with these immunoglobulin isotypes did not change significantly throughout the period of study.

Effect of treatment with dexamethasone on the shedding of BEC

Preliminary studies suggested that dexamethasone treatment (0·1 mg/kg body weight daily) for a period of 5 days reduced the shedding of BEC-specific immune complexes in faeces (data not shown). Further experiments were therefore conducted in order to determine the effect of dexamethasone treatment (0·1 mg/kg body weight daily) for a period of 11 days on virus shedding from four cows (Fig. 3). Following dexamethasone treatment, BEC-specific immune complexes could no longer be detected in faecal samples from three of the experimental cows, (Fig. 3). One animal (Fig. 3*d*) intermittently shed immune complexes on days 6, 7, 11 and 14. Cows 83-76 and 83-72 which were shedding high titres of immune complexes prior to dexamethasone ceased shedding detectable levels 4 and 6 days, respectively, after the commencement of treatment. In contrast, cows 83-99 and 83-155 which were only shedding low titres of immune complexes ceased shedding detectable levels immediately after treatment began. Shedding of BEC-specific immune complexes resumed in all animals within 18 days of the cessation of dexamethasone treatment. Free virus was detected sporadically from all animals throughout the entire period of experimentation, with the exception of 83-99 from which no free virus was detected. Low titres of coproantibody could also occasionally be detected in all animals; however, significant titres were detected in samples only from 83-155 on days 13, 15 and 16 (Fig. 3*d*). Dexamethasone treatment appeared to have no significant effect on the titres of BEC-specific serum antibody as measured by the ELISA. There was however a tenfold reduction in VN antibody in the sera of cows 83-72 and 83-76 between the beginning and end of the experimental period (Fig. 3*a*, *b*).

Table 1. *Association of coronavirus-specific immune complexes with immunoglobulin isotypes*

Cow no.	Week	$\log_{10}$ Titre of immune complexes associated with immunoglobulin isotypes			
		IgM	IgA	IgG_1	IgG_2
83-72	1	2·4	1·8	2·4	2·4
	6	3·0	3·0	3·0	3·0
	12	1·8	<0·6	1·8	1·8
83-73	1	3·6	2·4	3·0	3·0
	6	4·2	3·0	3·6	4·2
	12	2·4	2·4	3·0	3·0
83-76	1	2·4	1·2	2·4	2·4
	6	3·0	2·4	3·0	3·0
	12	1·8	<0·6	1·8	1·8
83-80	1	3·6	1·8	3·0	3·0
	6	4·2	3·0	3·0	3·0
	12	3·0	3·0	3·0	3·0
83-99	1	2·4	2·4	3·0	2·4
	6	2·4	2·4	2·4	2·4
	12	1·8	<0·6	2·4	1·8

Table 2. *Anti-coronavirus immunoglobulin isotype titres in sera from cows chronically shedding BEC*

Cow no.	Week	$\log_{10}$ Anti-coronavirus immunoglobulin isotype titres in sera			
		IgM	IgA	IgG_1	IgG_2
83-72	1	2·6	2·3	3·8	3·5
	6	2·9	2·3	4·1	4·1
	12	3·2	2·6	3·5	4·1
83-73	1	3·2	2·9	3·5	3·8
	6	3·2	2·6	3·5	3·8
	12	2·9	2·9	3·5	3·5
83-76	1	2·9	2·6	4·7	3·5
	6	2·9	2·9	3·8	3·8
	12	3·2	2·9	3·2	4·1
83-80	1	3·2	2·9	3·5	3·2
	6	3·2	2·6	3·5	3·2
	12	2·6	2·9	3·8	3·8
83-99	1	3·5	2·9	3·8	4·4
	6	3·2	2·9	3·8	4·1
	12	3·2	2·6	3·5	4·1
Conv a*		<1·0	<1·0	3·5	2·6
Conv b*		<1·0	<1·0	3·5	3·2

* Sera from convalescent calves 8 weeks after experimental infection with BEC.

