Antibodies Against Mimotopes of Simian Virus 40 Large T Antigen, the Oncoprotein, in Serum Samples From Elderly Healthy Subjects

ELISA MAZZONI,1 GIOVANNI GUERRA,2 MARIA VITTORIA CASALI,3
SILVIA PIETROBON,1 ILARIA BONONI,1 ANDREA PUOZZO,1 ANDREA TAGLIAPIETRA,1
PIER FRANCESCO NOCINI,4 MAURO TOGNON,1* AND FERNANDA MARTINI1*

1Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, Laboratories of Cell Biology and Molecular Genetics, School of Medicine, University of Ferrara, Ferrara, Italy
2Clinical Laboratory Analysis, University Hospital, Ferrara, Italy
3General Headquarter, San Marino City Hospital, Republic of San Marino
4Department of Surgery, School of Medicine, University of Verona, Verona, Italy

Simian Virus 40 (SV40), a monkey polyomavirus, was administered to human populations by early anti-polioylitis vaccines contaminated by this small DNA tumor virus. Data on SV40 infection in humans remain controversial. Elderly subjects represent an interesting cohort to investigate, because they were not immunized with SV40-contaminated vaccines. Taking advantage of the Italian population, the second oldest worldwide, elderly subjects (n = 237) up to 100 years old were enrolled in this study. Their sera were analyzed, by ELISA tests with synthetic peptides mimicking the viral epitopes, for IgG antibodies reacting with SV40 large Tumor antigen (Tag), the viral oncoprotein. An overall seroprevalence of 22% was revealed in subjects aged 66–100 years, ranging from 19% in individuals 66–74 years old, to 24% in subjects 82–100 years old, with a lower SV40 titer detected in the oldest group. Our data show that: (i) SV40 infection is not frequent in old individuals; (ii) the infection rate increases in elderly with the age; (iii) the antibody titer of SV40 Tag decreases with the age. In conclusion, SV40 infection seems to spread in old subjects independently from SV40-contaminated vaccines. This study seems to confirm that SV40 is also a human virus.


Human Polyomaviruses (HPyV) are non-enveloped small DNA viruses with a genome of approximately 5.2 kb in size. Although differences exist among HPyVs, in general their early region encodes for the Large T (Tag/LT) and Small T (tag/ST) antigens, whereas in the late region there are three main genes encoding for structural polypeptides, the viral capsid proteins 1, 2, and 3 (VP 1-2-3). VP 2 and 3 genes partially overlap. The HPyV genome, a double strand circularly closed DNA, contains a non-coding control region (NCCR) between the early (ER) and late regions (LR) (DeCaprio and Garcea, 2013; White et al., 2013). So far, 13 HPyVs have been identified, including the recently discovered human polyomaviruses 10–13 (HPyV10-HPyV13 or NJPyV) which were detected on the stools of children and liver specimens (DeCaprio and Garcea, 2013; Korup et al., 2013; White et al., 2013; Mishra et al., 2014). These HPyVs have not been proved to be associated with human diseases, whereas BKPyV, JCPyV, Trichosyphilis spinulosa polyomavirus (TSPyV), and Merkel cell polyomavirus (MCV) are etiological agents of different viral diseases, including Merkel Cell Carcinoma (MCC) (Jiang et al., 2009). It has been reported that HPyV associated diseases may arise more frequently in immune-compromised hosts, who are often elderly subjects (Barbanti-Brodano et al., 2006).

SV40 was experimentally characterized as a transforming and oncogenic virus (Butel and Lednický, 1999; Barbanti-Brodano et al., 2004b; Martini et al., 2007). More recently, SV40 sequences (Martini et al., 1996, 2002; Butel et al., 1999a;

Mauro Tognon and Fernanda Martini authors contributed equally in this study.

Conflicts of interest: The authors declare there are no potential conflicts of interest.

Contract grant sponsor: A.S.L.E.M., Repubblica di San Marino contract 2015-3 to M.T.
Contract grant sponsor: Associazione Italiana per la Ricerca sul Cancro (AIRC);
Contract grant number: IG-16046.
Contract grant sponsor: Fondazione Buzzi UNICEM.
Contract grant sponsor: Regione Emilia-Romagna.
Contract grant sponsor: Fondazione Cassa di Risparmio di Cento.
Contract grant sponsor: University Hospital of Ferrara.
Contract grant sponsor: University of Ferrara, FAR.

