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Antigenicity and pathogenicity characteristics of molecularly cloned chicken anaemia virus isolates obtained after multiple cell culture passages

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Summary. The Cux-1 isolate of chicken anaemia virus (CAV), which had received 310 (P310) cell culture passages, was substantially less pathogenic than virus that had been passaged 13 times (P13). Molecularly cloned virus isolates, selected from the P310 and P13 virus populations using recombinant DNA cloning and transfection procedures, reacted differently with 4 CAV-specific monoclonal antibodies (MAbs), which had been raised to low-passage Cux-1 virus. In contrast to the strong immunofluorescence (IF) reactivities exhibited by all P13 cloned isolates tested, 80% and 57% of the P310 cloned isolates reacted weakly with MAbs 2A9 and 4H4, which are directed against conformational epitopes on the capsid protein, VP1. Sequence analysis of the VP1 coding regions possessed by ten P310 and two P13 cloned isolates showed that 6 amino acid changes within VP1 had been selected by multiple-cell culture passage. One of these at position 89 in VP1 appeared to be crucial for determining reactivity with MAb 2A9. Of nine P310 cloned isolates evaluated, 8 were substantially attenuated compared to the low-passage Cux-1 virus pool. It is concluded that the individual virus variants comprising the P310 virus pool differ with regards to their antigenicity and pathogenicity.

Introduction

Chicken anaemia virus (CAV) is an economically important avian pathogen, which has been shown to have a worldwide distribution [7]. Vertical transmission of the virus through the egg from infected breeder flocks can result in a clinical disease characterised by anaemia, haemorrhages and lymphoid depletion and associated with increased mortality in 10–14 day old chicks. The virus can also be transmitted horizontally when maternal antibody has disappeared, resulting

in a sub-clinical disease that adversely affects growth and profitability in broiler flocks [12]. The virus can be grown in a Marek's disease virus transformed chicken lymphoblastoid cell line MDCC-MSB1 which has enabled characterisation of the physical and biological properties of the virus and elucidation of its molecular biology [15, 22]. Based on its morphology and circular single-stranded DNA genome, the virus has been classified in the animal virus family *Circoviridae* [5].

A long-term aim of our research is the development of a live attenuated CAV vaccine. However, all the naturally-occurring, geographically-distinct field isolates tested to date belong to the same serotype and all appear to be pathogenic in an experimental disease model [2, 9, 10]. This model involves inoculating 1-day-old specific pathogen free (SPF) chicks by the intramuscular (i.m.) route and assessing the chicks after 14 days for anaemia and lesions in the bone marrow and thymus. In an earlier paper, we reported that the Cux-1 isolate of CAV becomes reduced in pathogenicity following multiple passage of the virus in MDCC-MSB1 cells. We also described the molecular cloning of an attenuated isolate, cloned isolate 10, from the Cux-1 CAV pools that had been passaged 173 times in cell culture [20]. Cloned isolate 10 proved to be unstably attenuated and unsuitable for live vaccine development [20]. More recent work has shown that Cux-1 virus, that has received over 300 cell culture passages, is substantially attenuated [21]. With the view to elucidating the basis of the attenuation possessed by very highly-passaged virus, we are investigating the biological and genetic diversity of the virus isolates that comprise the virus pool that has been passaged 310 times (P310) in MDCC-MSB1 cells. In this paper we report that cloned virus isolates, selected from the P310 virus pool by recombinant DNA cloning and transfection methodologies, differ in terms of their monoclonal antibody (MAb) reactivities and pathogenicities. In addition, differences in the MAb reactivities of the P310 cloned isolates are related to amino acid differences that occur in the translated sequences of the capsid protein ORF.

Materials and methods

Cells and virus growth

The Cux-1 isolate of CAV and the MDCC-MSB1 cells were supplied by Dr. V. von Bulow (Free University of Berlin, Berlin, Germany). Virus pools that had been passaged in MDCC-MSB1 cells 13 times (P13) and 310 times (P310) since the receipt of the Cux-1 isolate in our laboratory were produced as described by Todd et al. [21]. Briefly, this involved passaging virus-infected cells at 2–3 day intervals into fresh cells or medium using reagents and conditions described previously [8]. The infectivity titres of virus pools including those of selected P13 and P310 cloned isolates were determined as described previously [25].

Cloning of CAV replicative form (RF) DNA

DNAs were extracted from MDCC-MSB1 cells that had been separately infected with either the P13 or P310 Cux-1 virus pools using the Hirt procedure [4] as described previouly [23]. The double-stranded (ds) P310 and P13 replicative form (RF) DNA populations, treated with the restriction endonuclease *Pst* l to generate linear 2.3 kb RF fragments, were separately isolated by electroelution following size fractionation using agarose gel electrophoresis [6]

and separately ligated into the multiple cloning site of pBlueScript SK+ (Stratagene, UK). Recombinant plasmids containing individual CAV RF DNAs were isolated from bacterial clones obtained following transformation into *E. coli* (JM 109).