Effect of dexamethasone on the production of BEC in vitro

In order to determine whether dexamethasone had a direct effect on coronavirus replication or release, coronavirus-infected cultures were treated with dexamethasone and assayed for virus production. In no instance was there any observed effect on either total virus or extracellular virus produced in the presence of dexamethasone (Table 3).

Effect of treatment with dexamethasone on bovine leukocyte numbers and activity

Since dexamethasone had no effect on virus production *in vitro* that would explain the alteration in virus shedding in cattle, studies on the effect of dexamethasone on bovine immune responses were initiated. Haematological analysis of peripheral blood revealed that treatment

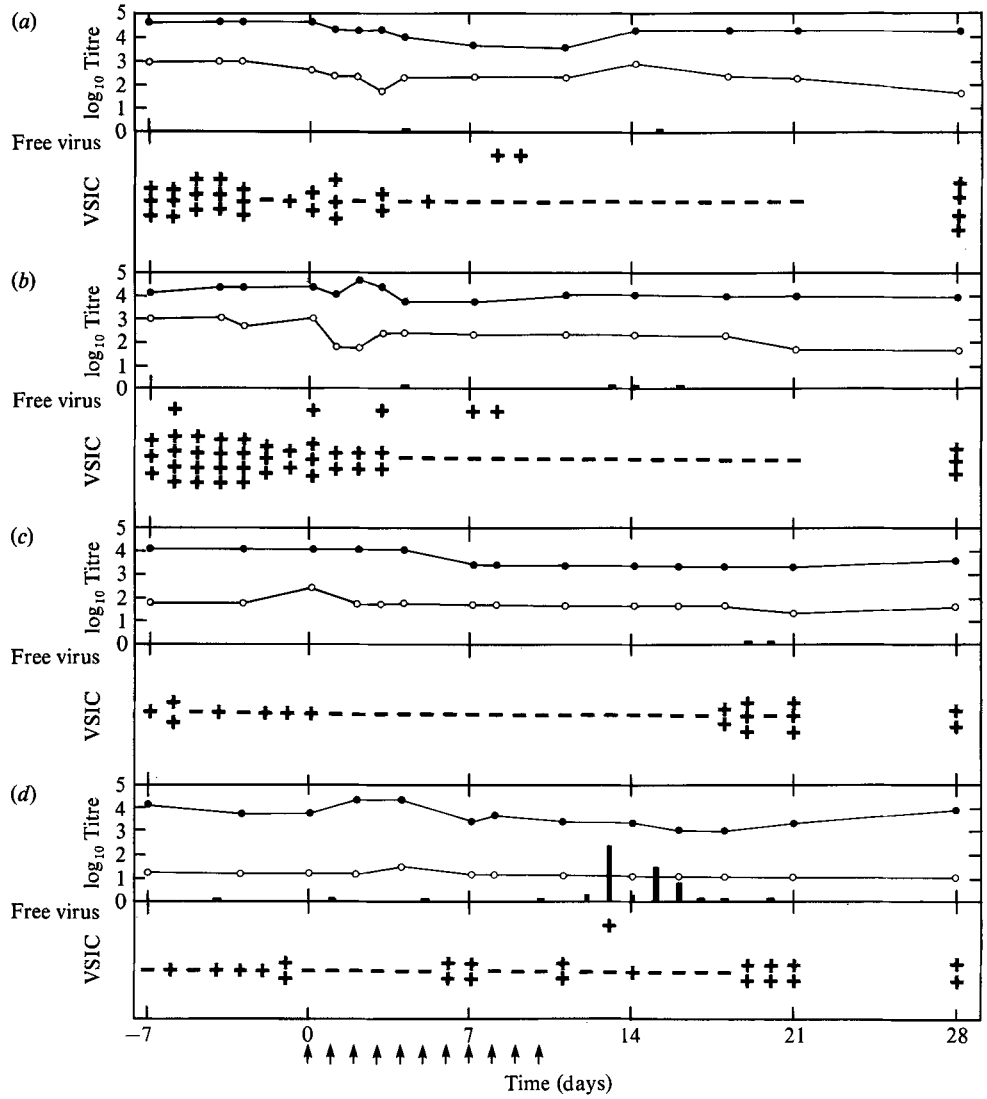

Fig. 3. Effect of dexamethasone treatment on virus shedding from four cows, (*a*) 83-72, (*b*) 83-76, (*c*) 83-99 and (*d*) 83-155. Arrows indicate days on which cows were treated with dexamethasone (0·1 mg/kg body weight). Virus-specific immune complexes (VSIC) titres: −, none detected; +, 1 in 4; + +, 1 in 16; + + +, 1 in 64; + + + +, ⩾1 in 256. Free virus + indicates days on which free virus was detected in faecal samples. Antibody titres: ●, serum antibody measured by ELISA; ○, serum antibody measured by virus neutralization; vertical bars measure coproantibody detected by ELISA.

with dexamethasone induced an immediate leukophilia, due to a significant increase in the number of neutrophilic granulocytes. This response appeared to be biphasic with peaks on days 2 and 11 (Fig. 4). Following termination of drug administration the cell number immediately declined towards normal levels (Fig. 4). The number of lymphocytes decreased within 2 to 4 days of treatment, but appeared to stabilize at or slightly under the normal (pretreatment) level around day 7. Monocyte numbers tended to increase slightly during the treatment period, whereas the number of eosinophils decreased to zero, and only reappeared in one animal (83-155, Fig. 4*d*) after termination of dexamethasone administration.

