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Nous dédions ce travail à notre ami Raoul Scherrer, trop tôt disparu, en souvenir de notre collaboration efficace, de ses critiques et de ses encouragements.

J.F. Vautherot, J. Laporte

We dedicate this work to our friend Raoul Scherrer, too soon passed away, in memory of our collaboration, his criticisms and his encouragements.

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Abstract

Hybrid cells secreting monoclonal antibodies against Bovine Enteric Coronavirus (BECV strain G110) were obtained by fusion between SP2/0 myeloma cells and splenocytes of mice hyperimmunized with purified virus. Specificity of 8 from the 12 monoclonal antibodies was established to be anti-GP105 by immunochemical staining of viral polypeptides and by immunoprecipitation of radioactive-labelled viral proteins. The reactivity of three BECV isolates (strain G110, F15 and NCDCV) with the different monoclonal antibodies was studied by indirect immunofluorescence (IIF), ELISA, hemagglutination-inhibition and seroneutralization. G110 and F15 interacted in a similar manner with the monoclonal antibodies, but NCDCV failed to react with A9 monoclonal antibody. IIF test allowed us to detect another difference between U.K. BECV isolate and other BECV, in that the U.K. isolate did not react with F7 monoclonal antibody. No differences could be seen by IIF in the reactivity of Danish BECV isolate, Human Enteric Coronavirus (HECV), Bovine Respiratory Coronavirus (BRCV) and our BECV strains, with the 12 monoclonal antibodies. Human Respiratory Coronavirus (OC43), Porcine Hemagglutinating Encephalitis Virus (HEV) and BECV share common antigenic determinants which are outlined by the 6 non-neutralizing monoclonal antibodies. On the basis of the reactivity of coronaviruses with anti-BECV monoclonal antibodies, a distinction can be easily made between BECV and other coronaviruses, and differences were also detected within the BECV isolates. Interestingly HECV were found to be more closely related to BECV than to human OC43. These results show that monoclonal antibodies are of valuable help for biochemical and antigenic studies as well as for diagnostic procedures.

Coronaviruses are enveloped, positively-stranded RNA viruses which can cause respiratory, enteric and generalized diseases in mammals and birds (Tyrrell *et al.*, 1978). These virions are large (100 to 150 nm in diameter) pleomorphic, and possess surface peplomers 20 nm long which are characteristically club-shaped (Tyrrell *et al.*, 1978). Bovine enteric coronavirus is related to this group of virus by morphological appearance (Stair *et al.*, 1972), antigenic relationships (Pedersen *et al.*, 1978) and polypeptide composition (Laporte and Bobulesco, 1981 ; King and Brian, 1982).

Bovine enteric coronavirus is constituted of at least five structural polypeptides, four of which are glycosylated (Laporte and Bobulesco, 1981; Bobulesco, 1983). The highest molecular weight glycoprotein (GP125, MW: 125 000 daltons) is reduced to 65 000 polypeptide chains by 2-Mercapto-ethanol (2ME) and, together with GP105 and GP100 (MW: 105 000 and 100 000 daltons respectively), constitute the outer projections of the virus (Laporte and Bobulesco, 1981). GP26 (MW: 26 000 daltons) is more deeply embedded in the virion envelope (Laporte and Bobulesco, 1981; Bobulesco, 1983), interacts with the internal ribonucleoprotein (Bobulesco, 1983) and may play an important role in the intracellular viral maturation (Holmes *et al.*, 1981). VP50 (MW: 50 000 daltons), the major protein of BECV, is a phosphorylated protein (Laporte and Bobulesco, 1981) associated with the viral genome (Laporte and Bobulesco, 1981; Bobulesco, 1983). Structural sites that are responsible for virus adsorption on cells and for viral agglutination of rat red blood cells (RRBC) have not yet been clearly defined. However, these two activities are located on the GP^s constituting the peplomers (Bobulesco, 1983).

Current evidence indicates that there are two groups of coronaviruses that affect mammals, as defined by their antigenic properties (Pedersen *et al.*, 1978; Wege *et al.*, 1982):

— OC43 and related viruses which are BECV, HEV (Haemagglutinating encephalitis virus), MHV3 (Mouse hepatitis virus), HECV (Human enteric coronavirus), SDAV (Sialodacryoadenitis virus), DVIM (Diarrhœa virus of infant mice);

— 229E group of viruses to which belong TGE (Transmissible gastroenteritis virus), FIP (Feline infectious peritonitis) and CCV (Canine enteric coronavirus). Common antigenic determinants exist within each group (Gerna *et al.*, 1981; Horzinek *et al.*, 1982). However, the only antigenic similarity between OC43 related viruses and 229E is a common determinant on the nucleoprotein (VP50) of MHV3 and 229E viruses (Hasony, 1982).

