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Specific antibodies reacting with simian virus 40 capsid protein mimotopes in serum samples from healthy blood donors

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ABSTRACT

Simian virus 40 (SV40), a small DNA tumor virus, was inadvertently administered to human populations with the use of contaminated vaccines. SV40 sequences have mainly been detected in healthy individuals and cancer patients using polymerase chain reaction techniques. However, some studies have failed to reveal the presence of SV40 in human specimens. These conflicting results indicate the need for new research to verify whether SV40 is circulating in humans. Mimotopes from SV40 structural peptides were tested to investigate for specific reactions to human sera antibodies. An indirect enzyme-linked immunosorbent assay with synthetic peptides from SV40 viral capsid proteins 1-2-3 (VPs 1-2-3) was set up and employed to test 855 serum samples from healthy blood donors. Data from immunologic assays indicate that serum antibodies against SV40 VP mimotopes are detectable, although with a low titer, in blood donors 18 to 65 years old. The overall prevalence of serum samples that reacted with the 2 SV40 VP peptides was 18%. The strong points for this novel method include the simplicity of its approach and the potential to discriminate between SV40-specific antibody responses and to draw correlations between responses to the 2 independent SV40 peptides. These data suggest that SV40, or a yet undetected closely related polyomavirus, is circulating in human populations, but with lower prevalence than that of the ubiquitous BK and JC human polyomaviruses.

1. Introduction

Simian virus 40 (SV40) is a small DNA tumor virus of approximately 5.2 kb. SV40 was inadvertently administered to human populations worldwide by contaminated vaccines produced in SV40 naturally infected monkey kidney cells [1,2].

Soon after its isolation, SV40 was characterized as a transforming and tumorigenic viral agent [1,2]. Cell transformation and tumorigenesis by SV40 is induced by 2 oncoproteins, the large T antigen (Tag) and the small t antigen (tag), which target key cellular proteins such as the tumor suppressor p53 and pRB family proteins, inactivating their functions [1,2].

Several studies, mainly carried out using polymerase chain reaction (PCR) techniques, have suggested that SV40 may have been contagiously transmitted in humans by horizontal infection, independent of the earlier administration of SV40-contaminated vaccines[3,4]. However, the presence of SV40 in human populations before the administration of contaminated vaccines cannot be excluded.

SV40 sequences have been detected at low prevalence in blood samples from healthy donors [5–9] by PCR techniques, suggesting that human cells are only in part permissive for its multiplication. Many investigations have detected SV40 footprints not only in hospitalized children and patients affected by tumors and other diseases, but also in normal subjects of differing ages [10–18]. However, contrasting reports have appeared in the literature regarding the presence of SV40 in humans and its association with cancers. As a consequence of these results, considerable debate has developed in the scientific community [2,4].

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A

Pep A	KGSCPGAAPKKPKEPV
Pep B	NPDEHQKGLSKSLAAEKQFTDDSP
Pep C	INQDIPRLTSQELERRTQRYLRD
Pep D	DSIQQVTERWEAQSQSPNVQSG
Pep E	KRKLSRGSSQKTKGTSASAKARHK

B

Pep VP B		Similarity* (%)	Pep VP C		Similarity* (%)
Pep B	NPDEHQKGLSKSLAAEKQFTDDSP		Pep C	IQNDIPRLTSQELERRTQRYLRD	
SV40	NPDEHQKGLSKSLAAEKQFTDDSP	100	SV40	IQNDIPRLTSQELERRTQRYLRD	100
BKV	DPDENLRGFSLKLSAENDFSSDSP	50	SA12	VRDDLPRLTSQEIERRTQRFFRD	70
JCV	DPDEHLRGFSKSISISDTFESDSP	50	BKV	IRDDIPSITSQELQRRTERFFRD	61
SA12	DANEHLRGYSQRVTCDVTFENDAP	29	JCV	VRDDLPALTSQEIQRRTQKLFVE	48
MCV	SPDLPTTSNWYTYTYDLQPKGSSP	20	KIV	YYNAGSTVVNRVLSDEIQRLLRD	30
TSV	NPHYKQKLTKDGAFPVECWCPDPS	20	HPyV6	GQYCIPQWLLFVLEELDKEIKED	26
LPV	NNIPSEDLYGYSNSINTAFSKASD	17	HPyV7	GIWTSYYRTGRELIQRTATRELA	22
KIV	NTTAAQDGREPTPHYWSISSAIHD	13	WUV	YYNTGRTVVNRAVSEELQRLLGD	22
WUV	NAEGTTPHYWSISSPLKTAEAANV	8	LPV	LYGDITPTWEVELNKLEKEEDGP	17
HPyV6	------------------------	0	HPyV9	IQLRQQYRSRGELPPTREQFEYQ	8
HPyV7	------------------------	0	MCV	------------------------	0
HPvV9	------------------------	0	TSV	------------------------	0