In addition to having an effect on cell populations, dexamethasone administration also resulted in a depression of the blastogenic response of PBL to PHA (Fig. 5). This effect was seen

Table 3. *Effect of dexamethasone on the production of bovine enteric coronavirus* in vitro

Multiplicity of infection	Molarity of dexamethasone	HA titre/ml	Infectivity titre (p.f.u./ml)
Cell-free virus			
1·0	0	$4·1 \times 10^4$	$3·0 \times 10^6$
1·0	10^{-5}	$4·1 \times 10^4$	$6·4 \times 10^6$
1·0	10^{-6}	$2·0 \times 10^4$	$5·2 \times 10^6$
1·0	10^{-7}	$4·1 \times 10^4$	$5·8 \times 10^6$
0·01	0	$1·0 \times 10^4$	$3·4 \times 10^6$
0·01	10^{-5}	$1·0 \times 10^4$	$5·8 \times 10^6$
0·01	10^{-6}	$1·0 \times 10^4$	$2·6 \times 10^6$
0·01	10^{-7}	$2·0 \times 10^4$	$3·4 \times 10^6$
Cell-associated virus			
1·0	0	$2·6 \times 10^3$	$1·6 \times 10^6$
1·0	10^{-5}	$5·1 \times 10^3$	$2·8 \times 10^6$
1·0	10^{-6}	$5·1 \times 10^3$	$1·8 \times 10^6$
1·0	10^{-7}	$5·1 \times 10^3$	$1·0 \times 10^6$
0·01	0	$5·1 \times 10^3$	$2·2 \times 10^6$
0·01	10^{-5}	$5·1 \times 10^3$	$4·2 \times 10^6$
0·01	10^{-6}	$5·1 \times 10^3$	$3·2 \times 10^6$
0·01	10^{-7}	$1·0 \times 10^4$	$2·0 \times 10^6$

within 2 to 4 days of treatment and remained low throughout the observation period in two cows (83-99 and 83-155), whereas restitution was seen in 83-72 (partial) and 83-76 (complete). Dexamethasone also altered both random and directed migration of PMN. Initially there was a slight increase but thereafter both decreased progressively and to a similar extent (i.e. the migration index remained unchanged: Fig. 6), until several days after termination of dexamethasone treatment. The respiratory burst of PMN was also affected by dexamethasone administration (Fig. 7). The activity decreased and resumed immediately following initiation and withdrawal of treatment, respectively. These changes in activity were closely accompanied by shifts in the relative proportions of mature PMN and bandforms (Table 4).

