Characterization of mAbs to chicken anemia virus and epitope mapping on its viral protein, VP1

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Three (MoCAV/F2, MoCAV/F8 and MoCAV/F11) of four mouse mAbs established against the A2/76 strain of chicken anemia virus (CAV) showed neutralization activity. Immunoprecipitation showed a band at ~50 kDa in A2/76-infected cell lysates by neutralizing mAbs, corresponding to the 50 kDa capsid protein (VP1) of CAV, and the mAbs reacted with recombinant VP1 proteins expressed in Cos7 cells. MoCAV/F2 and MoCAV/F8 neutralized the 14 CAV strains tested, whereas MoCAV/F11 did not neutralize five of the strains, indicating distinct antigenic variation amongst the strains. In blocking immunofluorescence tests with the A2/76-infected cells, binding of MoCAV/F11 was not inhibited by the other mAbs. MoCAV/F2 inhibited the binding of MoCAV/F8 to the antigens and vice versa, suggesting that the two mAbs recognized the same epitope. However, mutations were found in different parts of VP1 of the escape mutants of each mAb: EsCAV/F2 (deletion of T89+A90), EsCAV/F8 (I261T) and EsCAV/F11 (E144G). Thus, the epitopes recognized by MoCAV/F2 and MoCAV/F8 seemed to be topographically close in the VP1 structure, suggesting that VP1 has at least two different neutralizing epitopes. However, MoCAV/F8 did not react with EsCAV/F2 or EsCAV/F8, suggesting that binding of MoCAV/F8 to the epitope requires coexistence of the epitope recognized by MoCAV/F2. In addition, MoCAV/F2, with a titre of 1:12 800 to the parent strain, neutralized EsCAV/F2 and EsCAV/F8 with low titres of 32 and 152, respectively. The similarity of the reactivity of MoCAV/F2 and MoCAV/F8 to VP1 may also suggest the existence of a single epitope recognized by these mAbs.

INTRODUCTION

Chicken anemia virus (CAV), which was first isolated in Japan (Yuasa et al., 1979), is known to be ubiquitous worldwide. CAV causes a disease characterized by severe anaemia, retarded growth, intramuscular and subcutaneous haemorrhage, lymphoid depletion and increased mortality in young chickens (Taniguchi et al., 1982; Yuasa et al., 1979).

CAV is a non-enveloped virus and is classified in the genus Gyrovirus of the family Circoviridae (King et al., 2011). The viral genome consists of a negative-sense, circular, single-stranded 2.3 kb DNA genome with three partially overlapping ORFs (ORF1, ORF2 and ORF3) encoding the proteins VP1 (52 kDa), VP2 (24 kDa) and VP3 (14 kDa), respectively. VP1 is the only structural protein known to form the viral capsid (Todd et al., 1990). Although the function of the non-structural VP2 protein is unknown, it has been proposed that VP2 may act as a scaffold protein during virion assembly to facilitate the correct conformation of VP1 (Noteborn et al., 1998). The non-structural VP3 protein, also called apoptin, causes apoptosis in chicken thymocytes and chicken lymphoblastoid T-cell lines (Noteborn et al., 1994).

All CAV isolates belong to one serotype (McNulty, 1991; Yuasa & Imai, 1986) and the amino acid composition of VP1 is considered to be highly conserved, although Renshaw et al.
(1996) reported a hypervariable region within VP1 (aa 139–151) and that amino acid changes within this region influenced the rate of virus replication in cell cultures.

In a phylogenetic analysis of full-length deduced VP1 amino acid sequences, three distinct clusters were reported and a common signature amino acid profile (I/T75, L97, Q139 and Q144) could be identified only for genetic group I (Ducatez et al., 2006; Islam et al., 2002). Another major amino acid profile (V75, M97, K139 and E144) was also found (Hailemariam et al., 2008; van Santen et al., 2001). However, there is a lack of information related to the antigenicity of CAV strains belonging to these genetic groups.

There have been some reports on the production of mAbs to CAV, particularly focusing on those with neutralizing activity. McNulty et al. (1990) reported that mAbs to a Cux-1 strain showed three staining types in infected cells in immunofluorescent staining and only three of four type 1 mAbs had neutralizing activity. However, the epitopes recognized by these mAbs were not analysed. Immunofluorescent staining with the mAbs indicated antigenic differences amongst the five CAV strains tested (McNulty et al., 1990). In another study, eight mAbs were generated but lacked virus-neutralizing activity (Chandratilleke et al., 1991). Recently, one VP1-specific mAb was established by immunization of mice with truncated recombinant VP1; however, its virus-neutralizing activity was not evaluated (Lien et al., 2012). Thus, the neutralizing epitopes of CAV remain poorly understood. Scott et al. (1999) reported that most of the molecularly cloned viruses derived from Cux-1 after 310 cell culture passages showed weak reactivity to the neutralizing 2A9 mAb (McNulty et al., 1990) compared with the low-passaged cloned viruses and the amino acid at position 89 in VP1 appeared to be crucial for determining its reactivity with 2A9 mAb.

