Generation of Merkel Cell Polyomavirus (MCV)-Like Particles and Their Application to Detection of MCV Antibodies[†]

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The genome of a new human polyomavirus, known as Merkel cell polyomavirus (MCV), has recently been reported to be integrated within the cellular DNA of Merkel cell carcinoma (MCC), a rare human skin cancer. To investigate MCV seroprevalence in the general population, we expressed three different MCV VP1 in insect cells using recombinant baculoviruses. Viruslike particles (VLPs) were obtained with only one of the three VP1 genes. High-titer antibodies against VP1 VLPs were detected in mice immunized with MCV VLPs, and limited cross-reactivity was observed with BK polyomavirus (BKV) and lymphotropic polyomavirus (LPV). MCV antibodies were detected in 77% of the general population, with no variations according to age.

Polyomaviruses are small, nonenveloped DNA viruses, with a double-stranded circular DNA genome of ~5 kbp packaged within a capsid about 45 nm in diameter. The polyomavirus capsid is composed of three structural proteins: VP1, the major capsid protein, and VP2 and VP3, the minor capsid proteins. Twenty members of the polyomavirus family have been identified to date (24) and, with the exception of the murine pneumotropic polyomavirus and the avian polyomavirus, primary infection is generally asymptomatic. Five polyomaviruses infect humans, including the ubiquitous BK polyomavirus (BKV) and JC polyomavirus (JCV), which cause persistent and/or latent infections and the recently identified KI and WU polyomaviruses isolated from pulmonary secretions (1, 6). A new polyomavirus, the Merkel cell polyomavirus (MCV), was recently discovered in human Merkel cell carcinomas (MCC) (4). MCC is a relatively rare skin cancer in elderly or immunosuppressed patients and is one of the most lethal skin cancers (8). The annual incidence rate of this aggressive primary cutaneous neuroendocrine carcinoma in the United States was reported to be 0.44 per 100,000 inhabitants in 2001 and tripled between 1986 and 2001 (8), and this trend is continuing (7). An incidence of 0.13 cases per 100,000 was recently reported in France (15). Clonal integration of the MCV genome within the tumor genome (4) and the deletions and/or mutations observed within the T antigen gene (17) have suggested a direct oncogenic role for MCV. However, the prevalence and pathogenic-

ity of this newly discovered MCV have yet to be fully investigated.

The aim of the study was to produce MCV viruslike particles (VLPs) and to investigate the presence of MCV antibodies in the general population of Europe. MCV VLPs were obtained with only one of the three MCV VP1 strains investigated, and these VLPs were used to investigate cross-reactivity against other polyomaviruses and for the determination of the prevalence of MCV antibodies in the general European population.

MATERIALS AND METHODS

Generation of VLPs for MCV, BKV, and LPV polyomaviruses. Expression of the VP1 protein was performed using the MCC350 VP1 prototype sequence and the VP1 sequences amplified from two French MCC patients (MKT-21 and MKT-26) (EMBL FM864207 and FM864209, respectively) (21). MCC350 VP1 coding sequence was obtained by total synthesis with a codon usage adapted for expression in *Spodoptera frugiperda* cells (Geneart, Regensburg, Germany) (EMBL FN178624). The VP1 coding sequences were cloned under the control of the polyhedrin promoter between BamHI and HindIII restriction sites of the baculovirus double expression vector pFastBacDual. Recombinant baculoviruses were generated by using the Bac-to-Bac system (Invitrogen/Fisher Scientific, Illkirch, France). The production of BKV VP1 VLPs has been described previously (20), and the production of LPV VLPs was performed by expression of the LPV *Spodoptera frugiperda* codon-adapted VP1 sequence obtained by total synthesis (Geneart, EMBL FN178623). Sf21 cells, maintained in SF900II medium (Invitrogen), were infected with the different baculoviruses. VLPs were purified as described previously and the presence of VLPs was analyzed by electron microscopy (19, 20). The VLPs produced were quantified by determination of the mean number of particles observed per field (calculated from three to six micrographs).

Monoclonal and polyclonal antibodies. Sera from mice immunized with MCV MKT-21, BKV, and LPV VLPs and MCC350 VP1 were used to evaluate cross-reactivity among polyomaviruses. An anti-MCV VP1 monoclonal antibody (MAB) from a mouse immunized with MCC350 VP1 was used to detect VP1 proteins. This anti-MCC350 MAB was produced as previously described (5) and was directed against a linear cross-reactive epitope also present on BKV and JCV VP1 (data not shown).

