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Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2

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Summary. The production, preliminary characterisation and applications of monoclonal antibodies (mabs) against six porcine circovirus 2 isolates are described. A total of 14 stable hybridomas were produced, of which 7 were characterised. All of the mabs characterised were of IgG isotype. All the mabs tested reacted by IIF with acetone-fixed cell cultures infected with PCV2 isolates from Canada, France, Spain, Denmark, USA and UK. No cross-reactivity with a porcine circovirus 1 field isolate was demonstrated using the panel of mabs tested. In addition, one of the seven mabs tested demonstrated neutralising activity against PCV2 isolates from Canada and France. The use of selected PCV2-specific mabs for the development of virus detection methodologies is described.

Introduction

Porcine circovirus 2 (PCV2) is a small (17 nm), non-enveloped, icosahedral virus, containing a single-stranded, circular DNA genome of 1,768 nt [12]. PCV2 is genomically and antigenically distinct from the PCV contaminant of the PK/15 continuous pig kidney cell line, now designated PCV1 [12]. PCV1 is generally accepted to be non-pathogenic [2]. Recently, PCV2 has increasingly been associated with various disease syndromes in pigs worldwide [2], including postweaning multisystemic wasting syndrome (PMWS) [6] and porcine dermatitis and nephropathy syndrome (PDNS) [13]. Monoclonal antibodies (mabs) to PCV1 have been produced in this laboratory [4] and used for the preliminary characterisation of PCV2 isolates from diseased pigs [7]. However, to date, there have

been no detailed reports in the literature of the production of PCV2-specific mabs. The production and characterisation of PCV2-specific mabs are a prerequisite for the study of the biology of PCV2. In addition, such characterised mab reagents would be useful for the diagnosis of PCV2 infections and investigations on the pathogenesis of PCV2-associated diseases. The availability of specific mabs that could differentiate between PCV1 and PCV2 viruses would be of use to workers in this field.

This communication describes the production and preliminary characterisation of mouse mabs to PCV2. Some applications of these mabs are also described.

Materials and methods

Viruses

A PCV1 (3384) pool was prepared as previously described [1]. Previously characterised PCV2 isolates [5] from Canada (1010), France (48285), Denmark (1017), Spain (1019), USA (999) and Northern Ireland (5549) were used to prepare separate virus pools. Briefly, continuous pig kidney cells (PK/15), known to be free from PCV1 and PCV2, were dispersed from confluent cultures using trypsin-versene and resuspended in minimal essential medium containing Earle's salts supplemented with 10% foetal bovine serum (FBS) and 100 ug/ml gentamicin (growth medium) at a concentration of 5×10^4 cells/ml. Three-millilitres of each virus inoculum was added to 37 ml of this cell suspension and seeded in 20 ml volumes into two 75 cm^2 cell culture flasks. After 18 h incubation at 37 °C the resulting semi-confluent monolayers were treated with 300 mM D-glucosamine in Hanks basal salt solution for 30 min. After a further 48 h incubation at 37 °C one of the flasks for each inoculum was subjected to 3 freeze/thaw cycles. The remaining flask for each inoculum was passaged by dispersion of the cell monolayer using trypsine-versene and resuspension of the cells in growth medium at a concentration of 5×10^4 cells/ml. A 5 ml volume of the cell lysate derived from the freeze/thaw cycles was then added to 45 ml of the appropriate freshly dispersed cell suspension (superinfection) and the resulting mixture seeded in 20 ml volumes into two 75 cm^2 flasks and in a 6 ml volume into one 55 mm diameter petri dish containing 11 mm diameter circular coverslips. Following incubation and glucosamine treatment as described above, the coverslip cultures for each inoculum were harvested, fixed in acetone for 10 min at room temperature and the presence of PCV2 antigens determined by indirect immunofluorescence (IIF) staining. Corresponding flasks were either harvested at this time if a high incidence of PCV2 antigen-positive cells were detected, or the cultures were subjected to further passages, superinfection and monitoring by IIF until an appropriate level of PCV2 antigen-positive cells were observed. When harvesting virus isolates, the flasks were frozen and thawed three times and the cell lysate sonicated on maximum power for 30 sec. The resulting virus pools were then aliquoted and stored at −80 °C.

Mouse immunisation

BALB/c mice were used for all studies. Serum samples were taken from all mice prior to immunisation and at selected intervals throughout the immunisation schedules and tested for the presence of PCV2 antibody by IIF [15]. The immunisation protocols were designed dependent on the level of PCV2 antibody detected in these test bleeds. Details of individual fusions were as follows:

Fusion 1 (F190): A 200 ul volume of the Canadian PCV isolate (1002), which had been partially purified through a sucrose gradient, was mixed with an equal volume of purified saponin (Quil A; Superfos Biosector, Denmark) and injected subcutaneously (S/C) into two

mice. This immunisation was repeated 4 weeks later and after a further 10 days the mice were given 200 ul of virus without adjuvant intraperitoneally (I/P). After a further 8 weeks, one mouse was injected with a further 200 ul of virus without adjuvant. This mouse was subsequently euthanized 3 days later and the spleen harvested.