mAbs could be very useful and powerful tools for understanding the pathogenesis, isolate characterization and epidemiology or for improving CAV diagnosis. In this paper, we describe the production and characterization of CAV mAbs, and the expected epitopes recognized by neutralizing mAbs. To the best of our knowledge, this is the first report on epitope mapping of VP1 using neutralizing mAbs. Furthermore, we also genetically characterized two antigenic CAV groups that were differentiated by the mAbs.

**RESULTS**

**Establishment of hybridomas secreting antibodies to CAV**

Four hybridomas secreting CAV antibodies were established from a mouse immunized with CAV, and were designated MoCAV/F2 (IgG1), MoCAV/F8 (IgG1), MoCAV/F11 (IgG2b) and MoCAV/E6 (IgG2a). Three of the mAbs (MoCAV/F2, MoCAV/F8 and MoCAV/F11) showed neutralizing activity at titres ranging from 1:12 800 to 1:25 600 (Table 1), but MoCAV/E6 did not.

The immunofluorescent staining patterns of the A2/76-infected MSB1 cells with the mAbs could be classified into two types when observed within 36 h post-infection (p.i.), as shown in Figs 1(a) and 2. Diffused, irregularly shaped granular staining with MoCAV/F2, MoCAV/F8 and MoCAV/F11 was observed in the enlarged infected cells, whereas scattered, spherically shaped antigens of various sizes were observed in the infected cells reacted with MoCAV/E6.

Immunoprecipitation showed that MoCAV/F2, MoCAV/F8 and MoCAV/F11 precipitated a protein band of an estimated size of 50 kDa, corresponding to the VP1 (50 kDa) protein, in the infected MSB1 cell lysates (Fig. 1c); however, MoCAV/E6 did not precipitate this protein and also failed to precipitate any other viral protein. An mAb to the nucleoprotein of influenza A virus was used as a control and did not precipitate CAV proteins.

The VP1 recombinant proteins expressed in Cos7 cells using a pcDNA3.1(+) vector reacted with neutralizing mAbs as well as anti-VP1 peptide antibodies (Fig. 1d), whilst they did not react with MoCAV/E6 (data not shown).

**Viral protein expression in A2/76-infected MSB1 cells**

The kinetics of the expression of viral antigens was examined using three neutralizing mAbs and MoCAV/E6 in infected MSB1 cells fixed at different time points (6, 12, 24, 36, 48, 60 and 72 h p.i.).

Although there was no fluorescent signal observed at 6 h p.i. (data not shown), positive immunofluorescence was observed with all of the mAbs at 12 h p.i.; however, MoCAV/E6 showed clearer and stronger fluorescence compared with the other mAbs (Fig. 1a).

The irregular-shaped small granules detected by the three neutralizing mAbs became stronger and clearer at 24 h p.i. than at 12 h p.i. and reached a maximum level at 36 h p.i., when they were distributed all over the cells. However, the staining pattern changed markedly toward misshapen fluorescent staining of various sizes at 60 (Fig. 1a) and 72 h p.i. (data not shown), which was most likely due to a cytopathic effect. Many infected cells with misshapen antigens seemed not to be intact. An anti-VP1 peptide antibody confirmed the presence of VP1 antigen in the infected cells.

The intensity of fluorescent signals detected by MoCAV/E6 peaked at 24 and 36 h p.i. MoCAV/E6 did not change its fluorescent staining pattern (scattered, spherical structures of various sizes) during the observation period, although the number of infected cells decreased and the signal became weaker at later time points of 60 (Fig. 1a) and 72 h p.i. (data not shown).

**Co-staining of A2/76-infected MSB1 cells with mAbs**

Co-staining patterns of A2/76-infected MSB1 cells with MoCAV/E6 and neutralizing mAbs were analysed with a
confocal microscope. Antigens detected by MoCAV/F11 and MoCAV/E6 were localized in the nuclei of infected cells as indicated by DAPI counterstaining (Fig. 1b). The merged image of antigens detected by both mAbs indicated that the antigen signals seemed to partially overlap. The same results were obtained in combinations of MoCAV/E6 with other mAbs (data not shown).

Blocking indirect immunofluorescent antibody test (IFAT)

Binding of fluorescein-conjugated MoCAV/F2 and MoCAV/F8 was mutually competitively blocked, whereas the F11 and E6 competitors did not block the binding of conjugated MoCAV/F2 and MoCAV/F8 (Fig. 2). However, fluorescein-conjugated MoCAV/F11 and MoCAV/E6 were not blocked by any competitor, except for homologous mAbs.