Fig. 1. (A) Amino acid sequences of 5 different peptides of viral proteins (VP) 1-2-3 of simian virus 40 (SV40). The peptides were selected on the basis of their low homology with the corresponding peptides from other human/simian polyomaviruses. Preliminary enzyme-linked immunosorbent assay indicated that only VP1 B and VP2/3 C peptides reacted with human serum antibodies without cross-reaction with the BKV and JCV immune-sera employed as controls. (B) Similarity between synthetic peptides specific to SV40 and other polyomavirus sequences: BKV (human polyomavirus BK), JCV (human polyomavirus JC), SA12 (simian agent virus 12), MCV (merkel cell polyomavirus), TSV (trichodysplasia spinulosa-associated polyomavirus), LPV (B-lymphotropic polyomavirus), KIV (KI polyomavirus), WUV (WU polyomavirus), HPyV 6 (human polyomavirus 6), HPyV 7 (human polyomavirus 7), and HPyV 9 (human Polyomavirus 9 (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=10624).

The problems related to SV40 infection in the human population and its contribution to human cancer have been summarized in an evaluation by the Immunization Safety Review Committee, which was established by the Institute of Medicine of the National Academies [19]. Epidemiologic studies conducted in the past were flawed by the difficulty in establishing which individuals had received contaminated vaccines, in determining the dosage of infectious SV40 present in different vaccine lots because of formalin inactivation of the poliovirus, which may have variably affected SV40 infectivity, and finally in observing large cohorts of subjects for several decades after virus exposure to monitor for cancer development. The Committee recommended the development of specific, sensitive serologic tests for SV40 and the use of standardized techniques, which should be accepted and shared by all laboratories involved in SV40 research. SV40 antibody detection had been attempted in several studies using serologic methods with SV40 antigens, but because of high protein homology among the 3 main polyomaviruses, SV40, BKV, and JCV, the results were always affected by some cross-reactivity [20–26].

Specific immunologic assays for the identification of SV40-seropositive healthy individuals and serum antibody reactivity to SV40 antigens are of paramount importance in revealing the prevalence of SV40 infection in humans.

In this investigation, serum samples from blood donors were analyzed for exposure to SV40 infection. Mimotopes [27,28] from SV40 viral proteins (VP) 1, 2, and 3 were used as synthetic peptides in an indirect enzyme-linked immunosorbent assay (ELISA), without using recombinant proteins. Computer-assisted analyses indicated that linear peptides may recognize specific antibodies elicited against linear/conformational SV40 VP mimotopes/epitopes. Immunologic data, obtained with new synthetic peptides that mimic the epitopes of the SV40 late region, indicate that specific SV40 antibodies can be detected in serum samples from healthy blood donors and that SV40 is circulating at a low prevalence in humans.

2. Subjects and methods

2.1. Human samples

A total of 855 serum samples were collected from blood donors in different Italian institutions in the 2005–2010 period. Sera were taken from discarded laboratory analysis samples. Anonymously collected sera were coded with indications of age and gender only. The project was approved by the County Ethical Committee, Ferrara, Italy. Different cohorts were homogeneously clustered by age.

2.2. Synthetic peptides

Computer-assisted analyses enabled 5 specific SV40 peptides (Fig. 1A) to be selected from the late viral region by comparing the 3 capsid proteins, VP 1-2-3 from SV40, with amino acids from human BK (BKV) and JC (JCV) polyomaviruses, which are highly homologous to SV40, as well as with other less homologous polyomaviruses (http://blast.ncbi.nlm.nih.gov; Figs. 1B and 2). Preliminary ELISA results indicated that only 2 of 5 peptides did not cross-react with the BKV and JCV hyperimmune sera employed. These 2 peptides belong to the viral capsid proteins VP1/VP2/VP3 (http://www.ncbi.nlm.nih.gov/nuccore; Fig. 3). The amino acid sequences of the 2 peptides, known as VP1 B and VP2/3 C, respectively, are as follows:

VP1 B: NH2- NPDEHQKGLSKSLAAEKQFTDDSP- COOH

VP2/3 C: NH2- IQNDIPRLTSQELERRTQRYLRD- COOH

VP1 B and VP2/3 C mimotopes were selected because they reacted specifically in indirect ELISA testing (see below) with rabbit hyperimmune serum that had been experimentally immunized with SV40 (positive control serum). BKV and JCV hyperimmune sera did not react with VP1 B and VP2/3 C peptides (negative control sera). The amino acid residues of the 2 specific SV40 VP peptides exhibit low homology with the BKV and JCV VPs (Fig. 1B). The human peptide hNPS, amino acid (aa) sequence SFRNGVGTGMKKTSFQRAKS, was employed as a negative control peptide [29].

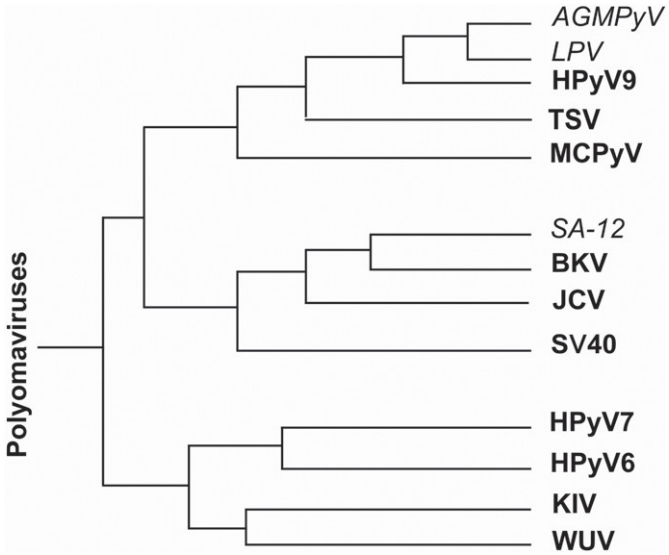

Fig. 2. Representation of the polyomaviruses phylogenetic tree. It is worth nothing that simian virus 40 is more closely related to JCV, BKV, and SA12 than to the other polyomaviruses. BKV (human polyomavirus BK), JCV (human polyomavirus JC), SA12 (simian agent virus 12), MCV (merkel cell polyomavirus), TSV (trichodysplasia spinulosa–associated polyomavirus), LPV (B-lymphotropic polyomavirus), KIV (KI polyomavirus), WUV (WU polyomavirus), HPyV 6 (human polyomavirus 6), HPyV 7 (human polyomavirus 7), and HPyV 9 (human polyomavirus 9 (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=10624).

The synthetic peptides were synthesized by standard procedures and purchased from UFPeptides s.r.l. (Ferrara, Italy).

2.3. Peptide in silico structural analysis

Peptide sequences from the SV40 capsid viral protein known as VP1 peptide B (VP1 B) and VP2/VP3 peptide C (VP2/3 C) were characterized toward stable secondary structure formation. Analysis was carried out using the PSIPRED server [30–32] and NetSurfP [33,34]. The results were matched to verify the coherence of inference output. Peptide sequences were mapped on native virion proteins to verify structural similarities. The SV40 VP1 capsidic protein structure is available in its pentameric form from the RCSB PDB database with PDB ID 3BWQ [35,36]. The SV40 capsid proteins VP1, VP2, and VP3, in their monomeric form, were obtained from computational prediction carried out using the I-TASSER server [37–39]. Molecular visualizations were performed by PyMOL (PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC, New York). Computational tools were available through the ExPASy server [40] (Fig. 4).

2.4. Control immune sera and immunologic tests

Hyperimmune sera against SV40 and BKV were obtained from rabbits that had been inoculated with purified viral stocks as performed previously [41–43]. The serum against JCV was kindly provided by Dr. Major (NIH, Bethesda, MD) [44]. Briefly, a New Zealand White male rabbit (Morini s.a.s., San Polo D'Enza, Italy), aged 10 weeks, was immunized subcutaneously on its back with purified SV40 or BKV, with a titer of 5×10^7 plaque-forming units (pfu)/mL or 5×10^3 hemagglutination units (HAU), respectively. SV40 or BKV, 10^7 pfu, diluted in 600 μL of emulsified Freund's complete adjuvant, was employed for the first injection and then twice, at 3-week intervals, with the same amount of virus in Freund's incomplete adjuvant. The rabbit was bled 10 days after the last injection.