*Correspondence to: Mauro Tognon or Fernanda Martini, Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, Laboratories of Cell Biology and Molecular Genetics, School of Medicine, University of Ferrara, Ferrara 44121, Italy.
E-mail: tgm@unife.it (M.T.); mrf@unife.it (F.M.)
Manuscript Received: 22 March 2016
Manuscript Accepted: 7 April 2016
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 00 Month 2016.
DOI: 10.1002/jcp.25405
Vanchiere et al., 2005a,b; Patel et al., 2008; Pancaldi et al., 2009) and SV40 antibodies (Jafar et al., 1998; Lundstig et al., 2005; Kean et al., 2009; Corallini et al., 2012; Mazzoni et al., 2012) have been detected in normal subjects of differing age, and in patients affected with different cancer types, mainly brain (Bergsagel et al., 1992; Lednicky et al., 1995; Martini et al., 1996) and bone tumors (Carbone et al., 1996; Lednicky et al., 1997; Gamberi et al., 2000; Yamamoto et al., 2000; Martini et al., 2002), different lymphoproliferative disorders (Martini et al., 1998; David et al., 2001; Shivapurkar et al., 2002; Vilchez et al., 2002; Dolcetti et al., 2003) and malignant pleural mesothelioma (Carbone et al., 1994; Galea-Salle et al., 1998; Martini et al., 2004), which are tumors of the same histotypes induced by SV40 in experimental animals (Martini et al., 2007). These studies indicate that SV40 is now circulating in humans. Due to a lack of conclusive epidemiologic surveys, the role of SV40 in human tumorigenesis is not yet fully understood. A recent WHO/IARC meeting established that SV40 is not classifiable as a carcinogenic viral agent in humans (Bouvard et al., 2012; WHO, 2013). Data from several studies, carried out mainly by PCR techniques and indirect ELISA assays, suggest that SV40 is now contagiously transmitted in humans by horizontal infection, independently from the administration of SV40-contaminated anti-polioimmunizations (Butel, 2012; Corallini et al., 2012; Tognon et al., 2016). However, other studies have not confirmed the presence of SV40 in human hosts (Barbanti-Brodano et al., 2004b). It is also possible that SV40 was already present in human populations before its introduction with the contaminated vaccines.

In order to carry out an epidemiologic survey with a novel immunologic method, we recently developed a specific, sensitive serologic assay to detect SV40 antibodies in human serum samples from normal subjects (Corallini et al., 2012) and patients affected by tumors of different histotypes (Mazzoni et al., 2012). Specific immunologic assays for identifying SV40-seropositive healthy individuals and serum antibody reactivity to SV40 antigens are of paramount importance in revealing the presence and prevalence of SV40 infection in humans. In particular, little information is available about SV40 infection in elderly healthy subjects. The presence of tumor viruses, including SV40, in older human hosts should be investigated due to their oncogenic potential. Indeed, it has been well established that elderly individuals are more prone to develop tumors than younger ones.

The aim of this study was to investigate and compare age-specific seroprevalence for SV40 antibodies and thus annotate this virus as a possible human polyomavirus. Serum samples from elderly healthy subjects were analyzed for exposure to SV40 infection with an innovative immunological test using two synthetic peptides mimic SV40 LT epitopes, they were employed as mimotopes in indirect ELISAs, (Tognon et al., 2016) similarly to what has recently been reported for SV40 VP antigens (Corallini et al., 2012; Mazzoni et al., 2012). Immuno logical data, obtained using these SV40 LT mimotopes, indicate that: (i) specific SV40 antibodies can be detected in serum samples from elderly healthy subjects; (ii) these individuals, aged between 66 and 100, showed different age cohorts prevalence; (iii) SV40 is circulating in humans, although at low prevalence and low titer, independently from SV40-contaminated vaccines.

**Materials and Methods**

**Study population**

Serum samples were collected from 273 elderly healthy individuals during 2014 and 2015 (Table 1). Sera were taken from discarded laboratory analysis samples before incineration and after routine analyses at the University Hospital, Ferrara, Italy. The hospital records annotated these serum samples as belonging to elderly healthy subjects. Indeed, parameters of blood analyses were all in the normal index range.

Sera were collected anonymously, coded with indications of age and gender only. The project was approved by the County Ethical Committee, Ferrara (Corallini et al., 2012; Mazzoni et al., 2012). Written informed consent was obtained from all participants at the time of hospital admission. All serum samples were stored at −20°C until testing.