Fusion 2 (F199): A 200 ul volume of a French PCV2 isolate (48285) was mixed with an equal volume Quil A of and injected S/C into 2 mice. This immunisation was repeated 4 weeks later and after a further 4 weeks the mice were given 200 ul of virus without adjuvant I/P. Two weeks later one mouse was given a further 200 ul of virus without adjuvant by an I/P route. This mouse was subsequently euthanized 3 days later and the spleen harvested.

Fusion 3 (F210): A 200 ul volume of a Canadian PCV2 virus (1010) was mixed with an equal volume of Quil A and injected S/C into 3 mice. This immunisation was repeated 3 weeks later. Seven weeks later, 200 ul of virus, which had been partially purified through a sucrose gradient, was mixed with an equal volume of adjuvant (PCSL) and administered I/P. One of the mice was given 200 ul of virus without adjuvant I/P ten days later. This mouse was subsequently euthanized 3 days after the final inoculation and the spleen harvested.

Fusion 4 (F217): A 100 ul volume of a French PCV2 isolate (48285) which had been partially purified through sucrose was mixed with an equal volume of Quil A and injected S/C into 3 mice. This immunisation was repeated 3 weeks later and after a further 4 days one of the mice was euthanized and the spleen harvested.

Production of monoclonal antibodies and screening for secreting hybridomas

Mouse spleen cells were fused with non-secretory mouse myeloma cells using standard procedures [8, 16]. Resulting hybridoma colonies (HC) were maintained on RPMI 1640 medium supplemented with 20% gamma globulin free horse serum. Supernatant fluids from established HC were removed and tested for the presence of homologous PCV2 specific antibodies by IIF. Undiluted supernatant fluids were applied to acetone-fixed infected cell cultures grown on multispot slides and incubated at 37 °C for 1 h. After a brief wash in PBS the cultures were stained with an FITC-labelled antibody to mouse immunoglobulins, again for 1 h at 37 °C and after a further wash in PBS the multispot slides were mounted in buffered glycerol saline and examined. Selected HC were then cloned twice by limiting dilution, expanded into 75 cm^2 tissue culture flasks, the supernatant fluid collected and immunoglobulin precipitated and concentrated by using ammonium sulphate.

Determination of monoclonal antibody class

A 1/10 dilution in phosphate buffered saline (PBS) (pH 7.2) of each mab was applied to acetone-fixed PCV2-infected cell culture preparations grown on circular coverslips for 1 h at 37 °C. After a 5 min wash in PBS 5 class and sub-class specific FITC-labelled antibodies to mouse immunoglobulins (IgM, IgG1, IgG2a, IgG2b, IgG3) (Nordic) were diluted 1/80 in PBS and applied separately to coverslip preparations for each of the mabs. Following a further 1 h incubation at 37 °C the coverslips were again washed in PBS, mounted in buffered glycerol saline and viewed using incident UV illumination.

Virus neutralising activity of monoclonal antibodies

Neutralising activity was determined using a microtiter plate serum neutralisation assay incorporating an indirect immunoperoxidase (IIP) staining protocol to determine virus replication. The neutralisation assay was performed in duplicate using two different PCV2 isolates (1010 and 48285). A 100 ul volume of serial ten-fold dilutions (10^{-1} to 10^{-6}) of PCV2 made in growth medium was added to a 96-well flat-bottom microtiter plate. The mabs were then diluted 1/3 in growth medium and an equal volume added in duplicate to each virus dilution

and the microtiter plates placed in an incubator at 37 °C for 3 h in an atmosphere of 5% CO_2 in air. Following this incubation 2×10^4 PK/15 cells freshly dispersed from confluent monolayers using trypsine-versene solution were added to each well and the microtiter plates returned to the incubator. After a further 72 h the microtiter plates were removed from the incubator and the supernatant fluids discarded. The cell monolayers were washed once with PBS followed by a single wash in 100% methanol after which the cell monolayers were immersed in 100% methanol for 10 min. Fixed cell cultures were air dried and 100 ul of a 1/1,000 dilution of a rabbit anti-PCV2 specific polyclonal antibody applied for 1 h at 37 °C. The plates were then washed 3 times in PBS and the immunostaining protocol completed using a Histostain-SP Bulk kit for rabbit primary antibody (Zymed; Cat. No. 95-6143B) and the enzyme substrate 3-amino-9-diethylcarbazole (Zymed Cat. No. 00-2007). Cell monolayers were examined and virus neutralisation indices determined by a reduction in virus titer in the presence of mabs when compared to virus titrations performed in the absence of mab or with the addition of an inappropriate mab [4]. Within individual wells, virus neutralisation was deemed to have occurred if a decrease of greater than 90% of the number of positive cells was observed in mab treated wells when compared to equivalent wells containing inappropriate or no mab.