DISCUSSION

Although ELISA proved to be a specific and reproducible assay for detection of coronavirus-specific immune complexes the sensitivity of this assay was restricted by two factors. Firstly, the assay can only detect unsaturated immune complexes and secondly, reactivity was inhibited in the presence of high concentrations of faecal material. The use of artificially produced immune complexes in the assay system suggested that immune complexes would only be detectable under conditions when free virus could also be detected. However, the results of this present study show that in the majority of natural cases, immune complexes could be detected in the absence of free virus.

The patterns of virus excretion found in the five animals studied indicate that they were all chronic shedders of BEC. Although these animals were selected for their shedding of BEC, the percentage of animals shedding BEC does appear to be high since over 70% of cows in a single herd were found to be shedding BEC-specific immune complexes. Whilst significant levels of coproantibody could not be detected, the presence of antibody in the intestine is indicated by the formation of the BEC-specific immune complexes. However, even though there is antibody in the intestinal lumen it appears to be insufficient to clear the virus infection. Whether this is a reflection of antibody avidity, quantity, or class of antibody remains to be determined. The observation that all immunoglobulin isotypes including IgM were represented in the immune complexes suggests continuous replication of coronavirus which in turn stimulates IgM and IgA (Vonderfecht & Osburn, 1982).

The immunosuppressive effect of dexamethasone administration was revealed by the decreased blastogenic response of PBL, and the reduced activity of PMN in accordance with previous findings in man (Chretien & Garagusi, 1972; Fauci, 1980) and cattle (Roth & Kaeberle, 1981; Roth *et al.*, 1982). The suppressive effect on leukocyte reactivity has been

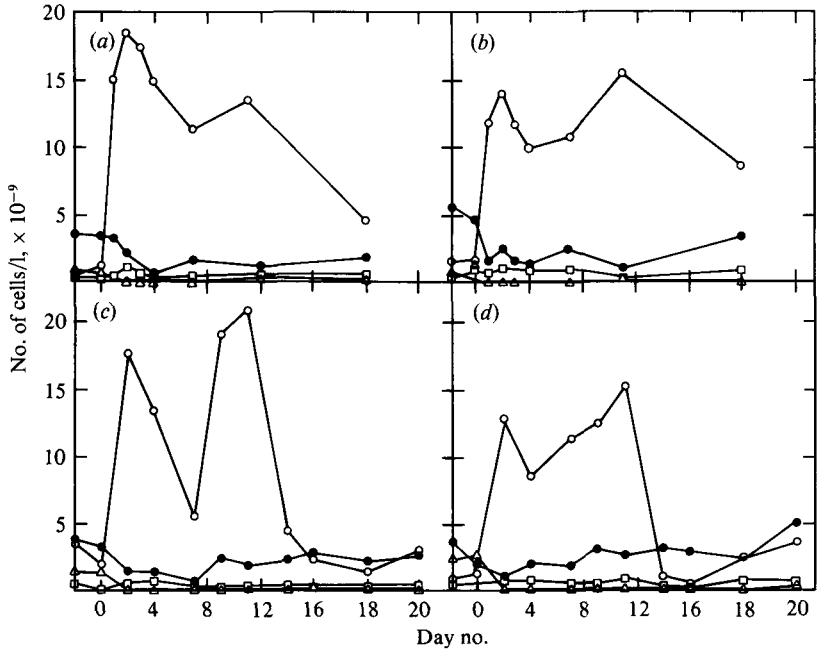

Fig. 4. Effect of dexamethasone administration on numbers of neutrophils (○), eosinophils (△), lymphocytes (●) and monocytes (□) in peripheral blood of cows 83-72 (*a*), 83-76 (*b*), 83-99 (*c*) and 83-155 (*d*).