Reactivity of neutralizing mAbs to heterologous CAV strains in the virus-neutralizing test (VNT)

MoCAV/F2 and MoCAV/F8 neutralized all of the CAV strains examined (Table 2). By contrast, MoCAV/F11 could not neutralize G3/78, G5/79, G6/79, NI/77 or HY/80. Thus, the CAV strains were divided antigenically into two distinct groups based on MoCAV/F11: mAb Group 1 included CAVs recognized by MoCAV/F11 and mAb Group 2 included CAVs not recognized by this mAb.

Phylogenetic analysis

Phylogenetic analysis of full-length deduced VP1 amino acid sequences of the two mAb antigenic group strains in comparison with other strains available in GenBank resulted in three distinct clusters (Fig. 3a). The amino acid profiles in VP1 sequences of CAV strains are shown in Fig. 3(b). mAb Group 2 strains (G5/79, G6/79, NI/77 and HY/80) with the amino acid profile of I75, L97, Q139 and Q144, which were not neutralized with MoCAV/F11, fell into cluster II, and they formed a single genetic group with TR20, Arg729, 704, CAV-E and SMSC-1 with the same profile. Although the G3/78 strain had the same profile, it was not used in phylogenetic analysis as its complete sequence was not conclusively determined due to the appearance of double peaks in some positions. Only six of all the strains classified in cluster II had a different profile (I/T/V75, L/M97, N/Q139 and H/Q144).

mAb Group 1 strains (A2/76, CAE26P4, 82-2, AO/77, A1/76, G7/91, IBA/94, NI/92, G1/74 and KY/80) with the amino acid profile of V75, M97, K139 and E144, which were neutralized by MoCAV/F11, fell into clusters I (V75, M97, K139 and E/D/N144) and III (V75, M97, K139 and E144).

Antigenic and genetic characterizations of escape mutants

One escape mutant was selected for each of MoCAV/F2, MoCAV/F8 and MoCAV/F11, designated EsCAV/F2, EsCAV/F8 and EsCAV/F11, respectively.

In IFATs of the MSB1 cells infected with escape mutants, MoCAV/F11 reacted with both EsCAV/F2 and EsCAV/F8, whilst both MoCAV/F2 and MoCAV/F8 recognized only EsCAV/F11. Chicken polyclonal antibody against A2/76 reacted with all of the escape mutants (Fig. 4).

MoCAV/F8 and MoCAV/F11 did not neutralize their corresponding escape mutants, EsCAV/F8 and EsCAV/F11 (Table 1). Moreover, MoCAV/F8 did not neutralize EsCAV/F2. By contrast, MoCAV/F2 neutralized both EsCAV/F2 and EsCAV/F8 with low dilutions of 1:32 and 1:152, respectively. IFATs were also conducted using escape mutants (EsCAV/F2 and EsCAV/F8) and neither MoCAV/F2 nor MoCAV/F8 reacted with the mutants at dilutions as low as 1:10 (data not shown).

Comparison of the VP1 amino acid sequence of the parent virus (A2/76) with those of the three escape mutants revealed the deletion of threonine (T) and alanine (A) at positions 89 and 90 (T89+A90) in EsCAV/F2, a single amino acid change of isoleucine (I) to T at position 261 (I261T) in EsCAV/F8, and a glutamic acid (E) to glycine (G) change at position 144 (E144G) in EsCAV/F11 (Table 1). Additionally, the three escape mutants did not show any amino acid changes in VP2 and VP3 in comparison with the parent virus (data not shown).
Fig. 1. (a) Viral protein expression kinetics in CAV A2/76-infected MSB1 cells. Immunofluorescent antibody tests (IFATs) were conducted to detect antigens using mAbs (MoCAV/F2, MoCAV/F8, MoCAV/F11 and MoCAV/E6) and a rabbit serum anti-VP1 peptide. Mouse normal ascitic fluid was used as a negative control. Infected cells were collected at 6, 12, 24, 36, 60 and 72 h post-infection.

(b) Neutralizing epitope mapping on VP1 of CAV A2/76.

(c) Western blot analysis showing the expression of VP1 and other viral proteins.

(d) Fluorescence microscopy images of VP1-expressing cells and mock cells.
The titre of EsCAV/F2 was reduced by 1.75 log compared with that of the parent A2/76 strain in MSB1 cells (Table 1).