Human serum and plasma samples. Plasma samples from 101 female students participating in a study on clearance of HPV performed at the Antwerp University,

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FIG. 1. Electron micrographs of viral structures obtained by expression of VP1 and VP1 plus VP2 genes from three MCV different strains and LPV in insect cells using recombinant baculoviruses. The preparations were applied to carbon coated grids and negatively stained with 1.5% uranyl acetate and observed at $\times 50,000$ nominal magnification with a JEOL 1010 electron microscope. Scale bars, 100 nm. Numbers: 1, 45-nm particles; 2, 30-nm particles; 3, 20-nm particles; 4, capsomers.

Antwerp, Belgium, were obtained. The Medical Ethics Board of the University of Antwerp approved the study protocol, and the participants provided informed consent for the HPV study. Serum samples from 194 healthy adult blood donors were obtained from the Blood Center and Clinical Analysis Laboratory of the city hospital of Ferrara, Italy, using a protocol approved by the local ethics committee. Consent from participants was not requested for BKV and MCV testing, and samples were therefore deidentified and analyzed anonymously.

Detection of anti-MCV, anti-BKV and anti-LPV antibodies by ELISA. Enzyme-linked immunosorbent assays (ELISAs) were performed as described previously (19). The MCV, BKV, and LPV antigen concentrations were determined by using mouse immune sera, and antigen saturation for all VLP preparations was reached using 200 ng of VLPs. Microtiter plates were therefore coated with 200 ng of MCV, BKV, or LPV VLPs per well. Sera were diluted 1:100 and peroxidase-conjugated goat anti-mouse immunoglobulin Fc (Sigma Aldrich) diluted 1:5,000 or peroxidase-conjugated anti-human IgG (Southern Biotech) diluted 1:10,000 was used to detect the binding of mice or human IgG, respectively. Endpoint antibody titers were determined as the last of serial 3-fold dilution that yielded a positive result. Since there is no obvious negative standard to measure lack of exposure to MCV, the cutoff value was determined by plotting the ranked net optical density (OD) individual values. A tendency curve was drawn from a second-degree polynomial regression for BKV. These representations evidenced an inflection point corresponding to 0.200 for both MCV and BKV. Thus, cutoff

values for all assays were set at 0.2, and the presented data are the means of two to three determinations. In VLP competition assays, preincubation of anti-MCV-positive sera with competition VLPs (MCV, BKV, and LPV) was done by mixing the sera diluted 1:100 with 2 μ g of VLPs in a final volume of 200 μ l.

Statistical methods. Proportional analysis between groups and correlation analysis between BKV and MCV reactivities were performed with the chi-square test and the Spearman test, respectively, using the XLStat software (Addinsoft, Paris, France).

RESULTS

Generation of VLPs for MCV, BKV, and LPV polyomaviruses. Immunoblotting with an anti-MCC350 VP1 monoclonal or polyclonal antibody as the primary antibody revealed the presence of a 40-kDa band in the nuclear fraction of insect cells infected by the three recombinant baculoviruses corresponding to the three MCV strains investigated (data not shown). The relative amounts of VP1 proteins were evaluated by Coomassie blue staining and varied from 1 for BKV to 10 for MKT-21 and MCC350 MCV strains. The self-assembly of the VP1 protein of MCV was observed only with one of three VP1 clones used (Fig. 1). A high number of VLPs, equivalent to HPV16 VLPs, were observed with the VP1 derived from the MKT-21 clone isolated from a French patient with MCC, but only protein aggregates were detected for VP1 proteins derived from clones MCC350 (4) and MKT-26 (21). VP1 proteins for BKV and LPV were detected at lower levels than MCV VP1 (1/2 to 1/4, respectively), and they self-assembled in various proportions in both large (45-nm) and small (20- to 35-nm) VLPs with the additional presence of free capsomers, as previously observed with murine polyomavirus (16).

Antibody cross-reactivity and immunogenicity of MCV VLPs using mouse monoclonal and polyclonal antibodies. The findings indicated that high titers of MCV antibodies were induced in mice immunized with MKT-21 VP1 VLPs (geometric mean titer [GMT] = 145,800) but not in mice immunized with nonassembled MCC350 VP1 protein (Table 1). This suggested that, as observed with HPV, VLPs composed of the major structural protein have the potential to induce high titers neutralizing antibody and thus may be potential components for an effective prophylactic MCV vaccine. ELISA for MCV, BKV, and LPV

TABLE 1. ELISA antibody titers against MCV, BKV, and LPV VLPs^a

Antibody (polyclonal mouse sera)	Sample no.	ELISA antibody titer		
		MCV (MKT-21)	BKV	LPV
Anti-MCV (MCC350)	1	100	100	300
Anti-MCV (MCC350)	2	300	300	300
Anti-MCV (MCC350)	3	300	300	300
Anti-MCV (MKT-21)	4	145,800	600	1,350
Anti-MCV (MKT-21)	5	145,800	200	450
Anti-MCV (MKT-21)	6	145,800	500	450
Anti-BKV	7	450	2,700	450
Anti-BKV	8	450	5,400	150
Anti-LPV	9	500	200	40,500
Nonimmunized	10	<100	<100	<100

^a Three serum samples were obtained from three mice immunized with MCC350 VP1 protein (samples 1 to 3), three from mice immunized with the MCV MKT-21 VP1 VLPs (samples 4 to 6), two from mice immunized with BKV VP1 VLPs (samples 7 and 8), and one with LPV VP1 VLPs (sample 9). Serum from a nonimmunized mouse was also investigated (sample 10).