Reactivity of monoclonal antibodies on acetone-fixed PCV2-infected cell cultures

Acetone-fixed PCV1 and PCV2 infected cell cultures were grown on multispot slides. One PCV1 isolate and 6 PCV2 isolates were used (Table 1). Three degreased sterile multispot-slides were placed into a 10 cm^2 square petri dish. Cells were dispersed from confluent PK/15 monolayers using trypsine-versene and resuspended in growth medium at a concentration 5×10^4 cells/ml. Seed virus was added to the cell suspension to give a multiplicity of infection of 0.1 and the resulting mixture was dispensed into the petri dishes containing multispot slides in 40 ml volumes. After 72 h incubation at 37 °C in 5% CO_2 the multispot slides were removed from the petri dishes, fixed in acetone for 10 min, air dried and stored at − 20 °C until required. Serial two-fold dilutions from 1/10 to 1/102,400 of the mabs were prepared in PBS and applied to the acetone-fixed virus-infected multispot slides. After 1 h incubation at 37 °C the multispot slides were washed twice in PBS and an FITC-conjugated antibody to mouse IgG applied. After an additional 1 h incubation the multispot slides were again washed twice in PBS, mounted in buffered glycerol saline and examined using UV illumination. The titer of each mab was determined as the highest dilution, which resulted in immunofluorescent staining.

Reactivity of monoclonal antibodies on acetone-fixed cryostat sections of tissues from infected pigs

Tissues were collected from colostrum-deprived (CD) pigs that had been inoculated at 2 days of age by an oral/nasal route with either the 1010 or 48285 PCV2 isolate. Twenty-one days after inoculation the pigs were necropsied and the tissues processed for cryostat sectioning. Cryostat sections of selected tissue known to contain abundant PCV2 antigen [3] were cut onto degreased glass slides and fixed in cold acetone for 10 min. Sections were allowed to air dry and a 1/100 dilution in PBS of selected mabs was applied for 1 h at 37 °C, after which, the IIF procedure was completed as described previously.

Dual immunolabelling of PCV2-infected cell cultures using monoclonal antibodies and a two-colour immunofluorescent protocol

PCV-2 infected cell cultures were grown on circular glass coverslips and fixed in acetone prior to immunostaining. Dual immunolabelling protocols were performed essentially as is

Table 1. Preliminary characterisation of mabs to PCV2

Mab	Isotype	Staining pattern in cell cultures	Reactivity on cryostat sections[a]	Reactivity on paraffin sections	Neutralising activity	Cross-indirect immunofluorescent staining of PCV-infected cell cultures						
						PCV1 3384	PCV2 1010	PCV2 48285	PCV2 1017	PCV2 1019	PCV2 5549	PCV2 999
F190 2B1	IgG 2b	1	Yes	Strong	Yes	< 50	3200	12000	3200	3200	6400	6400
F199 A11	IgG 1	2	No	No	No	< 50	12000[b]	**24000**	12000	24000	12000	24000
F199 B1	IgG 2a	1[c]	Yes	Weak	No	< 50	3200	**12000**	3200	6000	12000	3200
F210 A1	IgG 1	2	No	No	No	< 50	**48000**	24000	6000	24000	48000	12000
F210 D6	IgG 1	2	No	No	No	< 50	**48000**	48000	24000	48000	48000	48000
F217 E7	IgG 1	1	Yes	Strong	No	< 50	800	**800**	1600	800	1600	1600
F217 B6	IgG 1	1	Yes	Strong	No	< 50	1600	**800**	1600	800	800	1600

[a]F199 A11; F210 A1and F210 D6 do not react with PCV2 antigens in cryostat sections of acetone-fixed pig tissues. F190 2B1, F199 B1 and both F217 mabs give typical immunostaining of PCV2 antigens in tissue sections

[b]reciprocal of the last dilution of monoclonal antibody which gave positive indirect immunofluorescence staining; figures in bold are the antibody titres to homologous virus

[c]type 1 IIF staining pattern was observed as intense staining of the nucleus and cytoplasm; type 2 pattern was observed as intense staining of the nucleus only

described for IIF, except that combinations of mabs of differing isotypes were diluted 1/50 in PBS, mixed together and applied to a coverslip for 1 h at 37 °C. Following a brief wash in PBS, isotype specific anti-mouse antibodies labelled with FITC or texas red were diluted, pooled together and also applied for 1 h at 37 °C. After a further wash in PBS, the coverslip was mounted using Vectashield (Vector laboratories, Cat H-1000) and examined. Controls included individual mabs stained with appropriate and inappropriate anti-isotype conjugates separately.