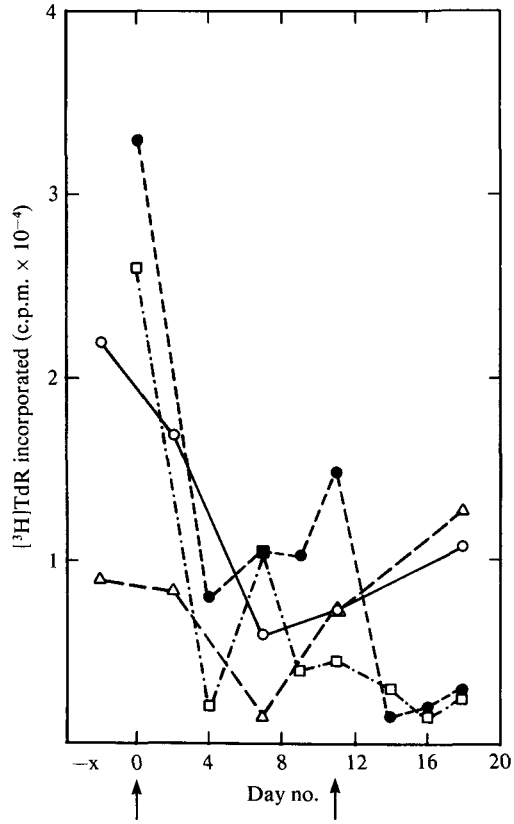

Fig. 5. Effect of dexamethasone treatment of cattle (○, 83-72; △, 83-76; □, 83-99; ●, 83-155) on the blastogenic response of PBL to PHA.

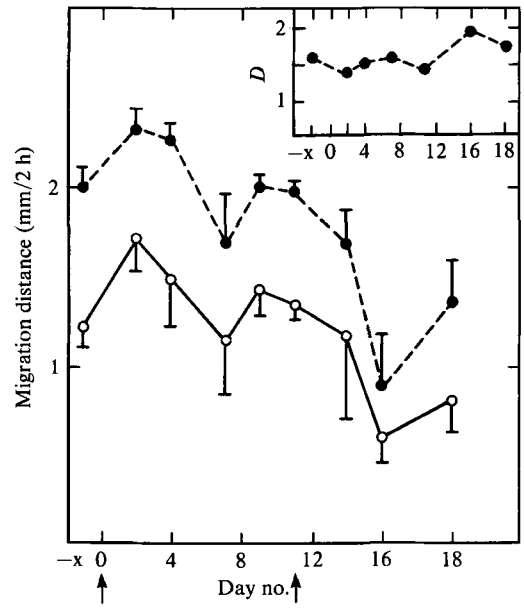

Fig. 6. Directed (●) and random (○) migratory activity of bovine peripheral blood PMN following *in vitro* administration of dexamethasone from day 0 to day 11. Inset shows the migration index (*D*).