Nucleotide sequence determination

The nucleotide sequences of the DNA encoding the VP1 ORF were determined for selected P310 and P13 cloned RFs. Thermal cycle sequencing (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin Elmer) was performed as described by the manufactures. Purified recombinant CAV RF plasmids, prepared from 3 ml bacterial cultures using the Wizard Minipreps DNA purification system (Promega) were used as templates. Sequencing was performed in both directions using primers based on the sequence of the low-passage Cux-1 CAV sequence reported by Meehan et al. [14; EMBL accession number M 81223]. In the forward direction, the primers used were
5′ CTTGATTACCACTACTCCCAGC 3′ (CAV VP1a),
5′CGGAAGGACTCATTCTGCCT 3′ (CAV VP1b),
5′ TTGCCGGTTCTTTAATCACC 3′ (CAV VP1c),
5′ TCATCACCGCTACTACAGCG 3′ (CAV VP1d),
5′ AGCTACATGTCAGCACCCG 3′ (CAV VP1e). Primers
5′ GGCTTGTGATTTAGACCCGT 3′ (CAV VP1revA),
5′ CGACGCGTGAGGTACTCTC 3′ (CAV VP1revB),
5′ TCTCGCCTTGTGGTGGTT 3′ (CAV vp1revC)
5′ CCCCAGTACATGGTGCTGTT 3′ (CAV VP1revD) and
5′ CCATCTTGACTTTCTGTGTACAGG 3′ (Eco rev) were used in the reverse direction. The VP1 ORF sequence of each cloned RF DNA was complied and analysed using the computer program DNASIS (Hitachi).

Transfection

Individual P13 and P310 cloned RF DNAs were used to transfect MDCC-MSB1 cells using an adaptation of the DEAE dextran protocol of Sompayrac and Danna [18], as described previously [23]. Infectious pools of molecularly cloned P13 and P310 CAV isolates were recovered after 5–9 passages of transfected cells as described by Todd et al. [20].

Immunofluorescence

Direct immunofluorescence (IF) using an FITC conjugated chicken antiserum to CAV was carried out as previously described [8]. The indirect immunofluorescence (IIF) test to detect CAV-specific antibody in chicken sera was carried out using MDCC-MSB1 cells infected with the P13 Cux-1 virus pool with serum dilutions in the range 1:100 to 1:8 000 as described by McNulty et al. [8]. IIF was also used to determine the reactivities of P310 and P13 cloned isolates with four CAV-specific MAbs, 2A9, 4H4, 1H1 and 3B1, which had been produced and characterised previously in our laboratory [11]. In this case, cells infected with selected P13 and P310 cloned virus isolates contained on multispots were reacted with dilutions of MAb in the range 1:100 to 1:80,000.

Experimental infection

One-day-old specific pathogen free (SPF) chicks housed in negative pressure isolators were used to evaluate the pathogenicities of selected CAV isolates as described previously [10]. Groups of 12–13 SPF chicks were inoculated intramuscularly (i.m.) using 0.2 ml of the virus pools that had infectivity titres ranging from $10^{6.75}$ to $10^{7.25}$ $TCID_{50}$/0.1 ml. A positive control

group inoculated with the P13 virus pool, and an uninoculated negative control group were also included. The birds were bled at 14 days post infection (p.i.) to allow estimation of their packed cell volumes (PCVs) by haematocrit. Birds were considered anaemic if their PCV was less than 27. Gross pathological changes, involving thymus atrophy and paleness of bone marrow, were recorded as 1+ and 2+ according to the severity of the lesion and a mean group "clinical score" estimated as described previously [21]. In a concurrent experiment, groups of eight 1-day-old SPF chicks were separately inoculated (i.m.) with 0.2 ml of P310 cloned virus isolates (numbers 27, 28, 31 and 33), pools of which had infectivity titres ranging from $10^{7.0}$ to $10^{7.25}$ $TCID_{50}$/0.1 ml. A control group was included which remained uninoculated. Serum samples were collected from these chicks at 4 weeks p.i. for serological testing.

Virus neutralisation

The abilities of the 2A9 MAb and an experimental chicken serum, which was obtained by experimentally infecting 5 week-old SPF chicks with low-passage Cux-1 virus and recovering a blood sample at 4 weeks p.i. [21], to neutralise different virus pools, including selected P310 cloned virus isolates, were investigated using an adaptation of the microtitre-plate based virus neutralisation protocol described previously [19]. Briefly, this involved titrating each virus pool, the endpoint being selected on the basis of the development of viral cytopathic effect (cpe) after two passages of the virus-infected cells. Based on this titre, 100 $TCID_{50}$ of each virus pool was incubated for 24 h at 4 °C with serial 2-fold dilutions of the MAb 2A9 (starting at 1:1 000 dilution of ascites) and the experimental chicken serum (starting undiluted). MDCC-MSB1 cells were then added to give a final concentration of 3×10^5 cells/ml and the mixtures incubated under growth conditions described previously [8]. The cells were passaged after 2–3 days into fresh growth medium and neutralisation titres determined after a further 2–3 days on the basis of viral cpe. This method was also used to determine the virus neutralisation titres of pooled serum samples collected from 4 week-old SPF chicks that had been infected at 1-day-old with selected P310 cloned isolates as described above. In this case, the low-passage P13 virus pool was used to assess neutralisation capability.