Reactivity of monoclonal antibodies on formalin-fixed paraffin embedded tissues from experimentally infected pigs

Tissues were removed at necropsy from pigs following experimental infection with the 1010 isolate of PCV2, fixed in 10% neutral buffered formalin (NBF) for 24–48 h and processed to paraffin using standard procedures. Tissue sections (4–5 um) were cut onto glass slides, dewaxed in xylene and washed twice in methylated spirit for 5 min. Endogenous peroxidase activity was neutralised by immersing the tissue sections in a solution of 0.5% hydrogen peroxide (H_2O_2) in methanol followed by a brief rinse in distilled water and two 10 min washes in 5 mM Tris-buffered saline (TBS) (pH 7.6). The sections were then incubated in a 0.05% Protease 14 solution in TBS for 15 min at 37 °C, rinsed in distilled water, followed by two 10 min washes in TBS, after which the sections were incubated with 10% normal goat serum for 30 min. The sections were then sequentially incubated with the respective PCV2 specific mab diluted 1/250 in TBS overnight at room temperature, followed by a biotinylated goat anti-mouse antibody (Zymed Histostain Mouse SP Kit Cat: 95-6543B) for 30 min at room temperature and a strepavidin peroxidase conjugate for 15 min, also at room temperature. Tissue sections were given two 10 min washes in TBS after each incubation. To complete the staining protocol the sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride (Vector laboratories Cat: SK-4100) for 2–7 min, washed in distilled water, counterstained with haematoxylin and permanently mounted.

Antigen detecting ELISA

Following preliminary studies, one mab was identified and used in the development of a PCV2-specific antigen detecting ELISA. PBS containing 0.05% Tween-20 (PBS-T) was used for washings. Four addition and incubation steps were used:

(a) Initially plates were coated overnight at 4 °C with a 1:2000 dilution, in carbonate/bicarbonate buffer (pH 9.6), of the immunoglobulin fraction of PCV2 mab FI90 2B1. Selected wells were coated with buffer only as controls.
(b) Then following 3 washes of coated ELISA plates, 100 ul of fractions from a PCV2 discontinuous CsCl gradient, diluted 1:100 in PBS-T, were added to the wells in duplicate and incubated for 1 h at 37 °C. Aliquots were also added to wells coated in buffer only. Plates were washed again as described above.
(c) Next a polyclonal rabbit antibody to PCV2 [5] was diluted 1:2000 in PBS-T and 100 μl aliquots added to all the wells for 1 h at 37 °C. Following this incubation, the plates were washed again.
(d) Finally a 1:10 dilution, in PBS-T, of biotinylated goat anti rabbit Ig (Zymed) was added to all wells for 1 h at 37 °C and the plates washed. This was followed by addition of 100 μl of a 1:25 dilution, in PBS-T, of strepavidin peroxidase conjugate (Zymed). The plates were washed again and 100 μl of TMB substrate added to all wells for 15 min at room temperature. Reactions were stopped by addition of 1 M H_2SO_4 and plates read at 450 nm.

The specificity of the PCV2 antigen capture ELISA was confirmed by testing against purified preparations of field isolates of PCV1 and the PCV1 contaminant of PK/15 cells as well as preparations of 6 PCV2 isolates from N America and Europe. Infectivity titres of all samples tested on the ELISA were determined by inoculation of serial ten-fold dilutions into PK/15 cells for 96 h and monitoring for virus infectivity by IIF as described previously.

Results

Mouse immunisation

All serum samples taken from the mice prior to immunisation were negative for antibodies to PCV2 at a 1:10 dilution by IIF.

F190: Serum antibody levels of 1:100 to PCV2 were first detected in these mice 7 days after the second S/C inoculation. The mice were bled again 5 weeks after the first I/P inoculation, when one of the mice had an antibody titre > 1:10,000 by IIF. This mouse was boosted again, euthanized and the spleen removed for fusion.

F199: Serum antibody levels to PCV2 of 1:100 were first detected in these mice at one week after the second S/C immunisation. The mice were bled again one week after the third immunisation by the I/P route when PCV2 antibody titres had increased to 1:10,000. One of these mice was given a further I/P inoculation, euthanized 3 days later and the spleen removed for fusion.

F210: PCV2 antibody titres in serum, ranging from 1:100 to 1:1000, were detected in blood samples collected from these mice 7 days after the second S/C inoculation. All mice were again bled 10 days after immunising with sucrose gradient purified virus when PCV2 antibody titres > 1:10,000 were detected in blood samples from all mice. One of these mice was given a further I/P inoculation, euthanized 3 days later, and the spleen removed for fusion.