The experiments with rabbits were performed in compliance with the relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki. The study was approved by the County Ethical Committee.

The anti-BKV immune serum was titered using the hemagglutination inhibition (HAI) test employing human erythrocytes from the 0, Rh^+ group[42]. The endpoint antibody titer of the BKV antiserum, determined by serial dilution, was 12,800 HAU. Anti SV40 serum was titered by neutralization assay [10,43]. In brief, serial dilution of titered SV40 stock (10^7 pfu/mL) was mixed with heat-inactivated immune rabbit serum. Specifically, 1 mL of the solution containing SV40 (5×10^4 pfu in 5 and 995 μL of serum) was incubated for 30 minutes at 37°C and then used as an inoculum in permissive CV-1 monkey kidney cells grown in 25-cm^2 flasks (3×10^6cells). After 2 hours of adsorbtion, the inoculum was discarded and the cells were overlaid with Dulbecco's modified Eagle's medium (DMEM) with 2% fetal calf serum. The cytopathic effect (cpe) resulting from SV40 multiplication was inspected from day 10 to day 15. SV40 hyperimmune rabbit serum was able to inhibit cpe in CV-1 cells completely. The endpoint dilution of the serum was considered the titer of the anti-SV40 serum still able to inhibit SV40 cpe. Negative and positive controls were uninfected and SV40-infected CV-1 cells, respectively.

2.5. Indirect ELISA

An indirect ELISA was developed and set up to detect specific antibodies against SV40 in human sera. ELISA plates were coated with synthetic peptides, which were employed as mimotopes, corresponding to specific SV40 polypeptides encoded by the late viral DNA region. Specifically, the 2 synthetic peptides VP1 B and VP 2/3 C were from VP1 andVP2/VP3, respectively.

2.5.1. Peptide coating: plates and blocking

Ninety-six well flat-bottom wells (Nunc-immuno plate PolySorp, CelBio, Milan, Italy) were coated with 5 μg of the selected peptide for each well, which were diluted in 100 μL of coating buffer (Candor Bioscience, Weissensberg, Germany). The plates were incubated at 4°C for 16 hours, allowing the peptide to cover the bottom well completely. The plates were rinsed 3 times with washing buffer (Candor Bioscience). This procedure eliminates uncoated peptide. Blocking was made with 200 μL/well of the blocking solution (Candor Bioscience) at 37°C for 90 minutes. This procedure allows for well saturation.

2.5.2. Primary antibody adding

To eliminate the residual blocking solution, the plates were rinsed again 3 times with the washing buffer (Candor Bioscience) in a washing apparatus (Model Wellwash 4MK2, Thermo Electron Corp, Vantaa, Finland). Then, different wells were covered with 100 μL containing the following sera: positive control represented by the immune rabbit serum containing anti-SV40 antibodies, negative controls represented by the immune sera with anti-BKV and anti-JCV antibodies and normal rabbit serum, and human serum samples under analysis diluted at 1:20 and other dilutions up to 1:160 in Low Cross-Buffer (Candor Bioscience). Each sample was analyzed 3 times. Additional controls were represented in each plate by a well with the secondary antibody and 2 other wells that were void of both primary and secondary antibodies. The plate was incubated at 37°C for 90 minutes.

2.5.3. Secondary antibody adding

After 90 minutes of incubation, a new triple rinsing cycle was repeated as described above. Then, the secondary antibody solution was added to each well. The solution contained a goat antihuman or antirabbit immunoglobulin G (IgG) heavy (H) and light (L) chain–specific peroxidase conjugate (Calbiochem-Merck, Darmstadt, Germany) diluted 1:10,000 in Low Cross-Buffer (Candor Bioscience). The reaction mixture was incubated at room temperature for 90 minutes.