Accession numbers

The GenBank accession numbers relating to the nucleotide sequences of the CAV VP1 ORFs specified by ten P310 cloned isolates and two P13 cloned isolates are AJ 133507; AJ 133508; AJ 133509; AJ 133510; AJ 133511; AJ 133512; AJ 133513; AJ 133514; AJ 133515; AJ 133516; AJ 133517 and AJ 133518.

Results

Pathogenicity of P13 and P310 virus pools

Pools of P13 and P310 Cux-1 virus isolate, which possessed infectivity titres of $10^{6.75}$$TCID_{50}$/0.1 ml respectively, were evaluated for pathogenicity following inoculation of 1-day-old SPF chicks (Table 1). The results obtained from two individual experiments (A and B) indicated that the highly-passaged P310 virus pool was substantially less pathogenic than the low-passage P13 virus pool. This was evident from the lower number of chicks exhibiting anaemia, the higher mean PCVs, which were similar to those found with the uninoculated chicks, and the reduced severity of lesions observed when compared with chicks that had been inoculated with the P13 virus.

Table 1. Pathogenicity evaluation of P13 and P310 pools

Experiment	Group	Titre of inoculum ($TCID_{50}$/0.1 ml)	Mean PCV ±standard deviation	Number anaemic[a] (%)	Thymic atrophy	Paleness of bone marrow	Clinical score (mean)
A	Uninoculated	–	30.72±2.00	0/11 (0%)	– –	– –	0.00
	P13 pool	$10^{6.75}$	13.75±6.70	11/12 (92%)	3×1+ 6×2+	2×1+ 9×2+	2.42
	P310 pool	$10^{6.75}$	29.33±4.60	2/12 (17%)	2×1+ –	1×1+ 1×2+	0.42
B	Uninoculated	–	33.33±1.44	0/12 (0%)	– –	– –	0.00
	P13 pool	$10^{6.75}$	21.75±6.22	10/12 (83%)	3×1+ 2×2+	3×1+ 7×2+	2.00
	P310 pool	$10^{6.75}$	31.50±3.21	2/12 (17%)	1×1+ –	2×1+ –	0.25

[a]Anaemia is defined as a PCV value lower than 27

Production of P310 and P13 cloned virus isolates

Following cloning of the virus-specific RFs using pBluescript, six P13 and thirty P310 cloned RFs were randomly selected and transfected into MDCC-MSB1 cells to produce infectious virus isolates. Most of the P310 cloned RFs transfected produced viral cpe after six or seven passages, allowing small working pools to be produced after approximately eight or nine passages. In contemporaneous experiments we found that transfection with P310 cloned RFs required one passage less to produce cpe than their P13 equivalents. The infectivities of twelve P310 cloned isolates and six P13 cloned isolates were determined and with one exception, P310 cloned isolate 7, all were found to be in the range $10^{6.25}$ to $10^{7.25}$ $TCID_{50}$/0.1 ml. Cloned isolate 7, which had a titre of $10^{5.25}TCID_{50}$/0.1ml, required 4 extra passages in cells upon transfection to produce a small working pool, compared to other P310 and P13 cloned isolates.

IIF reactivities of P310 and P13 cloned isolates with CAV MAbs

Six P13 and thirty P310 cloned isolates produced by transfection of cloned viral RF DNAs were screened for reactivity with each of the four MAbs. Screening dilutions of each MAb that produced strong, easily-detectable, CAV-specific staining patterns were pre-determined for each MAb by titration, using MDCC-MSB1 cells infected with the P13 virus pool (Table 2). All of the P310 and P13 cloned isolates reacted strongly by direct IF using a CAV positive chicken serum conjugated to FITC. All of the cloned isolates also reacted strongly with CAV MAbs 3B1 and 1H1 at their screening dilutions. However, while MAbs 4H4 and 2A9 reacted strongly with all six P13 cloned isolates examined, only thirteen

Table 2. Reactivity of P13 and P310 cloned isolates with CAV MAb[a] panel

Group	Number investigated	Number reacting strongly at screening dilution				
		Direct conjugate	3B1 1:10 000	1H1 1:4 000	4H4 1:2 000	2A9 1:10 000
P13 cloned isolates	6	6	6	6	6	6
P310 cloned isolates	30	30	30	30	13	6