F217: Serum antibodies to PCV2 of 1:100 were first detected in these mice 10 days after the second S/C immunisation. The mice were re-bled 10 weeks later, when PCV2 antibody titres > 1:1000 were seen in all mice. One of these mice was euthanized without an additional immunisation and the spleen removed for fusion.

Detection of secreting hybridoma colonies

Microscopic examination demonstrated 343, 623, 531 and 340 actively dividing HC in F190, F199, F210 and F217 respectively. Following IIF testing of supernatant fluids from all of these colonies, 40 supernatants from F190, 42 supernatants from F199, 8 supernatants from F210 and 33 supernatants from F217 gave strong immunofluorescent staining on PCV2-infected cell cultures and not with uninfected PK/15 cells. Four HC from each fusion were selected for further cloning. The selection of HC for cloning was based on differing immunofluorescence staining patterns, and that the colonies contained highly efficient secretory cells as indicated by immunofluorescence staining intensity. Stable second clones were produced from 14 representative HC, expanded and the antibody precipitated.

Preliminary characterisation studies

Preliminary studies performed on the 14 mabs selected for characterisation demonstrated that many appeared to be very similar and the results of seven are presented (Table 1). Using the IIF test all seven mabs were shown to belong to subclasses of IgG (Table 1). Using a constant antibody-varying virus neutralisation test a neutralisation index of 4 was determined for one of the mabs (F190). No neutralising activity was detected with any of the remaining mabs against either the 1010 or 48285 PCV2 isolates.

Reactivity of monoclonal antibodies on acetone-fixed PCV2-infected cell cultures

The titres obtained following IIF staining of PCV2-infected acetone-fixed cell cultures with the mabs is shown in Table 1. In general, all of the mabs reacted with heterologous virus antigens to a titre within a 2 to 4 fold difference to that obtained with homologous virus antigens. None of the mabs reacted with PCV1 (3384) virus antigens. Two distinct IIF staining patterns were seen in PCV2-infected cell cultures. A type 1 IIF staining pattern was typified by intense labelling of the nucleus and cytoplasm, similar to that seen with rabbit and pig polyclonal antibodies (Fig. 1a). A type 2 IIF staining pattern was typified by intense staining of the nucleus only. Mabs derived from F210 immunostained all of the PCV2 viruses tested in a type 2 pattern (Fig. 1b). Mabs from F217 immunolabelled all the viruses tested in a type 1 pattern. One of the mabs (A11) from F199 gave intense nuclear staining only (type 2), similar to that seen with both the F210 mabs. The second mab from F199 (B1) gave typical type 1 staining on all of the PCV2-infected cell cultures examined.

Dual immunolabelling of PCV2-infected cell cultures using monoclonal antibodies and a two-colour immunofluorescent protocol

The results of a dual immunofluorescent staining protocol performed on 48285 and 1010 PCV2 antigens in acetone-fixed cell cultures using mabs producing type 1 staining (F199 B1) and type 2 staining (F210 A1) demonstrated that these mabs were immunostaining PCV2 antigens in the same cells (Fig. 2a and b).

Reactivity of monoclonal antibodies on acetone and formalin-fixed tissue sections from infected pigs

The suitability of all of the mabs for application in immunolabelling procedures was determined and is summarised in Table 1.

The reactivity of all mabs was determined for PCV2 antigens (1010 and 48285) in acetone-fixed cryostat sections of tissues obtained from experimentally infected pigs. Two mabs from F217 (E7, B6), one mab from F190 2B1 and mab F199 B1 immunostained PCV2 antigen in acetone-fixed, cryostat sections from pigs experimentally infected with PCV2 (Fig. 3a, b and c). The remaining mabs tested (F199 A11, F210 A1, F210 D6) did not immunostain PCV2 antigens in