TABLE 2. Prevalence of anti-MCV and anti-BKV antibodies observed in Belgium and Italy

Country	Age (yr)	No. of antibody-positive subjects/total no. of subjects examined (%)					
		Anti-MCV antibody			Anti-BKV antibody		
		Male	Female	Total	Male	Female	Total
Belgium	18–25		78/101 (77)	78/101 (77)		92/101 (92)	92/101 (92)
Italy	18–39	28/32 (88)	26/42 (62)	54/74 (73)	29/32 (91)	32/42 (76)	61/74 (82)
	40–64	27/30 (90)	40/50 (80)	67/80 (84)	23/30 (77)	29/50 (58)	52/80 (65)
	65–85	14/20 (70)	15/20 (75)	29/40 (73)	10/20 (50)	13/20 (65)	23/40 (58)
	Total	69/82 (84)	81/112 (72)	150/194 (77)	62/82 (76)	74/112 (66)	136/194 (70)
Total				228/295 (77)			228/295 (77)

allows testing of possible cross-reactivity between VP1 proteins. Very high levels of anti-MCV antibody titers were detected in mice immunized with MCV VLPs (GMT = 145,800). However, low antibody titers of cross-reactive antibodies were also detected against BKV VLPs (GMT = 392) and LPV VLPs (GMT = 649) representing 2.6 and 4.4% of the reactivity against MCV VLPs. Similarly, two mouse polyclonal antibodies reacted strongly against BKV (GMT of 3,818), and also against MCV (GMT = 450, 11.7%) and LPV (GMT = 260, 6.8%). Moreover, a mouse polyclonal antibody against LPV VLPs evidenced a low reactivity against MCV and BKV, representing 1.2 and 0.5% of the reactivity against LPV VLPs, respectively. Thus, relatively limited cross-reactivity with BKV and LPV was observed, although there is a 56 to 65% similarity in the VP1 amino acid sequence of these polyomaviruses.

Seroprevalence of anti-MCV and anti-BKV antibodies in adult population groups from Belgium and Italy. Anti-MCV and anti-BKV antibodies were both detected in 77% of the 295 blood samples investigated (Table 2). No statistical difference in anti-MCV antibody detection was observed according to sex or country. Likewise, no difference in MCV seroprevalence was observed with increasing age, in contrast to results observed for BKV, for which a seroprevalence of 84% was observed in 18- to 39-year-old subjects, decreasing to 58% in 65- to 85-year-old subjects ($P < 0.01$). To confirm the specificity of the MCV assay, competition studies using MCV, BKV, and LPV VLPs were conducted on eight human anti-MCV-positive sera (Table 3). Preincubation of the sera with MCV VLPs dramatically reduced the reactivity of all anti-MCV-positive sera, whereas preincubation with BKV and LPV VLPs had no effect on MCV reactivity. In addition, no positive correlation was found when we compared

the MCV IgG reactivity (OD values) and the BKV IgG reactivity (see Fig. S1 in the supplemental material). Taken together, and in agreement with animal immunization data, these findings indicate that the BKV and MCV VLP IgG responses were specific and that cross-reactivity was not a major concern.

DISCUSSION

Self-assembly of the VP1 protein of MCV was observed in only one of the three VP1 clones derived from three different MCV isolates. A high number of VLPs was observed with the VP1 derived from the MKT-21 clone isolated from a French patient with MCC, but only protein aggregates were detected for VP1 proteins derived from clone MCC-350 (4) and MKT-26 (21). It should be noted that coexpression of VP2 did not rescue assembly into VLPs (data not shown). Assembly-deficient VP1 protein was also observed by Pastrana et al. (14) using a mammalian expression system. These mutations within the VP1 sequences observed in the MCV viral genome from cancerous cells probably arose during tumorigenesis and may contribute to the immunological escape of MCV-positive cells. In addition, it was shown by Shuda et al. (17) that mutations or deletion within the T antigen observed in tumor cells can impair viral DNA replication. In agreement with this, VP1 protein is not detected in MCC cells (14), suggesting that MCC cells escape from the immunological surveillance by not producing this highly immunogenic viral component.