DISCUSSION

Three mAbs (MoCAV/F2, MoCAV/F8 and MoCAV/F11) possessed neutralizing activity with A2/76 and were directed against VP1 in immunoprecipitation analysis, whilst MoCAV/E6 did not show neutralizing activity and did not immunoprecipitate any of the VPs (Fig. 1c). This result was confirmed using Cos7 cells with the VP1 recombinant proteins (Fig. 1d), whilst MoCAV/E6 did not react with the recombinant proteins (data not shown). Moreover, MoCAV/E6 showed a different staining pattern from the other mAbs, as shown in Figs 1(a) and 2. A co-staining study showed partial co-localizations of the antigens detected by MoCAV/E6 and VP1 neutralizing p.i., and used as antigens. Bar, 10 μm. (b) Co-staining of A2/76-infected MSB1 cells with mAbs. The infected cells were stained with a neutralizing mAb (MoCAV/F11) and a non-neutralizing mAb (MoCAV/E6), and then with IgG subclass-specific secondary antibodies labelled with Rhodamine for MoCAV/F11 and with FITC for MoCAV/E6. Infected cells collected at 36 h p.i. were used as antigens. Cell nuclei were counterstained with DAPI. The fluorescent signals were observed under a confocal microscope. Bar, 10 μm. (c) Immunoprecipitation analysis of A2/76-infected MSB1 cells. The infected or uninfected cell lysates collected at 48 h p.i. were biotin-labelled and immunoprecipitated with mAbs against CAV and against influenza A virus nucleoprotein (NP). The immunoprecipitated samples were analysed by SDS-PAGE and then the biotin-labelled proteins were transferred from a gel to a nitrocellulose membrane. Biotin-labelled viral proteins were detected by a streptavidin–HRP conjugate and visualized with chemiluminescent substrate. M, molecular mass standard. F2 (MoCAV/F2), F8 (MoCAV/F8), F11 (MoCAV/F11), E6 (MoCAV/E6) and nucleoprotein (negative control) were used to immunoprecipitate viral proteins in infected cells; F2 MSB1, F8 MSB1, F11 MSB1, E6 MSB1 and NP MSB1 indicate the treatment of uninfected cells with mAbs described above. (d) Reactivity of neutralizing mAbs with recombinant VP1 proteins expressed in Cos7 cells. IFATs were conducted using the VP1-expressing cells with mAbs (MoCAV/F2, MoCAV/F8 and MoCAV/F11) and anti-VP1 peptide antibody. Mouse normal ascitic fluid was used as a negative control. Mock cells that were transfected with pcDNA3.1(+) vector were also used as negative control. Bar, 10 μm.
Table 2. VNT with mAbs against various CAV strains

The VNT was performed using the $\alpha$-neutralization procedure described in Methods.

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mAbs (Fig. 1b). However, the time-course staining pattern of MoCAV/E6 was different from that of VP1 mAbs (Fig. 1a). Thus, MoCAV/E6 seemed not to recognize VP1, although further study should be performed to define the target protein of MoCAV/E6.

Douglas et al. (1995) reported that VP1 antigens in infected MSB1 cells were not detected using a VP1 non-neutralizing mAb (1H1) until 30 h p.i., although VP2 and VP3 antigens were detectable as early as 12 h p.i. By contrast, the present study showed that VP1 neutralizing mAbs could detect antigens as early as 12 h p.i., indicating that VP1 is produced early (Fig. 1a). The reason for this discrepancy between studies is unclear. Although the same MSB1 cells were used in both studies, the CAV strains (A2/76 and Cux-1) tested were different. However, as both strains do not appear to have different biological properties, the mAbs used in the experiment might have affected the results. Our mAbs showed neutralization activity, whereas mAb 1H1 lacks this activity. In addition, anti-VP1 peptide antibody results supported the early detection of VP1 antigens detected by the neutralizing mAbs (Fig. 1a).

Although several mAbs against CAV or recombinant VP1 proteins have been developed (Chandratilleke et al., 1991; Lien et al., 2012; McNulty et al., 1990), there is nonetheless a lack of important information related to the antigenicity of VP1, especially with respect to neutralizing epitopes. We identified the neutralizing epitopes on VP1. Blocking IFATs showed that the binding of MoCAV/F2 and MoCAV/F8 to the A2/76-infected cells was blocked by mutual competition between the mAbs (Fig. 2). Moreover, the two mutants (EsCAV/F2 and EsCAV/F8) did not react with either MoCAV/F2 or MoCAV/F8 (Fig. 4). These results suggested that these mAbs recognize the same epitope on VP1. However, VP1 amino acid analysis of these mutants revealed two different mutations in the epitopes: the deletion of T89+A90 in EsCAV/F2 VP1 and I261T in EsCAV/F8 VP1 (Table 1). Kaverin et al. (2002) revealed that the antigenic sites on the haemagglutinin molecule of the H5 subtype avian influenza virus have distinct amino acid sequences but are topographically close in the three-dimensional structure and partially overlap in reaction with mAbs. Therefore, it is likely that the antigenic sites, including T89+A90 and I261, are topographically close in the VP1 structure.