This report describes the characterization of monoclonal antibodies to BECV and their use to define two biological activities of the outer glycoproteins. The monoclonal antibodies were also utilized for analyzing the antigenic relationships between BECV and other coronaviruses.

Materials and Methods

Cell lines and viruses

HRT18 cells (Human rectal tumor cell-line established by Tompkins *et al.* (1974) were grown as previously described (Laporte *et al.*, 1980), except that tylosine (10 µg/ml) and lincomycine (200 µg/ml) were added to the medium instead of penicillin and streptomycin. All BECV isolates, G110 and F15 (Laporte *et al.*, 1979), NCDCV (Mebus *et al.*, 1973), Danish and British isolates (Bridger *et al.*, 1978), as well as HEV, OC43 and a bovine respiratory coronavirus (Thomas *et al.*, 1982) grow to high titres on HRT18 cells. For biochemical purposes, these viruses were produced on HRT18, cloned, and purified according to a method described previously (Laporte and Bobulesco, 1981).

Primary fetal bovine kidney cells (PBFK) were grown in Eagle's minimum essential medium (Eurobio, Paris, France), supplemented with 10 % fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin. These PFBK were used to propagate BECV G110 (L'Haridon *et al.*, 1981) and NCDCV (Mebus *et al.*, 1973). RPd cells a pig kidney cell line, kindly provided by H. Laude (INRA, Thiverval-Grignon, France), were cultivated as previously described (Laude, 1978) for the propagation of HEV or TGE virus.

Purification of the virus (BECV G110 strain), immunization schedules fusion procedure and partial characterization of monoclonal antibodies have been described elsewhere (Roseto *et al.*, 1982).

Immune precipitation

Specificities of monoclonal antibodies were determined by immunoprecipitation of ^{14}C amino-acid labelled viral polypeptides from infected cells. HRT18 confluent monolayers were infected by BECV strain G110 at a multiplicity of infection (m.o.i.) of three plaques forming units (PFU)/cell. At four hours post-infection, supernatant medium was discarded and replaced by fresh RPMI (Eurobio, France) medium containing 2 % FCS and 5 µgCi/ml of a ^{14}C labelled amino-acid mixture (50 mCi/mAt of C-CEA, France).

After incubating 16 h, cells were solubilized in 20 mM Tris buffer pH 7.2, containing 150 mM NaCl, 0.002 M EDTA, 1 % Triton, 1 % DOC and 1 % Aprotinin. The cytosol extract was centrifuged at 10 000 *g* for 30 min to remove cellular debris. Aliquots containing 2×10^5 TCA precipitable counts/min per 50 µl were stored at − 80 °C.

For immune precipitation, the cytosol extract was diluted 1 in 5 in 50 mM Tris, pH 7.2 containing 150 mM NaCl buffer, 5 mM EDTA, 1 mg/ml ovalbumine and 0.05 % NP40 (TEN-OVA). One hundred microliters of diluted cytosol extract were mixed with 100 µl of undiluted monoclonal antibody, or immune mouse serum (diluted 1/20), and incubated at 37 °C for 1 h. One hundred microliters of sheep-antimouse Ig (Institut Pasteur Production, Marnes-la-Coquette, France) diluted 1/1000 in TEN-OVA were added, and the mixture

was further incubated for 1 h at 37 °C. One hundred microliters of a 10 % suspension of heat-killed formalin fixed *Staphylococcus aureus* (Pansorbinn Calbochiem) previously washed three times in TEN-OVA were added and incubation was continued at 20 °C for 45 min. Bacteria were pelleted by centrifugation at 11000 *g* for 2 min and washed four times in TEN-OVA. The final pellet was resuspended in 3 % SDS sample-preparation buffer and heated at 100 °C for 2 min. Reduction of viral polypeptide was achieved by incorporation 5 % ME in sample buffer before boiling. Bacteria were removed by centrifugation and samples were analysed by electrophoresis on 8.75 % polyacrylamide slab gels (Roseto *et al.*, 1982). After drying the gels, precipitated radioactive material was revealed by autoradiography of the gels on Kodak NS2T film. Controls included precipitation of polypeptides from non-infected ^{14}C labelled HRT18 cells.