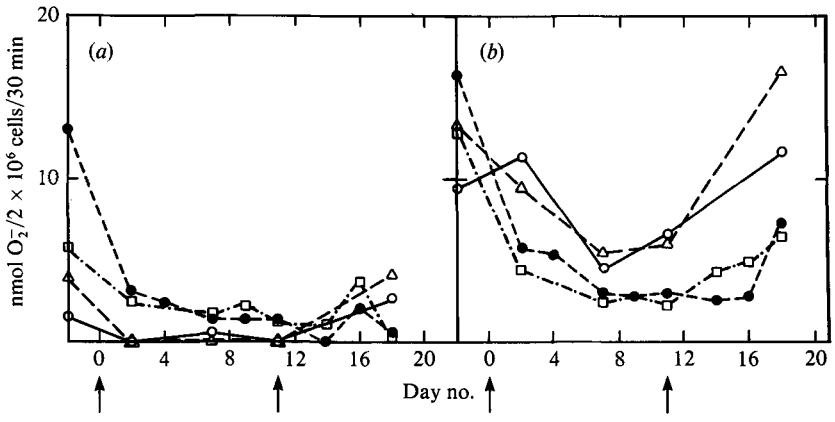

Fig. 7. Effect of dexamethasone treatment on the respiratory burst of peripheral blood PMN, as evaluated by superoxide anion (O_2^-) production in (*a*) 'resting' cells and (*b*) zymosan-activated cells of cows 83-72 (○), 83-76 (△), 83-99 (□) and 83-155 (●).

Table 4. *Relative proportions of mature neutrophils and bandforms during dexamethasone treatment*

Experimental day	Type of Neutrophil % Mature	% Band	±S.D.
−x	100·0	0·0	0·0
0	100·0	0·0	0·0
+2	73·6	26·3	17·1
+7	29·5	70·5	26·1
+9	26·5	73·5	10·6
+11	20·0	80·0	11·3
+14	33·0	67·0	26·8
+16	49·0	51·0	15·5

shown to occur by a number of different mechanisms. These may include depletion of cell populations due to redistribution, by suppression of accessory cell function or by direct effects on the cells. Thus corticosteroids may alter monocyte differentiation (Rinehart *et al.*, 1982) and expression of Fc receptors (Crabtre *et al.*, 1980) thereby interfering with macrophage accessory cell functions (Bielefeldt Ohmann *et al.*, 1983). In addition, production by T-lymphocytes of interleukin-2, which is necessary for a normal proliferative response to mitogens and antigens, may be inhibited (Crabtre *et al.*, 1980). The effect of dexamethasone on PMN is probably mediated by inhibition of microtubular functions involved in cell movement, and of energy-generating processes. Alternatively, the decrease may be due to an increase in the number of immature (bandforms) cells (Table 4).

Following treatment of cows with dexamethasone there was a reduction in shedding of BEC-specific complexes without an increase in the amount of free virus excreted. In fact, partial immunosuppression actually reduced the quantity of virus shed. Following termination of dexamethasone treatment the shedding resumed. A possible explanation for this phenomenon may be related to the mechanisms of virus release from infected enterocytes. If virus release from infected cells is mediated in part by an immune mechanism (e.g. antibody-dependent cell cytotoxicity by PMN), immunosuppression by dexamethasone treatment could lead to a reduction in the amount of virus released into the gut lumen and hence a reduction in the shedding of BEC-specific immune complexes. Alternatively, a reduction in the release of virus from infected cells due to a direct pharmacological action of dexamethasone, perhaps by altering the stability of cell membranes (Claman, 1975) could be postulated. The restitution of PMN migratory and cytotoxic activities commencing about 7 days after the final treatment with dexamethasone, appears to coincide with the reappearance of virus-specific immune complexes in the faeces, and this suggests that the first hypothesis may be correct. This is further supported by the *in vitro* observation that dexamethasone had no effect on release of coronavirus into the extracellular environment.

The present report clearly demonstrates that adult animals are often carriers of coronavirus which might serve as a source of infection for the susceptible neonate. Since much of this virus is complexed with antibody we are presently investigating the infectious nature of these complexes for neonates as well as whether a difference exists between the free virus shed periodically and the complexed virus.

We wish to thank the VIDO animal support staff for the services they provided, and to acknowledge the technical assistance of Donna Dent, Molly Denson and Jeanette Heise. Thanks are also due to Phyllis Zoerb for typing this manuscript. This work was supported by funds from the Alberta Research Council – Farming for the Future and the Medical Research Council of Canada. Published with permission of the Director of VIDO as Journal Series no. 36.

REFERENCES

ALEXANDER, D. J., GOUGH, R. E. & PATTISON, M. (1978). A long term study of the pathogenesis of infection of chickens with three strains of avian infectious bronchitis virus. *Research in Veterinary Science* **24**, 228–235.