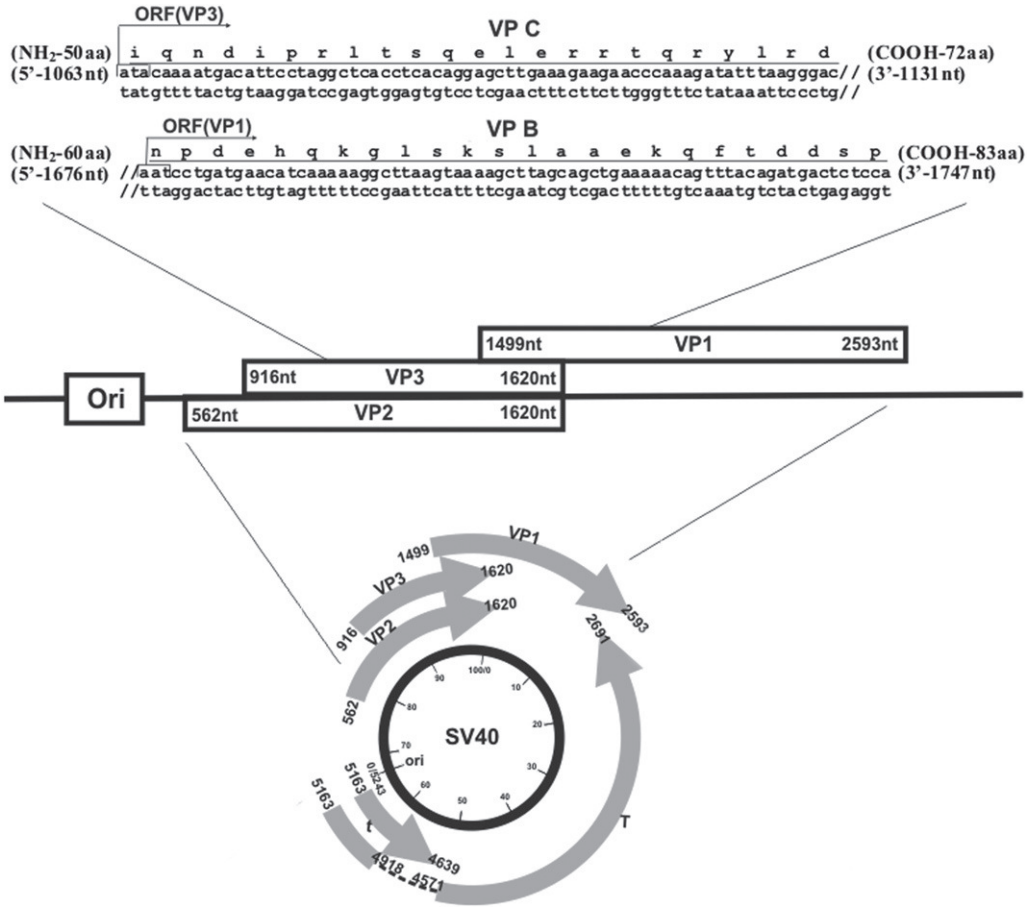

Fig. 3. Simian virus 40 (SV40) genome and the 2 selected peptides from the late region employed in indirect enzyme-linked immunosorbent assay. The circle at the bottom represents the SV40 genome, with the map unit from 0 to 100 running in a clockwise direction (inner circle, black). Ori is the origin of viral DNA replication, which is composed of 5,243 nucleotides, nt (0/5243). The nucleotide sequence and numbers refer to the SV40 776 wt strain (http://www.ncbi.nlm.nih.gov/genome). SV40 early and late genes are transcribed in both anticlockwise and clockwise directions, respectively (gray arrows); numbers indicate nt. The large T antigen (T) and small t antigen (t) are encoded by the early region (T antigen, exon 1, 5,163–4,918 nt, intron 1, 4,917–4,572 nt, and exon 2, 4,571–2,691 nt; t ag 5,163–4,639 nt), whereas viral protein (VP) 1–3 capsid proteins are codified by the late region (VP1, 1,499–2,593 nt; VP2, 562–1,620 nt;VP3, 916–1,620 nt). The late coding region is expanded in the upper part of the figure. The selected peptides, namely VP1 B and VP2/3 C, are indicated in the schematic representation of the late region, which encodes for the VP 1–3 capsid proteins. VP1 aa 60–83 (24 aa) and VP3 aa 50–72 (23 aa), respectively, together with the nt sequences.

2.5.4. Dye treatment and spectrophotometric reading

At the end of the incubation period, the plates were rinsed 3 times with the washer buffer and then treated with 100 μL of 2,2′-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid solution (Sigma–Aldrich, Milan, Italy), which reacted with the peroxidase enzyme for the color reaction. After 45 minutes of incubation, the colorimetric reaction was stopped with 100 μL of 0.1 M citric acid. The plate was then read by the spectrophotometer (Model Multiskan EX, Thermo Electron Corp) at a wavelength (λ) of 405 nm. This approach reveals the color intensity in wells where the immunocomplexes were formed by optical density (OD) based on the presence of specific antibodies that bind to the SV40 synthetic peptide/epitopes/mimotopes.