Haemagglutination inhibition (HAI)

At first, purified coronavirus (Laporte and Bobulesco, 1981) was titrated by an haemagglutination assay (HA). A virus suspension was prepared in phosphate buffer saline solution (PBSS) containing 2 mg/ml bovine serum albumin (BSA). Fifty microliters of the virus were serially diluted in a round-bottom microtitration plate and then mixed with 50 µl of a 0.8 % (v/v) suspension of washed rat red blood cells (RRBC) in PBSS containing 2 mg/ml BSA. Plates were kept at room temperature for 1 h and the HA titre of the virus suspension was determined as the last dilution giving fully agglutination of the RRBC.

For HAI, semi-purified monoclonal antibodies (TCF precipitated by ammonium sulfate at a final concentration of 50 % saturation at 4 °C and dialysed against PBSS) were absorbed with packed RRBC. Monoclonal antibodies were then diluted in PBSS containing 2 mg/ml BSA and 25 µl of each dilution were mixed with 25 µl of a viral suspension containing 4 HA units. Microtitration plates were incubated at 37 °C for 1 h. Fifty microliters of a 0.8 % RRBC suspension were added in each well, plates were incubated for a further hour at room temperature and the titres were determined.

ELISA

Detection and titration of monoclonal antibodies by ELISA was performed according to a method previously described for the detection of bovine antibodies against BECV (Vautherot *et al.*, 1981). Briefly, wells of microtitration plates (Microelisa, Dynatech) were coated with 0.5 µg of purified BECV in 100 µl of 50 mM pipes buffer pH 6.4, containing 150 mM NaCl. Plates were incubated overnight at 37 °C. Excess viral antigen was removed by four washes with PBSS containing 0.05 % Tween 20 (PBS-Tween), and unbound sites were

Table 1. Characterization of monoclonal antibodies.

Mo. Ab	Isotype	Specificity	Reciprocal titre										
			NT[a]			IIF[b]			HAI[c]			ELISA	
			G110	F15	NCDCV	G110	F15	NCDCV	G110	F15	NCDCV	G110	NCDCV
A9	IgG2b	GP 105	90	0	0	32	32	0	256	128	0	2100	0
A20	IgG2a	GP 105	4000	2000	4000	128	64	32	0	0	0	1400	1200
B5	IgG2a	GP 105	3200	2800	10000	128	64	128	0	0	0	2000	1500
C13	IgG1	GP 105	100	140	100	64	64	32	32	32	32	1000	2000
I7	IgG2a	GP 105	1000	1000	1000	32	32	32	0	0	0	3000	3500
J18	IgG2a	GP 105	5500	5500	4000	256	128	256	0	0	0	13000	20000
I16	IgG1	GP 105	0	0	0	256	128	64	0	0	0	2800	3000
F7	IgG1	GP 105	0	0	0	64	64	32	0	0	0	800	2500
E5	IgG2a		0	0	0	128	128	128	0	0	0	8000	9500
H7	IgG2a	–	0	0	0	256	256	128	0	0	0	5000	5000
H19	IgG2a	–	0	0	0	64	64	64	0	0	0	1800	2500
I12	IgG2a	–	0	0	0	128	64	64	0	0	0	3000	2500

a : Neutralization titres are expressed as the reciprocal of the highest antibody dilution giving a 50 % reduction in plaque number.

b Indirect immunofluorescence titres were expressed as the reciprocal of the highest antibody dilution giving a positive reaction.

c : Hemagglutination inhibition assay.

saturated by adding 100 µl of a 1 % gelatin (Bio-Mérieux, France) solution in 10 mM Tris pH 7.4, 150 mM NaCl buffer. After 1 h at 37 °C, the plates were washed four times with PBS-Tween and 100 µl of duplicate dilutions of monoclonal antibodies in PBS-Tween were added to the wells. Plates were incubated at 37 °C for 2 h and then washed four times in PBS-Tween. One hundred microliters of anti-mouse IgG serum conjugated to Horseradish peroxidase (Institut Pasteur Production, Marnes-la-Coquette, France) diluted 1/2000 in PBS-Tween were added to each well. Plates were incubated for an additional 2 h at 37 °C and then washed four times with PBS-Tween. Orthophenylene diamine (ODP) was used as peroxidase substrate at a concentration of 10 mg per 25 ml in 35 mM citrate 66 mM phosphate buffer, pH 5 containing 50 µl/25 ml of a 6 % solution of H_2O_2. The reaction was stopped after 12 min incubation at room temperature by adding 50 µl per well or 5N H_2SO_4. Optical densities were read with a Flow Multiskan titertek (Flow Laboratories SA, France).