**Simian virus 40 large T antigen mimotopes**

Computer assisted analyses allowed us to select two specific SV40 peptides from the early viral region by comparing the three LT antigens from SV40 with corresponding BKPyV and JCPyV amino acids, which are highly homologous to SV40, as well as with other, less homologous polyomaviruses (Tognon et al., 2016). Previous ELISA results indicated that the two SV40 LT peptides did not cross-react with the BKPyV and JCPyV hyperimmune sera, which were employed as controls (Tognon et al., 2016). The amino acid sequences of the two peptides, known as SV40 LT peptides A and D, respectively, are as follows:

| LT A: NH2-G S F Q A P Q S S Q S V H D H N Q P Y H I-COOH |
| LT D: NH2-H E T G I D S Q Q S G F Q A P Q S S Q S V H D-COOH |

LT A and LT D mimotopes were selected as they react specifically in indirect ELISA with the rabbit hyperimmune serum that had been experimentally immunized with SV40 Tag protein (the positive control serum; this serum sample was a generous gift from Dr. Janet S. Butel, Baylor College of Medicine, Houston, TX). BKPyV and JCPyV hyperimmune sera did not react with LT A or LT D peptides (negative control sera). The amino acid residues of the two specific SV40 LT peptides show low homology with BKPyV and JCPyV Lts (Tognon et al., 2016). The human neuropeptide S (hNPS), a.a. sequence SFRNGVGTGMKKTSFQRAKS, was employed as a negative control peptide (Guerrini et al., 2010). The synthetic peptides were synthesized using standard procedures and were purchased from UFPptides s.r.l., Ferrara, Italy (Tognon et al., 2016).

**Control immune sera**

Hyperimmune rabbit sera against SV40 LT (a generous gift from Prof. Janet Butel, Baylor College of Medicine), were obtained in rabbits that had been inoculated with purified LT protein, as previously reported (Tognon et al., 2016). Additional SV40-negative and SV40-positive human sera were taken from our collections (Corallini et al., 2012; Mazzoni et al., 2012) and employed as controls. The BKPyV hyperimmune serum was from our laboratories, whereas the JCPyV

**Table 1. Prevalence of immunoglobulin G antibodies reacting to SV40 LT mimotopes in serum samples from elderly healthy subjects**

<table>
<thead>
<tr>
<th>Age years</th>
<th>Number of sample</th>
<th>Male (%)</th>
<th>LT A</th>
<th>LTD</th>
<th>LT A + D</th>
</tr>
</thead>
<tbody>
<tr>
<td>66–74</td>
<td>106</td>
<td>63</td>
<td>25</td>
<td>25</td>
<td>20 (19)</td>
</tr>
<tr>
<td>75–81</td>
<td>80</td>
<td>40</td>
<td>22</td>
<td>23</td>
<td>19 (24)</td>
</tr>
<tr>
<td>82–100</td>
<td>87</td>
<td>27</td>
<td>21</td>
<td>24</td>
<td>21 (24)</td>
</tr>
<tr>
<td>66–100</td>
<td>273</td>
<td>49</td>
<td>70</td>
<td>26</td>
<td>60 (22)</td>
</tr>
</tbody>
</table>

Human sera were from elderly healthy subjects. Statistical analysis was performed with the Fischer’s exact test (P < 0.05).
Indirect enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was developed and standardized to detect specific antibodies against SV40 in human sera using LT A and D synthetic peptides (Tognon et al., 2016).

Peptide coating. Plates were coated with 5 μg of the selected peptide for each well and diluted in 100 μl of Coating Buffer (Candor Bioscience, Wangen, Germany) at 4°C for 16 h.

Peptide coating. Blocking was made with 200 μl/well of the Blocking Solution (Candor Bioscience) at 37°C for 90 min.

Primary antibody adding. Different wells were covered with 100 μl of serum sample, diluted with 1/20 Low Cross-Buffer (Candor Bioscience). Each plate contained positive-control to SV40, represented by hyperimmune rabbit sera with anti-SV40 LT antibodies, and three human serum samples found to be SV40-negative in our previous investigation (Corallini et al., 2012; Mazzoni et al., 2012).

Secondary antibody adding. The solution contained a goat anti-human or anti-rabbit IgG heavy and light chain specific peroxidase-conjugate (Calbiochem-Merck, Darmstadt, Germany) diluted 1:10,000 in Low Cross-Buffer (Randhawa et al., 2008).

Dye treatment and spectrophotometric reading. Samples were treated with 100 μl of 2.2-sazino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution (Sigma–Aldrich, Milan, Italy), for 45 min at RT, and then read on the spectrophotometer (Thermo Electron Corporation, model Multiskan EX, Vantaa, Finland) at a wavelength 405 nm (λ). The color intensity in wells where the immunocomplexes were formed was determined by optical density (OD).