BAILEY, O. T., PAPPENHEIMER, A. M., CHEEVER, F. S. & DANIELS, J. B. (1949). A murine virus (JHM) causing disseminated encephalomyelitis with extensive destruction of myelin. II. Pathology. *Journal of Experimental Medicine* **90**, 195–212.

BIELEFELDT OHMANN, H. & BABIUK, L. A. (1984*a*). Effect of bovine recombinant alpha interferon on inflammatory response of bovine phagocytes. *Journal of Interferon Research* **4**, 249–263.

BIELEFELDT OHMANN, H. & BABIUK, L. A. (1984*b*). *In vitro* generation of hydrogen peroxide and of superoxide by bovine polymorphonuclear neutrophilic granulocytes, blood monocytes and alveolar macrophages. *Inflammation* **8**, 251–275.

BIELEFELDT OHMANN, H., FILION, L. G. & BABIUK, L. A. (1983). Bovine monocytes and macrophages: an accessory role in suppressor cell generation by Con A and in lectin-induced proliferation. *Immunology* **50**, 189–199.

CHRETIEN, J. H. & GARAGUSI, V. F. (1972). Corticosteroid effect on phagocytosis and NBT reduction by human polymorphonuclear neutrophils. *Journal of the Reticuloendothelial Society* **11**, 358–367.

CLAMAN, H. N. (1975). How corticosteroids work. *Journal of Allergy and Clinical Immunology* **55**, 145–151.

CRABTRE, G. R., GILLIS, S., SMITH, K. A. & MUNCK, A. (1980). Mechanisms of glucocorticoid-induced immunosuppression: inhibitory effects on expression of the receptors and production of T-cell growth factor. *Journal of Steroid Biochemistry* **12**, 445–449.

CROUCH, C. F. & ACRES, S. D. (1984). Prevalence of rotavirus and coronavirus antigens in feces of normal cows. *Canadian Journal of Comparative Medicine* **48**, 340–342.

CROUCH, C. F. & RAYBOULD, T. J. G. (1983). Comparison of different antigen preparations as substrates for use in passive haemagglutination and enzyme-linked immunosorbent assays for the detection of antibody against bovine enteric coronavirus. *Journal of Clinical Microbiology* **18**, 146–149.

CROUCH, C. F., RAYBOULD, T. J. G. & ACRES, S. D. (1984). Monoclonal antibody capture-enzyme-linked immunosorbent assay for the detection of bovine enteric coronavirus. *Journal of Clinical Microbiology* **19**, 388–393.

ENGVALL, E. & PERLMANN, P. (1972). Enzyme-linked immunosorbent assay. III. Quantitation of specific antibodies by enzyme labelled anti-immunoglobulin in antigen coated tubes. *Journal of Immunology* **109**, 129–135.

FAUCI, A. S. (1980). Glucocorticoid effects on circulatory human mononuclear cells. *Journal of the Reticuloendothelial Society* **27**, 727–738.

GIELKENS, A. L. F. & ELLENS, D. J. (1981). Application of purified 5-aminosalicylic acid as substrate in enzyme-linked immunosorbent assay (ELISA). In *Laboratory Diagnosis in Neonatal Calf and Pig Diarrhoea (Current Topics in Veterinary Medicine and Animal Science, vol. 13)*, pp. 33–39. Edited by P. W. de Leeuw and P. A. M. Guinee. Boston: Martinus Nijhoff.

LAI, Y. L., JACOBY, R. O., BHATT, P. N. & JONAS, A. M. (1976). Keratoconjunctivitis associated with sialodacryoadenitis in rats. *Investigative Ophthalmology* **15**, 538–541.

LANGPAP, T. J., BERGELAND, M. E. & REED, D. E. (1979). Coronaviral enteritis of young calves: virologic and pathologic findings in naturally occurring infections. *American Journal of Veterinary Research* **40**, 1476–1478.