2.5.5. Cutoff determination

The cutoff in each assay was determined by the OD reading of the 2 negative controls, both for BKV and for JCV, and 2 OD values for the human peptide hNPS employed as assay controls added to the standard deviation and multiplied 3 times (+3 SD). Sera with antibodies against SV40 were considered VP positive upon reacting to both peptides from the late region. The reproducibility of the results was assessed with 3 replica experiments carried out by independent operators with no data variability.

2.6. Cells and viruses

Viral working stocks were obtained in Vero cells infected with the SV40 776 strain or BKV Gardner strain, as described previously [41–44]. JCV stock was purchased from ATCC. SV40 stock had a titer of 5×10^7 pfu/mL and was determined by the plaque assay as described previously [41]. BKV and JCV stocks were titered by the endpoint method to measure their hemagglutination activities for human erythrocytes, 0, Rh^+ type [42–45]. BKV and JCV had a titer of 5×10^3 and 3.2×10^3 HAU/mL, respectively.

2.7. SV40 neutralization assay with human serum samples

Permissive CV-1 monkey kidney cells were used for the neutralization assay on SV40 infectivity, as described [11], and modified slightly as follows: CV-1 cells were grown and propagated in DMEM containing 2× vitamins and aa, supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2. SV40 infectivity neutralization was carried out by incubating each human serum, diluted at 1:20 in phosphate-buffered saline (PBS), with 5×10^4 pfu of SV40 at 37°C for 30 minutes. Then, the suspension was added to the CV-1 cell monolayer for 2 hours at 37°C. The inoculum was removed and the monolayer washed 3 times with DMEM and overlaid with the medium containing 2% fetal bovine serum. Each sample was tested in duplicate. The neutralization