[a]MAbs 4H4 and 2A9 react with conformational epitopes on VP1, the CAV structural protein, whereas MAbs 1H1 and 3B1 react with linear epitopes on VP1 and VP3, a non-structural protein, respectively

(43%) of the thirty P310 cloned isolates reacted strongly with MAb 4H4 at the screening dilution and only six (20%) reacted strongly with MAb 2A9. With those P310 cloned isolates that failed to react strongly with MAbs 4H4 and2A9 at their screening dilutions, very weak or undetectable fluorescent staining was observed. This observed reduction in IIF reactivity with MAbs 4H4 and 2A9, associated with the majority of P310 cloned isolates, was further investigated. The results obtained by reacting four P13 and ten P310 cloned isolates with varying dilutions of the 4H4 and 2A9 MAbs indicated that, whereas all four P13 cloned isolates reacted strongly with both MAbs even at high dilution (1:40,000 or $\geq$ 1:80,000), the P310 cloned isolates behaved heterogeneously regarding their MAb reactivities (Table 3). Only three out of the ten P310 cloned isolates examined exhibited detectable staining with MAb 2Ab 2A9 at a 1:80 000 dilution or greater. Similarly, only one of the P310 cloned isolates examined reacted strongly with MAb 4H4 at or in excess of a 1:80,000 dilution. The remaining P310 cloned isolates were found to have reactivity endpoints for MAb 4H4 ranging from dilutions of 1:100 to 1:20,000. It was noted that of the three P310 cloned isolates that reacted strongly with MAb 2A9, only 1 exhibited strong reactivity with MAb 4H4.

Susceptibility of P310 cloned isolates to neutralisation

The ability of MAb 2A9 to neutralise the infectivity possessed by four selected P310 cloned isolates was investigated. The results indicated that cloned isolates 4 and 28, which reacted strongly with MAb 2A9 as determined by IIF, could be neutralised by high dilutions (1:256,000 and 1:512,000 respectively) of this MAb, whereas the weakly-reacting cloned isolates 27 and 31 by comparison required lower dilutions (1:4,000) of MAb 2A9 for neutralisation (Table 3). All four P310 cloned isolates were neutralised by high dilutions (1:2,048–1:16,384) of a convalescent antiserum obtained from SPF chicks experimentally infected with a low-passage Cux-1 CAV pool (Table 3). In an additional experiment, it

Table 3. IIF reactivities and virus neutralisation capabilities of CAV-specific MAbs[a] and antiserum with selected cloned isolates and P13 and P310 virus pools

Cloned isolate/ virus pools	Reciprocal of dilution at which MAbs produce detectable IF staining		Reciprocal of dilution at which MAb 2A9 and experimental chicken serum neutralise virus infectivity	
	2A9	4H4	2A9	Experimental chicken serum
P13 cloned isolate 1	≥80,000	40,000	nt	nt
P13 cloned isolate 2	≥80,000	40,000	nt	nt
P13 cloned isolate 3	≥80,000	≥80,000	nt	nt
P13 cloned isolate 4	≥80,000	≥80,000	nt	nt
P310 cloned isolate 7	1,000	1,000	nt	nt
P310 cloned isolate 18	10,000	10,000	nt	nt
P310 cloned isolate 19	10,000	1,000	nt	nt
P310 cloned isolate 26	1,000	10,000	nt	nt
P310 cloned isolate 33	≥80,000	1,000	nt	nt
P310 cloned isolate 34	1,000	1 000	nt	nt
P310 cloned isolate 4	≥80,000	20,000	256,000	2,048
P310 cloned isolate 28	≥80,000	≥80,000	512,000	16,384
P310 cloned isolate 27	1,000	1,000	4,000	4,096
P310 cloned isolate 31	1,000	100	4,000	8,192
P310 virus pool	≥80,000	40,000	2,000	nt
P13 virus pool	≥80,000	≥80,000	512,000	nt

nt Not tested

[a]MAbs 4H4 and 2A9 react with conformational epitopes on VP1, the CAV structural protein

was shown that the P13 virus pool could be neutralised by considerably higher dilutions of the 2A9 MAb than the P310 virus pool (Table 3).

Pathogenicities of P310 cloned virus isolates

The pathogenicities of 9 selected P310 cloned isolates were evaluated by inoculating 1-day-old SPF chicks in 2 separate experiments (A and B, Table 4). Eight of the nine P310 cloned isolates exhibited lower levels of pathogenicity than the P13 virus pool, which was used as the positive pathogenic control. This reduction in pathogenicity was evidenced by lower numbers of birds with anaemia, higher mean PCVs and reduced clinical scores. In experiment A, in which the pathogenic P13 control virus was found to exhibit moderate levels of pathogenicity, cloned isolates 18 and 26 appeared to be the least pathogenic in that none of the chicks experimentally infected with these isolates became anaemic. Also of note is the behaviour demonstrated by cloned isolate 34 which produced anaemia in one chick from the 12 infected and a very low clinical score in an experiment in which infection with the pathogenic P13 control virus produced anaemia in 100% of the inoculated chicks and a relatively high clinical score of 2.75. In contrast, P310 cloned isolate 33 appeared to be more pathogenic than the P13 virus pool.