Cut-off determination. Cut-off values were determined for each assay using the OD reading of the three negative control sera that were added to the standard deviation and multiplied three times (× 3SD). The three SV40 negative control sera were selected from those below the cut-off value determined with second-degree polynomial regression by plotting the ranked net OD individual values for each peptide. A tendency curve was drawn from a second-degree polynomial regression for Tag A and D peptides, as published before for MCPyV and BKPyV virus-like particles (VLPs) (Touze et al., 2010; Coursaget et al., 2013). Our representations revealed an inflection point at 0.18 for each peptide. Sera with antibodies against SV40 were considered Tag-positive if they reacted to both Tag peptides A and D three times in three independent ELISA tests. SV40 LT antibody titer was determined by serial dilutions of sera from 1:20 to 1:640 (Corallini et al., 2012). Statistical analyses were performed using Prism software (GraphPad, San Diego, CA). Data are presented as a percentage of the positive samples. Differences among proportions of small sample size were statistically analyzed using the Fischer’s exact test. The serologic profile of serum antibody reactivity to SV40 mimotopes was statistically analyzed using Anova and Newman–Keuls Multiple Comparison Test. P-values < 0.05 were considered statistically significant.

Results

SV40 seroprevalence in elderly healthy subjects

In our investigation, indirect ELISA was employed to test serum samples, taken from elderly healthy subjects aged 66–100 years old, which had been diluted at 1/20. The samples were tested for reactivity to two SV40 antigens of LT peptide. Serum samples, which reacted with the SV40 LT A mimotope, reached a prevalence of 24% for SV40 antibodies in the IgG class in elderly individuals aged between 66 and 74, 28% in individuals between 75 and 81 of age, and 26% in 82 and 100 year-old subjects. The overall prevalence of antibodies reacting to SV40 LT A mimotope in elderly healthy individuals aged 66–100 years old was 26% (70/273) (Table 1).

Subsequently, the same assay was employed to detect IgG class serum antibodies against SV40 LT D epitopes. It turned out that serum samples in the cohort of elderly healthy individuals, aged 66–74, reacted with the SV40 LT D peptide with a 24% prevalence. Furthermore, it increased in older subjects in the cohort of healthy subjects aged 75–81 to a 29% prevalence, while it was 28% in the cohort of aged 82–100. It is noteworthy, that the two synthetic peptides reacted with the sera under analysis with a similar prevalence level in the 3 age cohorts. Indeed, in subjects aged 66–100, the prevalence of antibodies against SV40 LT peptide D was 26% (72/273) (Table 1).

Conversely, seronegative samples for the SV40 LT A peptide failed to react with SV40 LT D epitopes. Exceptions were negligible and were represented by a few serum samples, which were found to be negative for LT A, while testing positive for LT D peptide, and vice versa. The difference was not statistically significant (P > 0.05) (Table 1).

In our study, sera were considered SV40 LT-positive when reacting with both A and D peptides. The overall prevalence both for A and D peptides by combining SV40 LT-positive sera was 22% (60/273) (Table 1;Fig. 1).

No positive results were obtained with human neuropeptide S, which was used as a control, with an OD of less than 0.1 (0.088–0.0923). This OD value is usually consistent with SV40-negative sera.

SV40 LT-positive sera tested by indirect ELISA, diluted at 1/20, had a general cut-off point in the 0.18 OD range according to the spectrophotometric reading. This cut-off point represents the value that discriminates SV40 LT-negative
(sample with OD below 0.18) from SV40-LT positive samples (OD above 0.18). The positive control, represented by the SV40 LT hyperimmune rabbit serum, had an OD of up to 2.5, whereas human negative controls, had an OD of <0.1. Six SV40 LT positive human sera from a previous investigation, with OD in the 0.28–0.30 range, were also enclosed as additional SV40-positive controls (Mazzoni et al., 2012).

A prevalence selection corresponding to 19%, 24%, and 24%, was detected in senior subjects within the cohort aged 66–74, 75–81, and 82–100 years old, respectively (Table 1; Fig. 1). However, the different prevalence of SV40 LT antibodies among the three cohorts of older individuals was not statistically significant.

The two indirect ELISA tests, with two distinct LT, A and D peptides, gave overlapping results, thus confirming the presence of antibodies against SV40 LT in human sera from elderly healthy subjects (Table 1; Fig. 1).