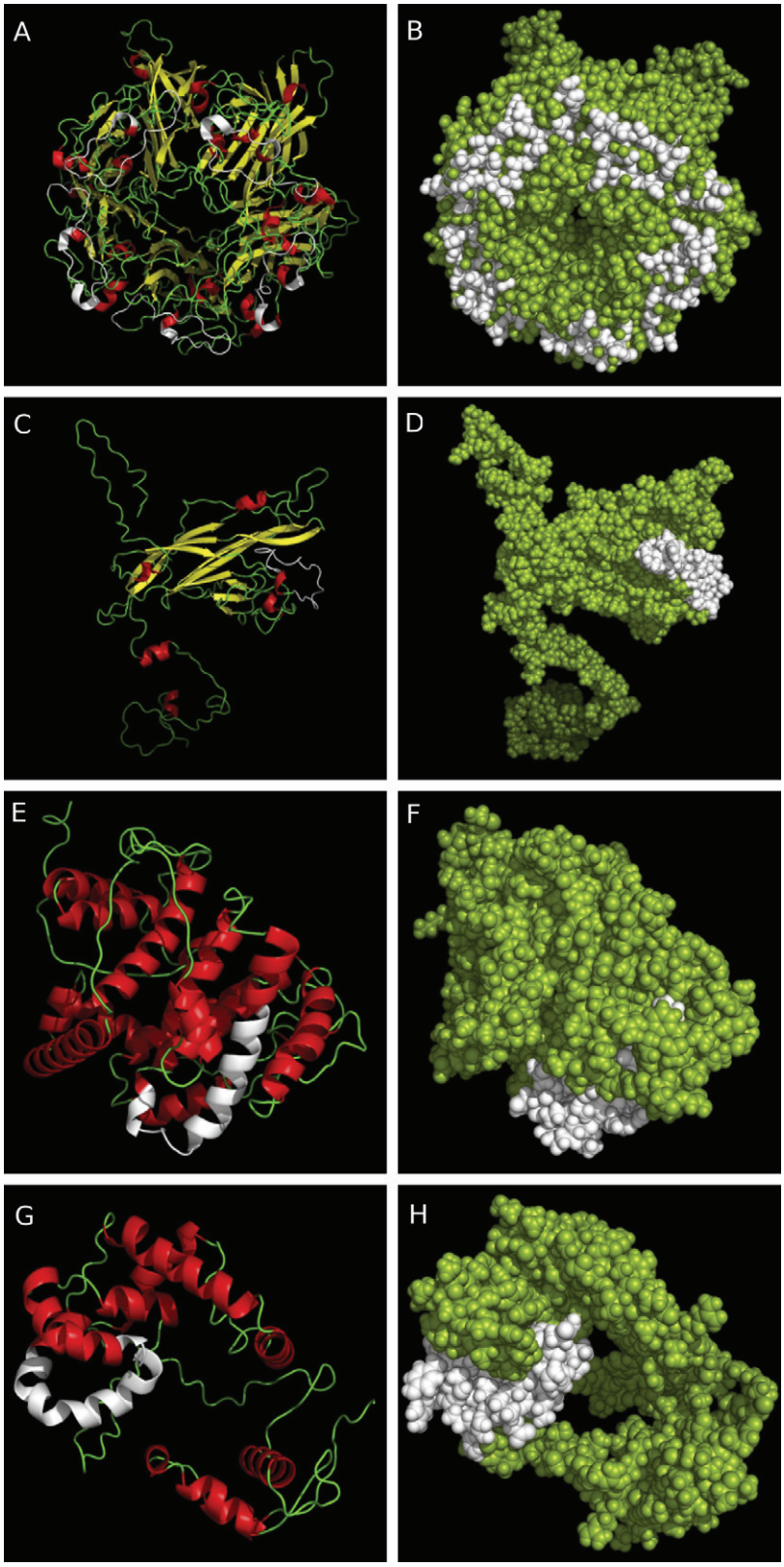

Fig. 4. Secondary and tertiary structure visualization (PyMOL program) of the results of the computational analysis carried out on synthetic polypeptides and simian virus 40 (SV40) native proteins. The column on the right illustrates the 3-dimensional sphere representation of the inferred protein structures where the linear peptides are mapped (white). (A and B) Viral protein (VP)-1 peptide B in pentameric form (database PDB ID: 3BWQ) and (C and D) in monomeric form. (E and F) VP2 peptide C. (G and H) VP3 peptide C. The VP1 peptide B maps on the BC loop of this viral capsid. The VP2/3 peptide C is represented 72 times in the SV40 virion, both in VP2 and in VP3 capsid proteins. VP3 representation is based on structural prediction.

assay included the following controls: (i) SV40 only in PBS, (ii) SV40 mixed with rabbit or human nonimmune serum, (iii) SV40 mixed with hyperimmune rabbit serum, and (iv) cells only in PBS. Cultures were observed using a light microscope for the presence of cpe for 3 weeks.

2.8. BKV and JCV hemagglutination and hemagglutination-inhibition assays

BKV and JCV HA and HAI titrations were carried out as described in detail elsewhere [42,43]. The HA titer was calculated based on the highest virus dilution that gave complete hemagglutination. The HAI titer was defined as the highest dilution of each serum sample that inhibited viral HA completely.

2.9. Statistical analysis

Statistical analyses were performed using Prism software (GraphPad, San Diego, CA). Data are presented as a percentage of the positive samples. The 95% confidence intervals of the percentage of positive samples are also reported. Differences among proportions were calculated by χ^2 testing for independence in the contingency tables. The small sample size was statistically analyzed using χ^2 with Yates' correction. The correlation between the OD value and the SV40 cpe was evaluated by nonparametric Spearman analysis, which indicates the Spearman r and p values.

3. Results

3.1. Computational analysis

Computational analysis indicated that the 2 linear VP1 B and VP2/3 C mimotopes are characterized by a stable secondary structure folding domain that can be identified as follows (PSIPRED server). The VP1 B peptide contains an α helix domain from aa 3 to 19 (*i.e.* 3DEHQKGLSKSLAAEKQF19). The VP2/3 C peptide also forms a stable secondary structure; indeed, an α helix domain is present from aa 5 to aa 22 (*i.e.*, 5IPRLTSQELERRTQRYLR22). Computational analysis is supported by statistical data. The analysis performed with the PSIPRED server returned a VP B predicted secondary structure where every aa residue is associated with a statistical confidence level. The structures obtained the highest statistical confidence rates for each residue. Overall, the secondary structure prediction methods employed achieved an average accuracy of 75%; thus, the model obtained can occur with this probability.