MARSOLAIS, G., ASSAF, R., MONTPETIT, C. & MAROIS, P. (1978). Diagnosis of viral agents associated with neonatal calf diarrhea. *Canadian Journal of Comparative Medicine* **42**, 168–171.

MEBUS, C. A., STAIR, E. L., RHODES, M. B. & TWIEHAUS, M. J. (1973). Pathology of neonatal calf diarrhea induced by a corona-like agent. *Veterinary Pathology* **10**, 45–64.

MENGELING, W. L. & CUTLIP, R. C. (1976). Pathogenicity of field isolates of hemagglutinating encephalomyelitis virus for neonatal pigs. *Journal of the American Veterinary Medical Association* **168**, 236–239.

MOORE, B., LEE, P., HEWISH, M., DIXON, B. & MUKHERJEE, T. (1977). Coronaviruses in a training centre for intellectually retarded. *Lancet* i, 261.

MORIN, M., LARIVIERE, S. & LALLIER, R. (1976). Pathological and microbiological observations made on spontaneous cases of acute neonatal calf diarrhea. *Canadian Journal of Comparative Medicine* **40**, 228–240.

RAYBOULD, T. J. G. & CHANTLER, S. (1979). Comparison of activation and blocking procedures in the use of defined antigen substrate spheres (DASS) for quantitative and visual assessment of conjugate activity. *Journal of Immunological Methods* **27**, 309–318.

RINEHART, J. J., WUEST, D. & ACKERMAN, G. A. (1982). Corticosteroid alteration of human monocyte to macrophage differentiation. *Journal of Immunology* **129**, 1436–1440.

RODAK, L., BABIUK, L. A. & ACRES, S. D. (1982). Detection by radioimmunoassay and enzyme-linked immunosorbent assay of coronavirus antibodies in bovine serum and lacteal secretions. *Journal of Clinical Microbiology* **16**, 34–40.

ROTH, J. A. & KAEBERLE, M. L. (1981). Effects of *in vivo* dexamethasone administration on *in vitro* bovine polymorphonuclear leukocyte function. *Infection and Immunity* **33**, 434–441.

ROTH, J. A., KAEBERLE, M. L. & HSU, W. H. (1982). Effects of ACTH administration on bovine polymorphonuclear leukocyte function and lymphocyte blastogenesis. *American Journal of Veterinary Research* **43**, 412–416.

SCHERRER, R., CORTHIER, G. & L'HARIDON, R. (1981). ELISA for the detection of rotavirus and rotavirus/antibody complexes in faeces. In *Laboratory Diagnosis in Neonatal Calf and Pig Diarrhoea (Current Topics in Veterinary Medicine and Animal Science, vol. 13)*, pp. 40–48. Edited by P. W. de Leeuw & P. A. M. Guinee. Boston: Martinus Nijhoff.

TYRRELL, D. A. J., ALEXANDER, D. J., ALMEIDA, J. D., CUNNINGHAM, C. H., EASTERDAY, B. C., GARWES, D. J., HIERHOLZER, J. C., KAPIKIAN, A., MCNAUGHTON, M. R. & MCINTOSH, K. (1978). Coronaviridae: second report. *Intervirology* **10**, 321–328.

UNDERDAHL, R., MEBUS, C. A. & TORRES-MEDINA, A. (1975). Recovery of transmissible gastroenteritis virus from chronically infected experimental pigs. *American Journal of Veterinary Research* **36**, 1473–1476.

VOLLER, A. & BIDWELL, D. (1975). A simple method for detecting antibodies to rubella. *British Journal of Experimental Pathology* **56**, 338–339.

VONDERFECHT, S. L. & OSBURN, B. I. (1982). Immunity to rotavirus in conventional neonatal calves. *Journal of Clinical Microbiology* **16**, 935–942.

(Received 28 September 1984)