Table 4. Evaluation of pathogenicity of cloned virus isolates

Experiment	Group	Titre of inoculum ($TCID_{50}$/0.1ml)	Mean PCV ±standard deviation	Number anaemic (%)[a]	Thymic atrophy	Paleness of bone marrow	Clinical score (mean)
A	Path control (P13)	$10^{6.75}$	24.33±8.36	6/12 (50%)	3×1+ 1×2+	3×1+ 3×2+	1.16
A	Uninoculated	–	32.25±1.76	0/12 (0%)	– –	– –	0.00
A	P310 Cl 18	$10^{7.25}$	30.75±3.36	1/12 (8%)	1×1+ –	3×1+ –	0.33
A	P310 Cl 26	$10^{7.00}$	31.00±2.73	0/12 (0%)	– –	3×1+ –	0.25
A	P310 Cl 27	$10^{7.00}$	28.75±4.11	3/12 (25%)	1×1+ –	3×1+ –	0.33
A	P310 Cl 28	$10^{7.00}$	28.17±5.49	2/12 (17%)	2×1+ 1×2+	2×1+ 2×2+	0.83
A	P310 Cl 31	$10^{7.00}$	30.09±3.24	2/11 (18%)	– –	3×1+ –	0.25
A	P310 Cl 33	$10^{7.25}$	23.08±7.25	8/12 (67%)	4×1+ 2×2+	8×1+ 3×2+	1.83
B	Path control (P13)	$10^{6.75}$	17.42±6.32	10/12 (83%)	3×1+ 5×2+	2×1+ 9×2+	2.75
B	Uninoculated	–	33.75±1.82	0/12 (0%)	– –	– –	0.00
B	P310 Cl 4	$10^{6.50}$	30.25±3.11	1/12 (8%)	1×1+ –	3×1+ 1×2+	0.50
B	P310 Cl 19	$10^{6.75}$	27.54±6.83	4/13 (31%)	1×1+ –	4×1+ 3×2+	0.92
B	P310 Cl 34	$10^{6.25}$	30.50±2.28	1/12 (8%)	– –	1×1+ –	0.08

[a] Anaemia is defined as a PCV value lower than 27

Antibody development following infection with P310 cloned isolates

Testing by IIF indicated that chicks that had been experimentally infected at 1-day-old with P310 cloned isolates (numbers 27, 28, 31 and 33) possessed CAV-specific antibody at 4 weeks p.i. Examination of the end-point dilutions at which IIF staining was detected showed that there were no substantial differences in the levels of CAV-specific antibody induced irrespective of the antigenic or pathogenic characteristics of the cloned isolates (data not shown). Pooled serum samples separately collected from groups of birds infected with each of the four P310 cloned isolates were found to have neutralisation titres of 1:1,024 or 1:2,048 when tested against the P13 virus pool, indicating that the P310 cloned isolates which reacted either weakly or strongly with MAb 2A9, induced similar levels of virus neutralising antibody.