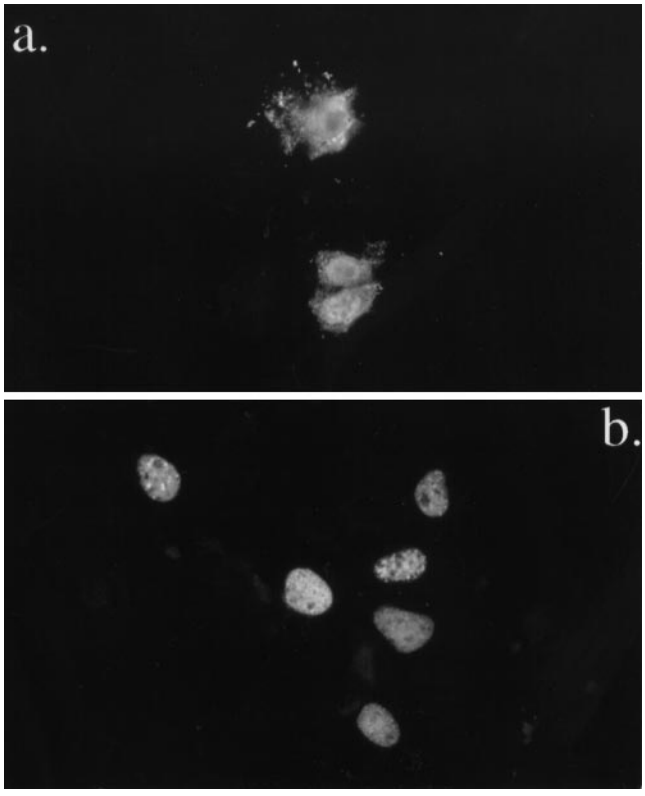

Fig. 1. Indirect immunofluorescent staining of PCV2-infected PK/15 cell cultures with monoclonal antibody (**a**) F199 B1 to PCV2 and monoclonal antibody (**b**) F210 A1 to PCV2. Note the two distinct staining patterns

these tissue sections. The reactivity of all the mabs for 1010 PCV2 antigens was also determined in formalin-fixed paraffin embedded tissues. The results obtained were similar to acetone-fixed cryostat sections. Immunostaining with the F190 and F217 mabs resulted in strong labelling of PCV2 antigens in the formalin-fixed tissues (Fig. 4). The F199 B1 mab gave only weak immunolabelling of the PCV2 antigens, with the remaining 3 mabs (F199 A11, F210 A1, F210 D6) not reacting with PCV2 antigens after formalin fixation.

Antigen capture ELISA

Based on results from earlier characterisation studies, including the finding that F190 2B1 was the only mab to demonstrate neutralising activity it was selected for the development of an antigen capture ELISA. Testing of 21 fractions from a PCV2 discontinuous CsCl gradient resulted in a peak of detection in fractions 6–8 of PCV2 antigen using the ELISA. This peak of detection correlated with a peak of PCV2 infectivity (Fig. 5) and to the highest concentration of virus particles seen by electron microscopy. No reactivity was detected in the ELISA when aliquots of PCV1 virus were tested. A high level of reactivity was detected with all isolates of PCV2 tested from N America and Europe (data not shown).

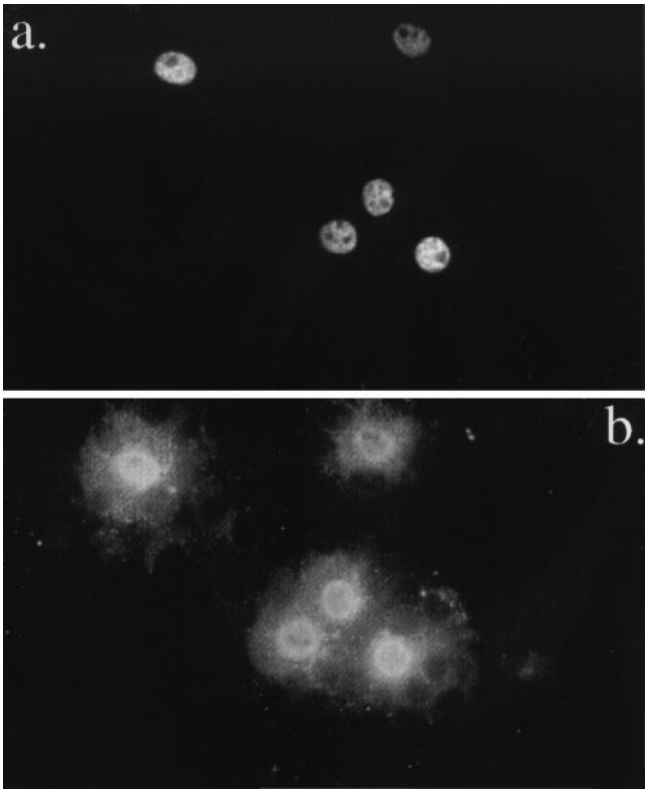

Fig. 2. Dual immunofluorescent staining of PCV2-infected PK/15 cell cultures with PCV2 monoclonal antibodies (**a**) F210 A1 (FITC) and (**b**) F199 B1 (Texas Red). Note that both monoclonal antibodies stain PCV2 antigens in the same cells in PCV2-infected cell cultures

Discussion

This is the first detailed description of the production, and characterisation of mabs to PCV2. Applications of some these mabs to the study of PCV2 infections are also described. In previous reports from our group, some applications of mabs to PCV2 have been presented [5, 7, 11, 15]. These include the use of specific PCV2 mabs for immunostaining of cell cultures and tissue sections and the use of a PCV2 mab in a PCV2-specific antibody detecting ELISA. This present communication expands these initial reports by reporting the results of comparative studies on an extended panel of PCV2 mabs. In total 123 HC, which gave strong reactivity against PCV2 by IIF, were obtained from the 4 fusions. However the results of IIF staining from F210 were disappointing, producing only 8 strongly reactive HC from the initial 531 actively dividing colonies. The reasons for this are unknown, but may be a reflection on the mouse immunisation regime used for this virus.