Scott et al. (2001) reported a variant virus, P310 2A9-resist, that resists neutralization by mAb 2A9 (McNulty et al., 1990), which was selected from a Cux-1 strain that had been passaged 310 times in MSB1 cells. Fluorescent VP1 antigens were not detectable by the mAb in the cells infected with P310 2A9-resist virus, even at low antibody dilutions (1:100), whereas the low-passage virus produced positive staining at high dilutions ($\geq$1:80 000). The authors suggested that the amino acid change at position 89 of VP1 was a key determinant of mAb 2A9 reactivity, because P310 2A9-resist virus, which has A89 instead of T89, produced no immunofluorescence (1:100). Although mAb 2A9 neutralized the P310 2A9-resist virus at very low dilutions (1:5), whether the mAb could react with antigens at lower dilutions than 1:100 in IFATs was not evaluated. In this study, a similar phenomenon was observed (Table 1). Although MoCAV/F2 did not neutralize EsCAV/F2 at low dilutions (1:100), unexpectedly, it was neutralized at even lower dilutions (1:32). Thus, these results suggest that the amino acid change of T89A in VP1 is not necessarily a key determinant of MoCAV/F2 reactivity, as EsCAV/F2 lacks T89+A90. These results raise questions as to why MoCAV/F2 could neutralize EsCAV/F2 at a high mAb concentration (Table 1). One possible explanation for this phenomenon may be that the epitope was not mutated completely; therefore, the corresponding mAb was still able to bind to it, but with weaker affinity. However, MoCAV/F2 also neutralized EsCAV/F8, which possesses T89+A90, at a high mAb concentration. Although the reason for this phenomenon is unclear, the results suggest that complete binding of MoCAV/F2 to the epitope might require the coexistence of an antigenic site including I261, which is recognized by MoCAV/F8. However, we could not explain why MoCAV/F2 did not recognize the antigens in the infected cells with EsCAV/F2 even when using very low dilutions (e.g. 1:10), which could be due to the low sensitivity of IFAT.

MoCAV/F8 could not only neutralize EsCAV/F8 but also EsCAV/F2 (Table 1). This unexpected phenomenon may indicate that the binding of MoCAV/F8 to the epitope requires coexistence of the epitope recognized by MoCAV/F2.

Kaverin et al. (2007) reported that some mAbs to the haemagglutinin molecule of the H5 subtype avian influenza virus have the ability to overlap two distinct antigenic sites.
Similarly, MoCAV/F2 and MoCAV/F8 might overlap two different antigenic sites that are close to each other in the three-dimensional VP1 structure; therefore, any change at the mAb-binding VP1 site could result in dramatic reduction or loss of mAb reactivity. However, the possibility of a single epitope recognized by these mAbs cannot be denied considering the similar reactivity of these VP1 mAbs, although the escape mutants of MoCAV/F2 and MoCAV/F8 displayed different amino acid mutations in VP1. In this study, only one escape mutant for each neutralizing mAb was examined. More escape mutants should be examined to obtain further information about antigenic epitopes in VP1.

Wang et al. (2009) reported that the VP1 gene had undergone positive selection and eight (75, 125, 139, 141, 144, 287, 370 and 447) positively selected amino acid sites were identified. In this study, only EsCAV/F11 showed an amino acid change corresponding to one of the positively selected amino acid sites (E144G). Thus, it is likely that the escape mutants were selected by antibody selection pressure, with the exception of EsCAV/F11.

Renshaw et al. (1996) indicated that one or both of the amino acid differences at positions 139 and 144 affected the rate of replication or the spread of infection in MSB1 subline cells. In this study, the amino acid change E144G did not affect the CAV replication rate in MSB1 cells (Table 1). Therefore, both of the amino acid changes at positions 139 and 144 might be required to affect CAV replication in MSB1 cells.

The CAV strains examined were phylogenetically grouped into three clusters (Fig. 3a, b). In cluster II, corresponding to group II described by Islam et al. (2002), 26 of the 32 strains had the amino acid profile I75, L97, Q139 and Q144, including all strains of mAb Group 2 that were not neutralized by MoCAV/F11. A2/76 (V75, M97, K139 and E144 profile) lost the neutralizing epitope recognized by MoCAV/F11 owing to the amino acid change E144G (Table 1). However, this change was not observed in the mAb Group 2 strains (I75, L97, Q139 and Q144 profile), which naturally lack the epitope recognized by MoCAV/F11. MoCAV/F11 reacted with the HK1/13 strain (V75, L97, N139 and Q144 profile, cluster II) in IFATs. Thus, the amino acid change at position 144 is not necessarily associated with the loss of binding ability to MoCAV/F11. However, further studies are required to determine whether all CAV strains (I75, L97, Q139 and Q144 profile) naturally lack the antigenic site(s) recognized by MoCAV/F11.