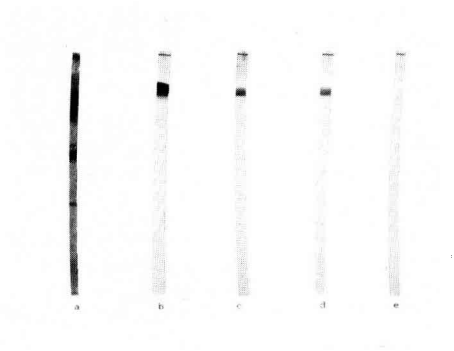

Fig. 1.— Immunoperoxidase staining of viral polypeptides.
Viral proteins were separated by electrophoresis on SDS polyacrylamide gels, and transferred on nitrocellulose sheet (Millipore) by transverse electrophoresis as described by Towbin *et al.* (1979). Nitrocellulose sheets were saturated with a 3 % solution of BSA in 10 mM Tris, pH: 7.4 buffer, 150 mM NaCl and stained using:
a. Hyperimmune mouse serum (dilution 1/1000)
b. I16 Monoclonal antibodies reacting with GP105
c. J18 Monoclonal antibodies reacting with GP105
d. F7 Monoclonal antibodies reacting with GP105
e. Monoclonal antibody of unknown specificity.

Anti-mouse serum conjugated to horseradish peroxidase was purchased from the Institut Pasteur Production. Controls included staining by conjugate alone, by anti-rotavirus monoclonal antibody and by an anti-cell monoclonal antibody (data not shown).

Results

Monoclonal antibodies isolation and characterization

Two fusions were made which yielded a total of 271 hybrid colonies. Twelve stable hybridomas were selected which secreted monoclonal antibodies to BECV (Roseto *et al.*, 1982) (table 1). Specificity controls were made, which included indirect immunofluorescence (IIF) testing of monoclonal antibodies on non-infected HRT18 monolayers and on PFBK cells that were infected with BECV G110. Ouchterlony immunodiffusion revealed that isotypes of monoclonal antibodies were 1 IgG2b, 3 IgG1 and 8 IgG2a (table 1) (Roseto *et al.*, 1982).

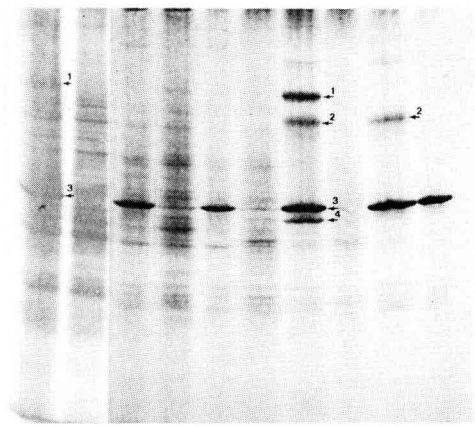

Fig. 2. Immunoprecipitation of ^{14}C amino-acids-labelled BECV polypeptides.

Lanes A and B: Electrophoresis of cytosol extract from infected (A) and control (B) HRT18 cells.

— Lanes C and D: Precipitation of infected (C) and control (D) cytosol extract by *S. aureus* alone (Non specific adsorption of VP50 (3) on the bacteria.)

— Lanes E and F: Precipitation of infected (E) and non-infected (E) cytosol extracts by *S. aureus* and anti-mouse IgG serum.

— Lanes G and H: Immune precipitation of infected (C) and control (H) cytosol extracts by an hyperimmune mouse serum (diluted 1/50). Viral polypeptides GP125 (1), GP105 (2), VP50 (3) and VP48 (4) are precipitated by hyperimmune mouse serum from infected cytosol extracts.

— Lane I: Precipitation of GP105 by monoclonal antibody I7.

— Lane J: Absence of precipitation by monoclonal antibody H19.

Specificity of the monoclonal antibodies was assayed by immunoperoxidase staining of separated viral polypeptides transferred on nitrocellulose sheets (Twobin *et al.*, 1979) and by immunoprecipitation. Immunoperoxidase staining was positive with six monoclonal antibodies which all reacted with a high molecular weight glycoprotein (fig. 1). However, this technique did not allow à precise characterization of monoclonal antibodies, due to impaired separation of high molecular weight GP as performed previously (Roseto *et al.*, 1982) and to possible SDS denaturation of antigenic determinants.