All the mabs to PCV2 characterised in this study reacted by IIF with the 6 other PCV2 isolates recovered from diseased pigs from N America and Europe but did not react with either a PCV1 field isolate (3384) or the PCV1 isolate from

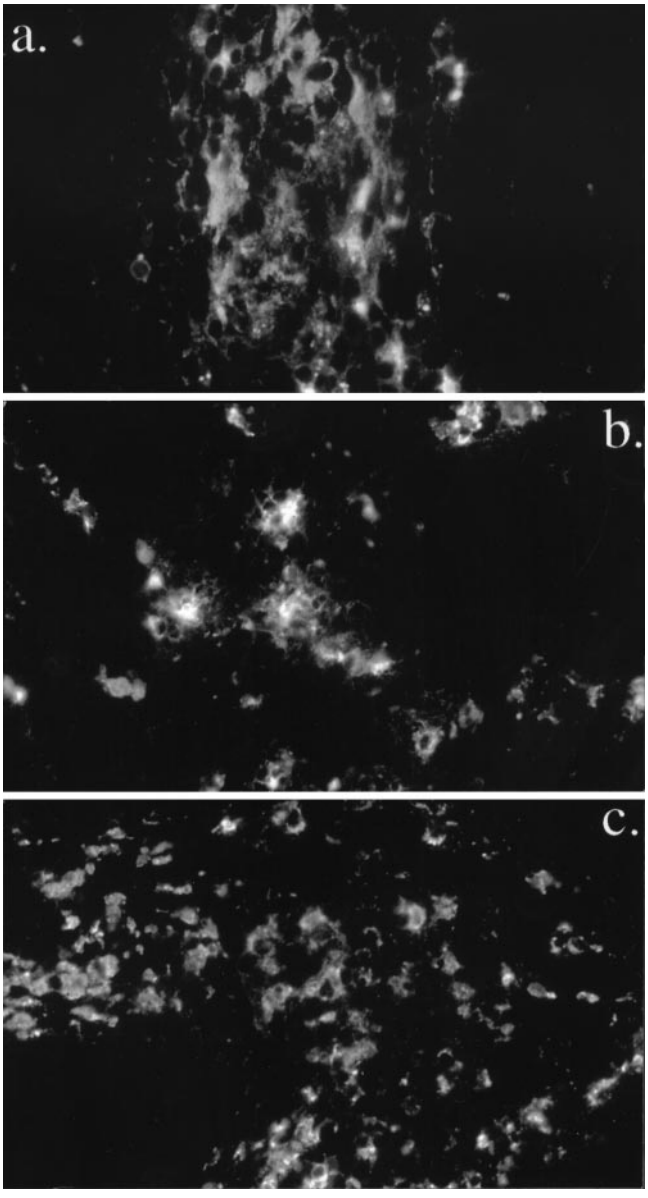

Fig. 3. Immunostaining of PCV2 antigens in acetone-fixed cryostat sections of tissues from pigs experimentally infected with PCV2. Monoclonal antibodies (**a**) F217 E7, (**b**) F190 B10 and (**c**) F199 B1

PK/15 cells (data not shown). Cross reactivity titres against heterologous PCV2 isolates were high, indicating that the mabs under study were directed against epitopes conserved between all these virus isolates, and hence suitable for use as broad-spectrum PCV2-specific reagents. Two distinct staining patterns of PCV2 antigen in acetone-fixed cell cultures were seen. Type 1, which was typified by nuclear and cytoplasmic staining in a “starburst” pattern, was consistent with that seen when PCV2-infected cell cultures were immunostained with polyclonal

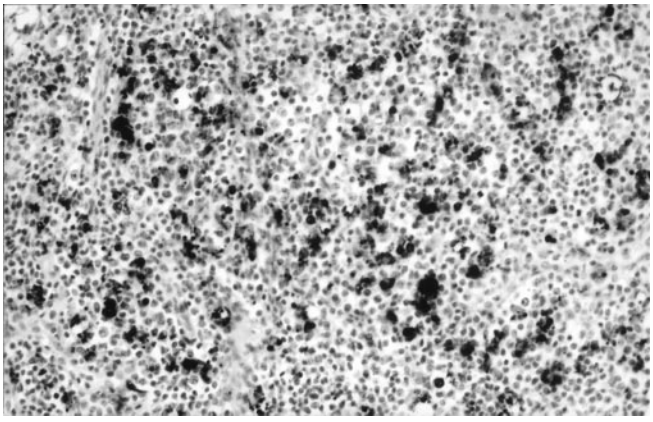

Fig. 4. Immunostaining of PCV2 antigen in formalin-fixed, paraffin-embedded lymph node tissue from a pig experimentally infected with PCV2 using monoclonal antibody F217 B6