Serologic profile of serum antibody reactivity to SV40 LT mimotopes

The sera had a mean value of approximately 0.1723–0.1735 when diluted at 1:20 in the cohorts of subjects aged 66–74 and 75–81, while it had a mean value of approximately 0.1836 in sera from individuals aged 82–100 years old. The serologic profiles are shown in Figure 2. The difference is not statistically significant across the three distinct cohorts of older individuals (P > 0.05).

Elderly individuals serum titer

To check the antibody titer, 20 randomly chosen sera with an OD in the 0.2–0.8 range from elderly individuals found to be SV40 LT-positive for both A and D peptides were serially diluted from 1/20 to 1/640 and further investigated by indirect ELISA. The assay indicated that these sera, carrying antibodies against SV40 LT, remained positive at a 1/160 dilution, with the OD above the cut-off value of 0.18 (Fig. 3).

Discussion

The prevalence of antibodies against SV40 LT peptides A and D, stratified by age, is shown in Table 1 and Figure 1. Overall, 22% of the study population had antibodies against SV40 LT. The prevalence appeared to stabilize for subjects who were 75–81 (24%) and 82–100 (24%), whereas it increased (>20%) when compared to the under 75 age group (19%). These immunologic data indicate that the SV40 infection rate increases in the elderly with age and suggest that different infection routes may occur (Lundstig et al., 2005; Corallini et al., 2012).

For the first time in this study, SV40 LT seroprevalence was determined in elderly healthy individuals with a trend that seems lower than recently reported for other human polyomaviruses, which are widespread in humans (Kean et al., 2009; Nicol et al., 2012). The lack of similar studies in other countries has not allowed us to compare the serologic profiles for SV40 LT in our sera with samples from other populations. However, a similar level of SV40 infection prevalence has been detected in previous investigations carried out in adults from Italy and the U.S., despite being obtained with different methods. It is worth noting that the U.S. data, which were obtained using the virus plaque neutralization assay (Jafar et al., 1998; Wong et al., 2013) did not differ substantially from those in our investigation, where specific SV40 mimotopes were employed (Corallini et al., 2012; Mazzoni et al., 2012). Both techniques are SV40 specific. A lower percentage of adults who tested positive for SV40 antibodies was reported in another study where SV40 VLPs were used as antigens (Carter et al., 2003). In this...
results was assessed with three replica experiments carried out by positive sera from elderly healthy individuals does not greatly differ dilution. This result indicates that the titer of SV40 LT antibodies in carry antibodies against SV40 LT which remain positive at a 1/160 dilution. The assay indicated that these sera investigated by indirect ELISA. The prevalence of SV40-positive individuals among healthy adults, as may occur (Patel et al., 2008). The low prevalence of elderly healthy individuals in the Italian population among elderly healthy individuals in the 66–100 age range, although at low prevalence and with a low titer. The presence of SV40 antibodies in senior subjects of different ages suggests that distinct virus transmission routes may occur (Patel et al., 2008). The low prevalence of elderly individuals exposed to SV40 is in agreement with the low prevalence of SV40-positive individuals among healthy adults, as shown in previous studies (Martini et al., 1996; Jafar et al., 1998; Lundstig et al., 2005; Kean et al., 2009; Pancaldi et al., 2009; Corallini et al., 2012; Mazzoni et al., 2012; Wong et al., 2013).

Interestingly, our data indicate that the human population we studied is too old to have been infected by SV40 through the early anti-polio vaccines contaminated by this virus, and suggest that SV40 is spreading in humans independently of those vaccines. Immunological data from this study and other investigations indicate that SV40, a monkey polyomavirus, is also a human virus. Alternatively, it may be that another, as yet undiscovered, polyomavirus is infecting humans. In this case, positive immunologic data could be due to a new virus, which is closely related to SV40.

Acknowledgments

We would like to thank Dr. Eugene O. Major, the Laboratory of Molecular Medicine and Neuroscience, the National Institute of Neurological Disorders and Stroke, Bethesda, MD, for the hyperimmune serum against JCV and Prof. Janet S. Butel, Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, for the hyperimmune serum against SV40. We wish to thank the Fondazione Veronesi, Milan, Italy for their post doctoral fellowship to Dr. Elisa Mazzoni. This study was supported, in part, by grants from A.S.L.E.M., Repubblica di San Marino; Associazione Italiana per la Ricerca sul Cancro (AIRC), project IGI-16046; Fondazione Buzzi UNICEM, Casale Monferrato; Regione Emilia-Romagna, Bologna; Fondazione Cassa di Risparmio di Cento, Cento; University Hospital of Ferrara, Ferrara; University of Ferrara, FAR projects, Ferrara, Italy

Literature Cited