Immunoprecipitation of ^{14}C labelled viral polypeptides was chosen to further analyse the monoclonal antibodies specificities (fig. 2). Immunoprecipitation showed that A9, A20, B5, C13, and J18 monoclonal antibodies (table 1) precipitated a glycoprotein of 105 000 daltons (GP105). This GP105 differed from the amine outer GP, GP125, in that the former was not affected by 2 ME treatment (data not shown). I16 and F7 dit not precipitate ^{14}C labelled viral proteins but they both reacted with GP105 as shown by immunoperoxidase staining of normal (fig. 1) and 2 ME treated viral proteins. Work is under progress to characterize monoclonal antibodies of yet unknown specificity.

The biological activities of monoclonal antibodies were assayed by neutralization and HAI. The six monoclonal antibodies which precipitated GP105 displayed neutralizing activity (table 1). I16 and F7 (anti-GP105) never neutralized BECV even when mouse ascitic fluids (MFA) were used instead of tissue culture fluids (TCF) (table 2). None of the remaining monoclonal antibodies (E5, H7, H19, I12) had any neutralizing activity (tables 1, 2). Agglutination of RRBC by BECV G110 was inhibited by two monoclonal antibodies A9 and C13 (table 1).

This preliminary characterization of monoclonal antibodies to BECV clearly showed that anti-PG105 monoclonal antibodies had two biological activities *in vitro* neutralization of the virus and inhibition of the haemagglutination induced by BECV. These activities, together with IIF and ELISA, were used to characterize the antigenic relationships within the BECVs and between BECV and other coronaviruses.

Antigenic comparison of BECV isolates

The reactivity of our monoclonal antibodies with BECV G110 and F15 (French isolates) and NCDCV (US isolates) was investigated by neutralization, IIF, HAI and ELISA (table 1). G110 and F15 could not be distinguished by the monoclonal antibodies, as they all reacted to a similar degree with the two viruses in all the assays (table 1). However, IIF revealed that monoclonal antibody A9 could not stain NCDCV infected cells (HRT18 or PFBK) (table 1). This result was confirmed by ELISA, HAI and neutralization performed with TCF (table 1) and by ELISA and IIF with MAF (table 3). The other monoclonal antibodies reacted to a same extent with G110 and NCDCV (table 1).

The British and Danish BECV isolates (Bridger *et al.*, 1978) kindly provided by Dr. Bridger, were compared to BECV G110 on the basis of their reactivity with the monoclonal antibodies, in an IIF assay (table 4). When grown on HRT18, no difference could be seen between G110 and the first two passages of the Danish isolate. During the first and the second passage of British BECV on HRT18, a declining number of infected cells was stained by F7 (table 4). Purified virus from the

Table 2. Comparison of biological activities of ascitic fluids (MAF) and tissue culture fluids (TCF) against BECV G110.

	MAF		TCF	
	NT	IIF	NT	IIF
A9	1×10^4	1×10^5	90	32
A20	2×10^5	10^5	4000	128
B5	5×10^5	2×10^5	3200	128
C13	1.8×10^4	NT	100	64
I7	2×10^5	5×10^4	2300	256
J18	10^6	1.2×10^4	5500	256
I16	< 100	1.2×10^4	0	256
F7	< 100	3.2×10^3	0	64
E5	< 100	5×10^4	0	128
H7	< 100	1.2×10^4	0	256
H19	< 100	1×10^5	0	64
I12	< 100	1.2×10^4	0	128

fourth passage was used as antigen in an ELISA and, again, no reaction occurred with monoclonal antibody F7 whereas the other monoclonal antibodies reacted with British and G110 BECVs to the same extent (data not shown).

The differences in the reactivity of British BECV and NCDCV to monoclonal antibody F7 and A9 were the only ones detectable in the group of BECV isolates tested.

Cross reactivity of monoclonal antibodies to BECV with other coronaviruses

Human enteric coronaviruses (HECV) (Laporte *et al.*, 1980) and a British respiratory coronavirus (BBRC) isolated on calf (Thomas *et al.*, 1982) were grown on HRT18 and infected cells were stained by IIF using the monoclonal antibodies. No differences could be seen between the three viruses, HECV, BBRC, and BECV G110. However, HEV and OC43, also grown in HRT18 differed from BECV in that only monoclonal antibodies E5, H7, H19, I12 and I16 reacted with GEV and OC43, the latter being also recognized by F7 (table 4). None of the monoclonal antibodies had any neutralizing activity against HEV.