In conclusion, we established four mAbs, three of which (MoCAV/F2, MoCAV/F8 and MoCAV/F11) had neutralizing activity and recognized the VP1 protein. Analysis of the escape mutants of the neutralizing mAbs revealed at least two neutralizing epitopes on the VP1 protein that, to the best of our knowledge, have not been reported previously. However, as the reactivity of MoCAV/F2 and MoCAV/F8 to VP1 was similar, the existence of a single epitope recognized by these mAbs cannot be denied. The CAV strains evaluated could be differentiated into two distinct antigenic groups by MoCAV/F11, which could be associated with specific amino acid profiles of VP1. Mutations in VP1 are known to affect pathogenicity in chickens or viral replication in cells. However, there is no consistent molecular biological evidence to explain the events and there are still many aspects that remain unresolved with respect to CAV biology.

METHODS

Cell culture. MDCC-MSB1 cells (MSB1 cells) were cultured in growth medium (GM) consisting of RPMI-1640 medium (Nissui Pharmaceutical) supplemented with 10% FBS and 1% Difco’s GF 21 growth factor (Wako Junyaku) in a humidified incubator with 5% CO2 at 39.5°C.

Virus. The following CAV strains were used: A1/76, A2/76, AO/77, G1/74, G3/78, G5/79, G6/79, KY/80 and N1/77 (Yuasa & Imai, 1986); HY/80, G7/91, IBA/94, N1/92 and HK1/13, which were isolated from diseased chicks infected with CAV (unpublished data); CAAS2-2 (Otaki et al., 1987); and 26P4, which was obtained from a vaccine (Intervet). CAV was propagated in MSB1 cells (Yuasa, 1983).

Viral titres were determined as described by Imai & Yuasa (1990). Briefly, 20 μl 10-fold virus dilution was added to wells of a 96-well microplate containing 200 μl MSB1 cells (2×105 cells ml-1). Four wells were used for each dilution. The inoculated cells were passaged every 3 days, in which 40 μl of the cell suspension was transferred to a new well including 200 μl GM. The negative wells were determined after eight passages. The cultures showing red colour (no cell growth) due to a cytopathic effect were regarded as CAV-positive (Yuasa, 1983). Virus titres were quantified as the TCID50 by the Behrens–Kärber method (Behrens & Kärber, 1934).

Mouse immunization and mAb production. A2/76 propagated in MSB1 cells was partially purified and concentrated as described previously (Imai et al., 1991), and then used as the inoculum in four BALB/c mice. The titre of the inoculum was ~1010 TCID50 ml-1. Each mouse was immunized with three intraperitoneal injections of 0.1 ml inoculum emulsified in Freund’s adjuvant every 3–4 weeks. One mouse showing the highest immunofluorescent titre was intravenously injected with 0.1 ml inoculum. Four days later, spleen cells were fused with P3 × 63Ag8U.1 myeloma cells in the presence of polyethylene glycol, and the fused cells were selected and cultivated in GM supplemented with hypoxanthine, aminopterin, thymidine, endothelial cell growth supplement (Becton Dickinson) and ITS (insulin–transferrin–selenium)-S supplement (Life Technologies) according to standard procedures. Antibody-positive hybridoma cells were selected by the IFAT (described below) and cloned two or three times by limiting dilution.

Ascites was obtained by intraperitoneal injection with ~107 hybridoma cells into a BALB/c mouse that had been primed with incomplete Freund’s adjuvant, as described previously (Harlow & Lane, 1988). Isotypes of mAbs were determined in an ELISA using a commercial kit (mouse mAb isotyping reagents; Sigma-Aldrich). All mouse studies were conducted in compliance with the institutional rules for the care and use of laboratory animals, and using protocols approved by the relevant committee at the institution.