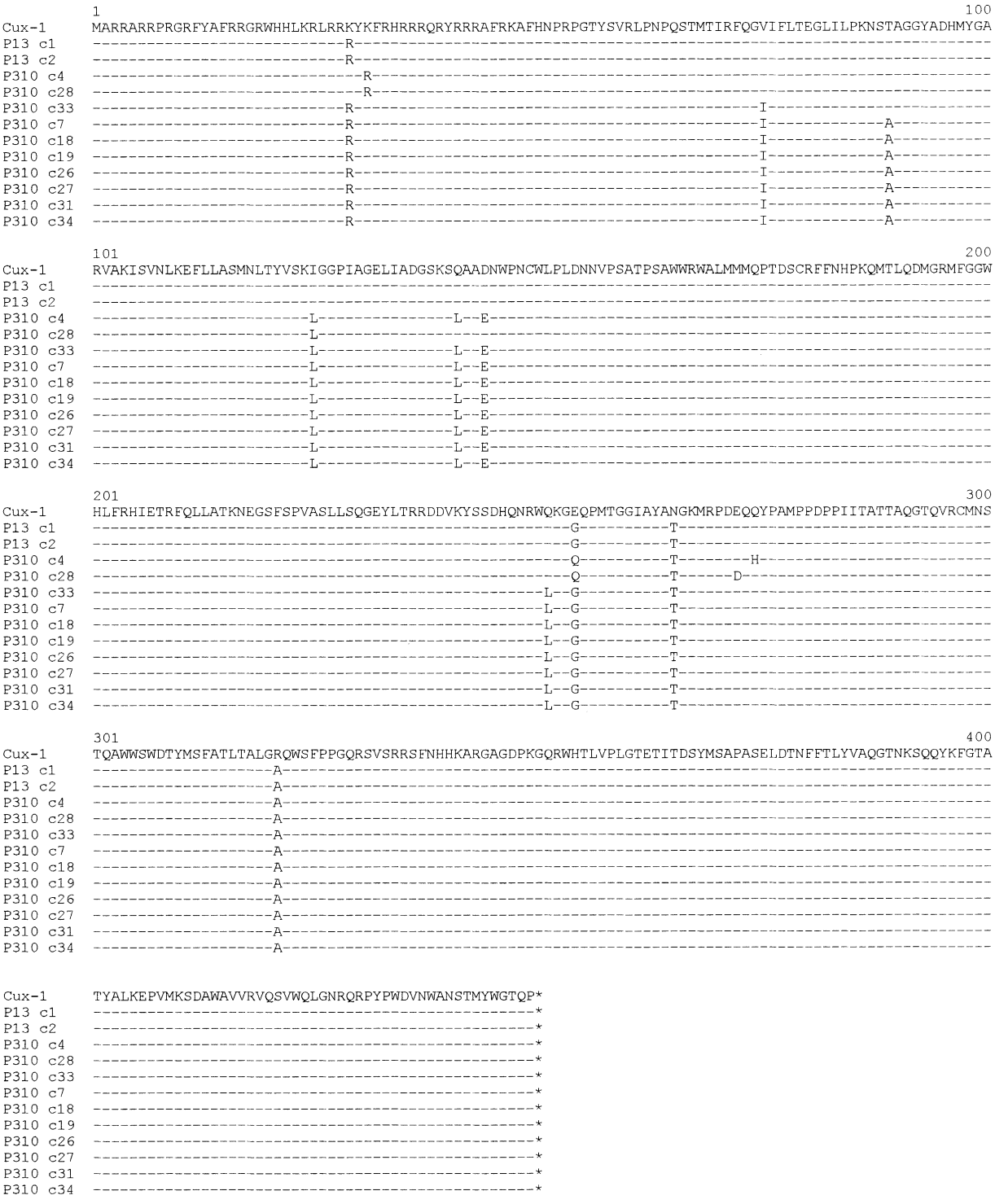

Fig. 1. VP1 amino acid sequences

CAV VP1 protein sequences

The RFs specifying ten P310 and two P13 cloned isolates were partially sequenced and the translated protein sequences of the VP1 ORFs deduced (Fig. 1). Comparisons indicated that the sequences of the P310 and P13 cloned isolates differed at 13 amino acid positions from that of the low-passage Cux-1 cloned isolate, cloned and sequenced previously in our laboratory [14]. When the sequences of the P310 and P13 cloned isolates were compared, amino acid differences were observed at 10 positions. Of these, the 6 differences at amino acid positions 75, 89, 125, 141, 144 and 251 occurred in at least 7 of the P310 cloned isolates suggesting that these changes were possessed by the predominant subpopulation within the P310 virus pool. In contrast, the differences at positions 31, 254, 272 and 274 occurred in either cloned isolate 4 and/or cloned isolate 28 only. It was noted that the P310 cloned isolates (numbers 7, 18, 19, 26, 27, 31 and 34), that have reduced MAb 2A9 reactivity, possessed all 6 amino acid changes shared by the predominant subpopulation. On the basis that the P310 cloned isolate 33 reacts strongly with MAb 2A9 and contained 5 of the 6 amino acid changes, it would appear that the amino acid change (threonine to alanine) at position 89 is crucial if reactivity with MAb 2A9 is to be reduced. The only P310 cloned isolate (number 28) tested that exhibited high reactivity with MAb 4H4 possessed only one (position 125) of the 6 amino acid changes possessed by the predominant subpopulation.

Discussion

Previous work in our laboratory showed that CAV pools that had received multiple cell culture passage became increasingly reduced in pathogenicity [20, 21]. In the present paper we confirm that CAV (Cux-1 isolate) that has been passaged 310 times in MDCC-MSB1 cells is substantially attenuated and report that this highly-passaged virus pool is biologically diverse with regard to the antigenicity and pathogenicity of the virus isolates that comprise the pool. We also report that multiple cell culture passage selects 6 capsid protein amino acid changes which affect the reactivity of the Cux-1 isolate of CAV with virus neutralising MAbs.