We investigated the reactivity of the 12 monoclonal antibodies against a coronavirus of the «229E group» TGE (Purdue strain, kindly provided by Dr. H. Laude, INRA, Thiverval-Grignon, France); none of the monoclonal antibodies stained the TGE-infected RPd-cells, whereas viral infected cells were detected by IIF with an anti-TGE serum.

Table 3. Characterization of antigenic differences within BECV isolates.

Monoclonal Antibody		ELISA titre		IFF titre		Protein concentrations mg/ml
		NCDCV	G110	NCDCV	G110	
A9	22[a]	0	5×10^5	0	2.5×10^4	8
	125[a]	0	4.5×10^5	0	1×10^4	13
E5	414[b]	2000	1900	128	128	2.1

a : *Ascitic fluid* precipitated by ammonium sulfate at a final concentration of 50 % saturation and dialysed against PBSS.
b : Tissue culture fluid.

Table 4. Cross reactivity of monoclonal antibodies to G110 strain with BECV isolates and HECV, BRCV, OC43, HEV and TGE coronaviruses (IIF).

	G110 (and F15)	NCDCV (o)	B. BCV (a)	D. BCV (b)	B. RCV (c)	HECV	OC43	HEV (*)	TGE
H9	+	–	+	+	+	+	–	–	–
A20	+	+	+	+	+	+	–	–	–
B5	+	+	+	+	+	+	–	–	–
C13	+	+	+	+	+	+	–	–	–
I7	+	+	+	+	+	+	–	–	–
J18	+	+	+	+	+	+	–	–	–
I16	+	+	+	+	+	+	+	+	–
F7	+	+	–	+	+	+	+	–	–
E5	+	+	+	+	+	+	+	+	–
H7	+	+	+	+	+	+	+	+	–
H19	+	+	+	+	+	+	+	+	–
I12	+	+	+	+	+	+	+	+	–

(o) Tested on HRT cells and on PBFK cells.
(*) Tested on HRT cells and on RPd cells (pig kidney).
(a) British and (b) Danish BECV isolates.
(c) British bovine respiratory coronavirus.

Conclusions

GP105, an outer glycoprotein which is insensible to 2-ME, interacted with monoclonal antibodies which neutralized the infectivity of BECV and inhibited the agglutination of RRBC by the virus. Further investigation on the biological role of the other outer GP needs to be conducted, but GP105 is involved in haemagglutination and *in vitro* virus cell interaction. Monoclonal antibodies helped to define at least three functional domains on the GP105, one recognized by monoclonal antibodies with neutralizing activity (A20, B5, I7, J18), a second one by monoclonal antibodies with neutralizing and HAI activities (A9 and C13) and the last one recognized by monoclonal antibodies which reacted with GP105 but displayed no biological activity (F7 and I16). Identical features have been demonstrated on the outer GP of Measles virus (Ter Meulen *et al.*, 1981), Vesicular stomatitis virus (Wolk *et al.*, 1982) and Orthomyxoviruses (Breschkin *et al.*, 1981).

BECV strains isolated in different laboratories did not differ greatly when compared on the basis of their reactivity with our monoclonal antibodies, except that US and UK isolates failed to react with monoclonal antibodies A9 and F7 respectively. BECV seems to be able to undergo minor antigenic variation leading to a change in the epitopes expressed on GP105. A bovine respiratory coronavirus isolated in England was found indistinguishable from BECV G110 but differed from the British BECV isolate as the former reacted positively with F7 monoclonal antibody.

HECV, a coronavirus isolated from diarrhœic babies (Laporte *et al.*, 1980) could not be differenciated from BECV by IIF. However, these HECV seem to be more closely related to BECV than to the human respiratory coronavirus OC43 (see below).

As seen with polyclonal antisera, HEV, OC43 and BECV share common antigenic determinants which were outlined by monoclonal antibodies E5, H7, H19, I12, F7 and I16. At least, two of these common epitopes recognized by I16 and F7 are located on GP105. Work is under progress to determine which polypeptides carry these OC43 group common epitopes.

As shown in this study, monoclonal antibodies are of valuable help in analyzing both the biological activities of viral polypeptides, and the precise antigenic relationships between coronaviruses.

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