Immunoprecipitation. An IgG fraction of the ascites including mAb was precipitated by 33% saturated ammonium sulphate and dialysed with PBS. Protein concentration of the semi-purified IgG mAb was determined using the Lowry method (Lowry et al., 1951).
A2/76-infected and uninfected MSB1 cells (negative control) were harvested at 48 h p.i. Immunoprecipitation was conducted according to the instructions of a commercial kit (Immunoprecipitation kit; Roche). Briefly, the cells (10^7 cells ml^-1) were lysed in 50 mM Tris/HCl (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40 and 0.5% sodium deoxycholate. The lysed cells were labelled with biotin-7-NHS.
Transfer of the proteins from the gel to a nitrocellulose membrane was conducted using a semi-dry apparatus (Biorad) and visualized with a chemiluminescent substrate using LAS-3000 (Fujifilm). A molecular mass standard with protein G–agarose (Sigma-Aldrich) and mouse IgG–agarose (Sigma-Aldrich) followed by incubation with 3 % BSA in PBS. The purified VP1 gene was ligated into a plasmid vector (Invitrogen) using a DNA Ligation Kit (EcoRI, 5' GAATTC; XhoI, 5' CTCGAG) and purified using a GeneClean II kit (MP Biomedicals). The purified VP1 gene was ligated into pCDNA3.1(+) plasmid vector (Invitrogen) using a DNA Ligation Kit (EZ-Link Sulfo-NHS-LC-Biotinylation kit; Thermo), according to manufacturer instructions. The lysates were precleared with Protein G–agarose and mouse IgG–agarose (Sigma-Aldrich) followed by incubation with mAb for at least 3 h at 4 °C. Then, 50 μl Protein G–agarose was added to the mixture and incubated for at least 3 h at 4 °C. An aliquot of 50 μl loading buffer [0.06 M Tris/HCl, pH 6.8; 10 % (w/v) glycerol; 2 % (w/v) SDS; 0.005 % (w/v) bromophenol blue] was added per complex after washing. The sample was boiled for 5 min and quenched on ice. The mAb to influenza A virus nucleoprotein (Serotec) was used as a negative control.

The samples obtained in immunoprecipitation were analysed using 17 % low bis-polyacrylamide slab gels according to Hirano (1989). Transfer of the proteins from the gel to a nitrocellulose membrane (0.22 μm pore size; Bio-Rad) was conducted using a semi-dry apparatus. Non-specific binding sites on the membrane were blocked by incubation with 3 % BSA in PBS.

### Biotin-labelled viral proteins

Biotin-labelled viral proteins were detected by a streptavidin–HRP conjugate (Sigma-Aldrich) and visualized with a chemiluminescent substrate using LAS-3000 (Fujifilm). A molecular mass standard with Precision Plus Protein WesternC Standards (Bio-Rad) was incubated with Precision StreptActin–HRP conjugate (Bio-Rad) and visualized as described above.

#### Expression of VP1 recombinant protein

The full coding gene of the VP1 protein was amplified using the following primers: CAV-VP1-F EcoRI, 5’-GGCGAGATCTGGCAAGCGCTCGAGCA-3’ and CAV-VP1-R Xhol, 5’-AATTCGAGTACGCTGCAGGCTCCCGC-3’ (italic characters show the site of restriction enzymes used). The PCR product was digested with restriction enzymes EcoRI and Xhol (Invitrogen), and purified using a GeneClean II kit (MP Biomedicals). The purified VP1 gene was ligated into a pCDNA3.1(+) plasmid vector (Invitrogen) using a DNA Ligation Kit.

### Cluster I

### Cluster II

### Cluster III
To express the VP1 protein in mammalian cells, 0.3 μg pcDNA3.1 (+) VP1 plasmid was characterized by restriction enzymes analysis (EcoRI and XhoI) and sequencing, and then purified using a EndoFree Plasmid kit (Qiagen). The constructed recombinant plasmid, pcDNA3.1 (+) VP1, was transfected into Cos7 cells cultivated in a Lab-Tek Chamber Slide (Nunc) by TransIT-LT1 Transfection Reagent (+) plasmids per well were transfected into Cos7 cells cultivated in a six-well plate and analyzed using a confocal microscope (Leica Microsystems).

To express the VP1 protein in mammalian cells, 0.3 μg pcDNA3.1 (+) VP1 plasmid per well was transfected into Cos7 cells cultivated in a Lab-Tek Chamber Slide (Nunc) by TransIT-LT1 Transfection Reagent (Mirus Bio) according to the manufacturer’s instruction. Mock cells were transfected with pcDNA3.1 (+) plasmid. At 36 h post-transfection, the cells were fixed with acetone for 10 min and used to react with mAbs in IFATs (described below).

IFAT. An IFAT using MSB1 cells was performed to detect CAV antigens or antibodies according to the method of Yuasa et al. (1985). Briefly, CAV-infected cells were smeared onto a glass microscope slide, dried and fixed with acetone for 10 min. The antigen slides were incubated with the culture supernatant of hybridoma cells, CAV mAb at ~3 μg ml⁻¹, rabbit antiserum to VP1 peptide (1 : 200) or chicken antiserum to A2/76 (1 : 40) and then with FITC-conjugated rabbit anti-mouse IgG (Rockland), goat anti-rabbit IgG (Sigma-Aldrich) or rabbit anti-chicken IgG (Rockland) at 37 °C for 30 min after washing with PBS (pH 7.4). In some experiments, a low dilution (1 : 10) of mAbs (MoCAV/F2 and MoCAV/F8) was used. Anti-VP1 peptide serum was prepared by immunizing a rabbit with a specific peptide (CWDVNWANSTMYWESQ; Qiagen). The fluorescent signal was observed under a confocal microscope (Leica Microsystems).