Amino acid mutations between MCC-350 and MKT-21 were observed at four different positions of the VP1 protein (288, 316, 366, and 422) and between MKT-26 and MKT-21 at two positions (181 and 362) (21). Although no single mutation could be identified as being responsible for the loss of ability to self-assemble into VLPs, the two most plausible explanations are that mutations within the backbone of the VP1 structure (position 181) affect its ability to self-assemble into VLPs, as has been reported for HPV-16 L1 protein (10), or that mutations at the C-terminal part of the MCV VP1 protein (positions 316 to 366) affect the size and shape of VLPs, as reported for simian virus 40 (SV40) (23). The yield of VLPs obtained with MKT-21 and MCC-339 strains and their strong immunogenicity in mice are compatible with the development of a prophylactic vaccine to prevent MCC (14; the present study). Considering the high proportion of MCV-infected adults and the high number of 5- to 10-year-old children positive for MCV antibodies (9), vaccination should be implemented during infancy or early childhood. However, the occurrence of MCC at an advanced age and the low incidence of this cancer make the industrial development of a VLP-based MCV vaccine improbable. However, this situation could be changed if

TABLE 3. MCV seroreactivity of eight anti-MCV-positive subjects after competition with polyomavirus VLPs^a

Subject	MCV seroreactivity after competition with VP1 VLPs (OD at 492 nm)			
	Mock	MCV	BKV	LPV
A	2.976	0.087	2.951	2.926
B	3.398	0.197	3.398	3.431
C	2.909	0.125	2.981	2.947
D	3.567	0.049	3.198	3.290
E	3.003	0.063	2.745	2.691
F	3.337	0.028	3.164	3.340
G	3.367	0.502	3.275	3.471
H	3.071	0.087	3.030	3.022

^a Sera were mixed at a final dilution of 1:100 with 2 µg of polyomavirus VLPs or buffer.

ongoing studies demonstrate that MCV infection is associated with others cancers or diseases.

Anti-MCV antibodies were detected in 77% of the adult populations of Belgium and Italy, and no variations were observed according to age or sex, confirming the results recently published by Kean et al. (9), Tolstov et al. (18), and Carter et al. (2). However, a higher proportion of anti-MCV subjects (75%) was observed compared to the 42 to 59% reported using capsomeres (2, 9). This difference could be attributed to differences in the prevalence of MCV in the United States and Europe or to differences in reactivity/antigenicity between capsomeres and VLPs. The low prevalence of MCV antibodies reported using MCC350 capsomeres is in agreement with our finding that this VP1 variant did not self-assemble into VLPs. Our results are more similar to the 63 to 64% recently reported by Tolstov et al. (18) in blood donors using MCC339 VLPs. MCV serology has been studied mainly in American and European populations using only two different MCV VP1 sequences. Additional studies are needed to investigate the existence of variations within the VP1 sequence on different continents and whether the VLPs derived from the different strains have different reactivity with sera of different geographical origins, as was previously shown for human papillomaviruses (13, 19), and also to investigate the possibility of the existence of serotypes as observed for BKV (11).

The seroprevalence against BKV observed in the adult populations from Belgium and Italy is consistent with previous reports (3, 9, 12). As reported by Kean et al. (9), our findings suggest that there is an age-related waning of BKV VP1 antibodies that does not occur for MCV (69.7% in subjects older than 70 years, compared to 87.3% in 21- to 50-year-old individuals). An increase in seroprevalence with age was observed for MCV antibodies, with values ranging from 20.5% in 1- to 5-year-old infants to 61.4% in adults older than 70 years. The results also confirmed that there is only low level of cross-reactivity between MCV and BKV and LPV (9, 18) and did not interfere in the detection of anti-VP1 VLP antibodies, as has been reported between BKV and SV40 (22). The low levels of cross-reactivity with other polyomaviruses were also confirmed in mice immunized with MCV VLPs.

In conclusion, the results obtained with MCV VLPs clearly suggest that they could be used as candidate antigens in serological tests and as antigenic components in prophylactic vaccines. Findings suggested that the large majority of persons who become infected with MCV do not develop MCC since MCC is a rare event, although the MCV prevalence is very high. Persistent MCV infection, sun exposure, and immunodeficiency are probably others factors that increase the risk of cancer development. However, given the high prevalence of MCV antibodies in the general population, serological support for the etiologic role of MCV in specific diseases may be difficult to establish. It appears from these preliminary studies that MCV infections have similarities to other polyomaviruses, such as benign initial infection at an early age, widespread prevalence in the population, and disease occurring only in the immunosuppressed and/or the elderly. MCV infection is very common, and the production of MCV VLPs opens up a way to perform investigations of the prevalence of MCV and to investigate its role in skin and other organ diseases and also to confirm the etiologic relationship between MCV and MCC.

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