We have used molecular cloning of the CAV RF DNA in conjunction with transfection procedures to select individual isolates from the P310 virus pool. This approach was chosen because the cell culture growth characteristics of CAV prevent conventional virus selection procedures such as plaque purification from being successfully used. Our finding that all 30 of the P310 cloned RFs selected produced infectious virus following transfection of MDCC-MSB1 cells suggests that the proportion of defective genomes within the 2.3 kbp RF population is relatively small. However, we cannot rule out the possibility that smaller defective RFs, with electrophoretic mobilities that were too great to be size fractionated as 2.3 kbp, are not being maintained in the highly-passaged population through the production of defective particles. Should high levels of defective particles exist within the P310 virus pool it is possible that they could contribute to the attenuation possessed by this highly-passaged pool. Although working pools of all six P13 and

11 of twelve P310 cloned isolates possessed similar high infectivity titres ($10^{6.25}$ – $10^{7.25}$ $TCID_{50}$/0.1ml), it was evident from contemporaneous experiments that the P310 cloned isolates produced visible cpe in MDCC-MSB1 cells at least one passage earlier than could be detected with P13 cloned isolates. This observation is consistent with the view that viruses within the P310 virus pool are better adapted for growth in MDCC-MSB1 cells than their P13 counterparts. The existence of the exceptional isolate, P310 cloned isolate 7, which attained an infectivity titre of $10^{5.25}$ $TCID_{50}$/0.1 ml and required 4 additional cell culture passages to produce a small working pool compared to other P310 cloned isolates, provided evidence that less well adapted isolates can also be maintained within the P310 virus pool.

All 6 of the P13 cloned isolates examined reacted strongly with each of the 4 CAV-specific MAbs at their pre-determined screening dilution. This was not unexpected since these MAbs were raised by immunising mice with a pool of the Cux-1 isolate of CAV, similar in passage number to P13. The thirty P310 cloned isolates tested also reacted strongly with MAbs 3B1 and 1H1 at their screening dilutions. The 3B1 MAb is directed against the viral VP3 or apoptin [24], while MAb 1H1 is directed against an epitope present on the capsid protein, VP1 [24]. The ability of these two MAbs to react with their respective proteins after Western blotting suggests that they recognise linear epitopes [24]. Neither MAb 3B1 nor 1H1 exhibits neutralising activity for CAV [11]. In contrast to the results obtained with the 3B1 and 1H1 MAbs, 80% and 57% of the P310 cloned isolates reacted weakly with MAbs 2A9 and 4H4 respectively (Table 2), both of which, on the basis of their lack of Western blot reactivity, are thought to react with conformational epitopes on the viral capsid (Todd; unpubl. res.). We have previously shown that MAb 2A9 possesses high levels of neutralising activity against a range of geographically different isolates whereas MAb 4H4 possesses relatively low levels of neutralising activity directed against the Cux-1 isolate only [11]. More recently, we have shown using IIF that MAb 2A9 reacts with all the geographically different CAV isolates that we have tested, whereas MAb 4H4 reacts with a very limited number of CAV isolates including Cux-1 (unpubl. res.). These results are consistent with those described by Snyder et al. [17], who reported the characterisation of virus-neutralising, CAV- specific MAbs, that react with group and strain specific viral epitopes.

It is probable that these neutralising MAbs react with VP1, encoded by the longest CAV ORF, since this is the only protein that can be detected in highly-purified particles [22]. Although, it was previously reported that small amounts of VP2 may be found in virus preparations [1], it is now thought that VP2 is a non-structural protein which may act as a scaffold protein in virion assembly [16]. The translated sequences of the VP1 ORFs possessed by selected P310 and P13 cloned isolates were determined in an attempt to identify which amino acid changes influenced MAb reactivity (Fig. 1). Comparisons indicated that the majority of the P310 cloned isolates differed from their low-passage counterparts at 6 amino acid positions (positions 75, 89, 125, 141, 144 and 251) suggesting that these changes were selected by multiple passages in MDCC-MSB1 cells. Of

these, the threonine to alanine change at position 89 appeared to be crucial for the observed reduction in reactivity with MAb 2A9. However, additional research involving in vitro mutagenesis would be required to demonstrate that the change at position 89 is solely responsible for reducing MAb 2A9 reactivity or whether one or more of the additional 5 amino acid changes is also required. Since P310 cloned isolate 28, which reacts strongly with MAb 4H4, possessed only 1 (position 125) of the 6 predominant amino acid changes, it is also difficult to predict which specific amino acid changes contribute to the altered reactivities with this MAb. However, the close sequence similarity between the strongly-reacting P310 cloned isolate 28 and P310 cloned isolate, 4, which exhibits reduced MAb 4H4 reactivity, make it likely that the changes at positions 141 and 144 have some effect. The fact that the majority of the virus variants comprising the P310 virus population exhibited altered reactivity with MAbs, that are known to react with conformational epitopes, suggests that viruses which possess capsid proteins with altered conformations are selected by multiple cell culture passages. A change in capsid protein conformation that would result in more efficient binding to the receptor present on MDCC-MSB1 cells provides one possible explanation for such a selection. It would be of interest, in some future investigation, to determine whether the capsid protein changes selected by multiple cell culture passages and possessed by representative P310 cloned isolates result in altered reactivities with the MAbs previously characterised by Snyder et al. [17].