Co-staining of A2/76-infected cells with mAbs was conducted to examine the localization of antigens. Briefly, the antigen slides were incubated with a combination of each of two mAbs as primary antibodies at 37 °C for 30 min and then with a combination of two isotype conjugates [rabbit anti-mouse IgG1–Rhodamine (Rockland) and goat anti-mouse IgG2a–FITC (Southern Biotechnology) or goat anti-mouse IgG2b–Rhodamine (Santa Cruz Biotechnology)] after washing with PBS. DAPI (Sigma-Aldrich) was used to counterstain the cell nuclei. The localization of antigens detected by mAbs was analyzed using a confocal microscope (Leica Microsystems).

Blocking IFAT. The A2/76-infected MSB1 cells prepared as described above were reacted with mAbs at 5 (MoCAV/F2, F8 or F11) or 200 μg ml⁻¹ (MoCAV/E6) for 30 min at 37 °C. After washing with PBS, mAbs that were labelled with isotype fluorescence using a Zenon mouse IgG labelling kit (Life Technologies) were reacted for 30 min. After washing, the fluorescent signal was observed under a fluorescence microscope.

VNT. A VNT was performed according to the microtest method of Imai & Yuasa (1990). Briefly, in the α-neutralization procedure (constant mAb, diluted virus), 10-fold stepwise dilutions of CAV were mixed with mAb (1 : 100) or GM (virus control) and the mixtures were incubated overnight at 4 °C. Then, 20 μl of each mixture was inoculated to each of four wells with 200 μl MSB1 cells (2 × 10⁵ cells ml⁻¹). The inoculated cells were passaged every 3 days. The virus titre of the mixture was determined as described above, and the neutralizing index was calculated based on the differences of virus titres (logₐ TCID₅₀) between the mixtures with mAb and the virus control.

In the β-neutralization procedure (constant virus, diluted mAb), serial twofold dilutions of mAb, beginning with a 1 : 100 dilution for the A2/76 strain or with a 1 : 2 dilution for the escape mutants, were mixed with an equal amount of CAV containing 200 TCID₅₀ (0.1 ml⁻¹). The mixture was incubated overnight at 4 °C and then inoculated into the wells containing cells, followed by cell passaging as described above. End-point titres corresponding to 50% neutralization were calculated by the Behrens–Kärber method. The reciprocal of the highest dilution of mAb neutralizing 50% of CAV was taken as the antibody titre.
Selection of escape mutants. The undiluted virus stock of the A2/76 strain (10^4 TCID_{50} ml^{-1}) was mixed with mAbs (1:10). Original antibody titres of the three mAbs used are shown in Table 1. After incubation for 1.5 h at 37 °C followed by overnight incubation at 4 °C, the mixture was inoculated into seven test tubes containing MSB1 cells (2 × 10^5 cells ml^{-1}) and the inoculated cells were passaged every 3 days up to eight times. The viruses that were not neutralized, indicated by the red colour of the culture, were cloned two or three times by limiting dilutions using MSB1 cells.

DNA extraction and PCR. Viral DNA was extracted from CAV-infected MSB1 cell culture fluids using a QIAamp DNA Blood Mini kit (Qiagen).

Primers used for sequencing of full-length CAV VP1 genes were selected based on the Cux-1 sequence (GenBank accession number M55918; Noteborn et al., 1991) and are available upon request. The PCR protocol was conducted as described previously (Imai et al., 1998), except that 50 °C was used as the annealing temperature.

Sequencing and phylogenetic analysis. VP1, VP2 and VP3 genes of escape mutants of A2/76 selected by mAbs, and VP1 gene sequences of CAV strains used in this study, except A2/76, G6/79, 26P4 and CAA 82-2, were determined by direct sequencing using a BigDye Terminator v3.1 cycle sequencing kit according to the manufacturer’s instructions (Life Technologies). Nucleotide sequencing was performed using an Applied Biosystems 3500 Genetic Analyzer (Life Technologies). VP1 gene sequences of A2/76, G6/79, 26P4 and CAA 82-2, and VP2 and VP3 gene sequences of A2/76 were obtained from GenBank.

Nucleotide sequences obtained were analysed using GENETYX version 10 software (GENETYX) and compared with other available sequences using the BLAST program. The nucleotides and translated amino acid sequences were aligned with CLUSTAL_W (Thompson et al., 1994). A phylogenetic tree of the VP1 gene was reconstructed using the maximum-likelihood method based on the Poisson correction model, supported by 500 bootstrap replicates. The tree was drawn to scale, with branch lengths corresponding to the number of substitutions per site. All positions containing alignment gaps and missing data were eliminated in complete deletion (complete deletion option). Evolutionary analyses were conducted in MEGA5 software (Tamura et al., 2011).

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