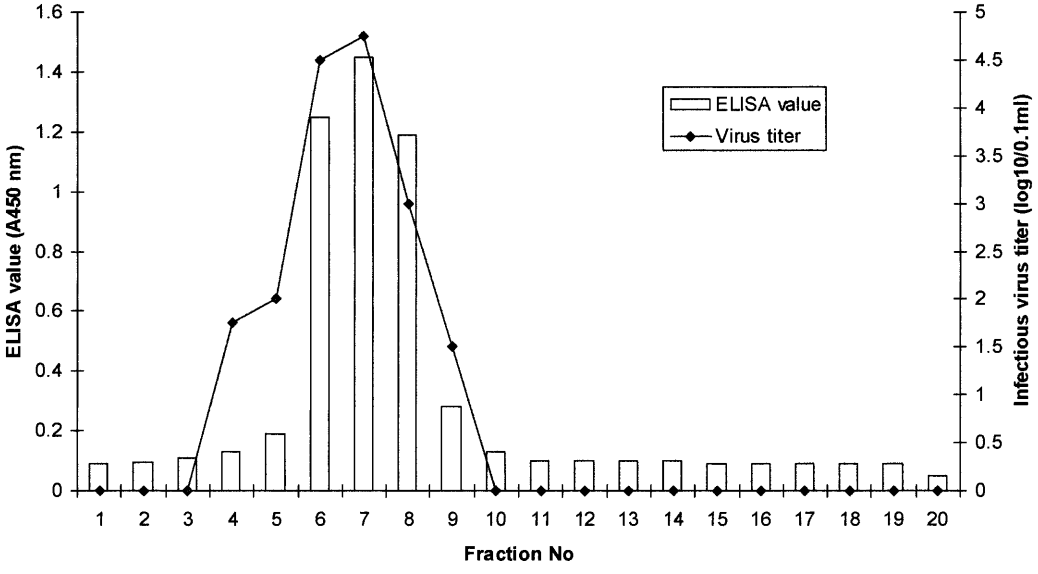

Fig. 5. Comparison of PCV2 infectivity titres and antigen capture ELISA results on 20 fractions from a discontinuous PCV2 CsCl gradient. Note that the peak of PCV2 infectivity corresponds to the peak of PCV2 reactivity in the antigen capture ELISA

hyperimmune rabbit sera or convalescent sera derived from PCV2 infected pigs. Type 2 staining was confined to the nucleus of PCV2-infected cells.

In a similar manner to results generated using PCV2-infected PK/15 coverslips, the reactivity of the panel of PCV2-specific mabs was shown to fall into two types of immunofluorescence staining, depending on the PCV2 ORF expressed in transfection studies. Type 1 staining (nuclear and cytoplasmic "starburst") was observed in ORF2 transfected constructs whereas type 2 IIF staining (nuclear) was observed following transfection with PCV2 ORF1 constructs. As has been

suggested for PCV1, PCV2 ORF2 is thought to represent the major structural protein [9].

The failure of mabs F199 A11, F210 A1, F210 D6 (type 2 staining ORF1) to detect PCV2 antigen in tissue sections from experimentally infected pigs was unexpected. It is known that ORF1 encodes the replication protein as an early event in the replication cycle of PCV [14] and the mabs in question would appear to have detected this protein in the nucleus of acetone-fixed cell cultures, but not in tissue samples from terminally ill pigs experimentally infected with PCV2. It is possible that the reactivity observed in PCV2 infected PK/15 cells with these mabs was against a PCV2-induced cellular protein produced in this cell line and not in infected pigs. Alternatively, it is possible that in terminally ill pigs infected with PCV2, either little or no expression of the replication protein is taking place or this protein is quickly degraded.

It is of particular interest to note that the restricted inoculation schedule used in the mice for F217 resulted in 33 strongly positive HC. Although the IIF antibody titres of supernatants derived from 2 of these HC were lower than those obtained from the other fusions, reflected in the low antibody titres immediately prior to the sacrifice of one mouse for fusion, these mabs were found to be the best reagents for immunostaining of PCV2 antigen in tissue sections from diseased pigs. This is probably a reflection of their reactivity against an epitope of the structural protein of PCV2, thought to be encoded by ORF2 [9, 10]. This result indicates the importance of assessing mab antibody suitability for different applications, even if antibody titres are comparatively low.

Mabs to newly recognised viruses, such as PCV2, serve as essential tools for diagnosis and the study of pathogenesis. The development of a PCV2-specific, mab-based antigen capture ELISA described in this paper for the detection of PCV2 in CsCl gradients will be of use in the preparation of purified virus preparations. Evaluation of this PCV2-specific antigen ELISA is ongoing to further assess the potential of this test in the quantitative detection of PCV2 virus in clinical samples as an aid to diagnosis.

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