Results from the virus neutralisation experiments showed that cloned isolates that react weakly with MAb 2A9 by IIF, were, not unexpectedly, poorly neutralised by this MAb. Also our finding that the P310 virus pool, as a whole, is much less well neutralised by MAb 2A9 than the P13 virus pool is consistent with our observation that the majority of isolates that comprise the P310 population react weakly with this MAb (Table 2). However, the fact that the P310 and P13 virus pools were neutralised to similar degrees by an antiserum derived from chicks, experimentally infected using a low-passage CAV, indicates that the epitope with which MAb 2A9 reacts is not the only epitope capable of eliciting neutralising antibody. The finding that P310 cloned isolates 27 and 31, which react weakly with MAb 2A9, induced similar levels of neutralising antibody as P310 cloned isolates 28 and 33, which react strongly with MAb 2A9, provides further evidence that this is the case. Our observation, that individual virus isolates from within the P310 virus pool varied with regard to their susceptibility to neutralisation by the MAb 2A9, has alerted us to the possibility that virus isolates that are non-reactive with MAb 2A9 may also exist and that these "monoclonal antibody escape mutants" could be selected by growing the P310 virus pool in the presence of this neutralising MAb. With other viruses, such as rabies, such mutants have been identified as having reduced pathogenicity [3].

The pathogenicity evaluations were determined in 2 separate experiments (Table 4 A and B) and showed that the majority of the P310 cloned isolates were attenuated to some degree when compared to the low-passage P13 virus pool used as the pathogenic control. It was noted, however, that the P13 virus pool exhibited different degrees of pathogenicity , in terms of the proportion of anaemic chicks

and clinical score, in each of the 2 experiments. Similar differences in susceptibility to CAV-induced disease were reported in an earlier paper form our laboratory [20]. In that study we speculated that, in the absence of protective, maternally derived antibody, genetic differences between the parental flocks, from which the chicks were supplied, may have been responsible for the differences in disease susceptibility [20]. Therefore, additional experiments, involving greater numbers of chicks, will be required to determine whether cloned isolates 18 and 26, which exhibited very low levels of pathogenicity in experiment A, together with cloned isolate 34, which exhibited similar low levels in experiment B, represent very attenuated isolates that are worthy of future evaluation as live vaccines. This investigation also showed that highly pathogenic variants, such as cloned isolate 33 exist within the P310 virus pool. Our finding that experimental infections of 1-day-old SPF chicks with selected P310 cloned isolates (numbers 27, 28, 31 and 33) induced similar levels of CAV antibody, as determined by IIF, indicated that virus isolates which varied with regards to their MAb reactivities and with regards to their pathogenicities (Tables 3 and 4), were capable of replicating within chicks.

The existence of highly pathogenic variants within its mixed population, and the possibility that such variants may be selected when the virus pool is grown in chicks, makes it highly unlikely that the P310 virus pool would constitute the basis for the development of a live vaccine. Rather, despite our previous results with cloned isolate 10 [20] in which reversion was observed, it is more likely that highly attenuated cloned isolates may be suitable for this purpose. Although it was noted that the cloned isolates, for example numbers 18, 26 and 34, exhibiting greatest attenuation reacted weakly with MAb 2A9, whilst the highly pathogenic cloned isolate 33 reacted strongly with this MAb, it is too early to speculate regarding a possible relationship between the reduction in pathogenicity and the observed reduction in MAb 2A9 reactivity. It is noted, for example, that P310 cloned isolate 4, which reacts strongly with MAb 2A9, exhibited substantial attenuation (Table 4). Earlier work has demonstrated that the attenuation exhibited by the highly-attenuated cloned isolate 10, molecularly cloned from a Cux-1 pool that had been passaged 173 times, was due to genetic changes that occurred throughout its genome [13]. This finding and the finding that cloned isolate 10 has been shown to react strongly with MAb 2A9 (unpubl. res.) indicate that other factors besides the amino acid changes that result in alterations in MAb reactivity are involved in reducing the pathogenicity of the isolates that comprise the P310 pool. Interestingly, the capsid protein (VP1) of the pathogenic cloned isolate 33 and those of the most attenuated P310 cloned isolates (for example number 34) differ only at amino acid position 89, which, we believe, may play a key role in dictating MAb 2A9 reactivity. Additional sequencing, which was not within the remit of this investigation, will be required to assess the extent of the sequence variation that may exist between the pathogenic P310 cloned isolate 33 and the attenuated cloned isolates before the significance of this amino acid change to pathogenicty can be determined. Since all field isolates so far tested react with MAb 2A9 (unpubl. res.) and are pathogenic, it is possible that the capsid

conformation required to maintain a MAb 2A9-reactive epitope might also be important for manifestation of full pathogenicity. Therefore, we cannot rule out the possibility, that further alterations in capsid conformation such as those required to abolish reactivity with MAb 2A9 completely, would not in itself stably attenuate the virus.

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