Tertiary structures of SV40 capsid VPs were selected from among those that had been computationally determined and presented *C* scores of -3.51 (VP1), -2.82 (VP2), and -4.43 (VP3). A confidence score named the *C* score is defined for estimating the accuracy of the 3-dimensional structure predictions. The *C* score is typically in the range [−5, 2], wherein a higher score reflects a model of better quality. Mapping the linear peptides on inferred protein structures indicated that the VP1 B mimotope in VP1 does not fold in any type of structure. Otherwise, the VP2/3 C mimotope folds in an α helix domain both in VP2 and in VP3 inferred proteins, which is a predicted structure. A stable α helix domain (aa 75–78) is exhibited by VP1 B when mapped on VP1 in its pentameric form (PDB ID: 3BWQ) for each monomer. A 3-dimensional graphic rendering of the peptides mapped onto the inferred structures indicated that several aa residues are exposed to the environment on the surface of the considered proteins (Fig. 4). It is possible that the physical configuration of selected polypeptides, although employed as linear peptides, may favor their immunologic reactivity as mimotopes.

3.2. Detection of SV40 antibodies by indirect ELISA

To verify whether human sera contain IgG antibodies that react to SV40 peptides and to determine the diffusion of SV40 infection in humans, indirect ELISA was set up using synthetic peptides that correspond to SV40 VP 1–3 mimotopes, together with an unrelated human peptide employed as a negative control.

In the first step of this investigation, indirect ELISA testing was employed to test serum samples taken from blood donors aged 18–65 years old that had been diluted at 1/20 for reactivity to SV40 epitopes from VP1, VP1 B peptide. Serum samples that reacted with the SV40 VP1 B mimotope reached an overall prevalence of 21.9%.

In the ELISA experiments, the human peptide hNPS [29] was employed as a negative control peptide. Data indicate that this negative control peptide does not react with 154 SV40-positive sera. The OD value was usually in the range of 0.088–0.098, which is consistent with the OD for SV40-negative sera.

The same assay was then addressed to detect IgG class serum antibodies against SV40 VP2/3 epitopes, which are known as VP2/3 C. Serum samples reacted with the SV40 VP2/3 C peptide with the same prevalence, 21.9%, as had been detected previously for the VP1 B peptide. Conversely, seronegative samples for the SV40 VP1 B peptide failed to react with SV40 VP2/3 C epitopes. The exceptions were negligible and were represented by a few serum samples found to be negative for VP1 B, but tested positive for VP2/3 C peptide and vice versa. The difference was not significant ($P > 0.05$; Table 1). Combining the SV40-positive sera, both for the VP1 B and for the VP2/3 C peptides, the overall prevalence was 18% (Table 1). No positive results were obtained with human peptide used as a control, which had an OD of less than 0.1. SV40-positive sera tested by indirect ELISA diluted at 1/20 had a general cutoff, by spectrophotometric reading, in the range of 0.17–0.19 OD. This cutoff represents the value that discriminates SV40-negative (sample below OD 0.17–0.19) from SV40-positive samples (above OD 0.17–0.19). The positive control, represented by the SV40 hyperimmune serum, had an OD of up to 1.8, whereas the 2 JCV and BKV hyperimmune sera, which were employed as negative controls, had an OD of less than 0.1.

A prevalence selection corresponding to 17, 22.7, and 20.5% within the cohort aged 18–65 years old was observed in subjects 18–30, 31–40, and 41–50 years old, respectively. Interestingly, the prevalence of serum antibodies against SV40 VPs declined in the cohort of individuals 51–65 years old, with a percentage of 12% (Table 1).

The 2 indirect ELISA tests, with 2 distinct VP peptides, gave overlapping results, thus confirming the presence of anti-SV40 VPs antibodies in human sera from blood donors (Table 1).

The SV40 seroprevalence of human sera was analyzed in detail for age, gender, and geographic region of the blood donors. ELISA data indicated that the prevalence of SV40 antibodies is similar in cohorts of individuals with the same age and gender, despite coming from different regions in Italy (data not shown).

In this investigation, the 12% prevalence of SV40 positive sera, which was detected in blood donors 51–65 years old, is lower than that detected in the other cohorts of healthy individuals (Table 1).

Table 1
Prevalence of immunoglobulin G antibodies reacting with simian virus 40 (SV40) viral peptide (VP) mimotopes

Age (years)	Number of samples	Number of positive samples (%)		
		VP B	VP C	VPs (B-C)
18–30	147	34 (23.1)	28 (19.0)	25 (17)
31–40	203	54 (26.6)	53 (26.1)	46 (22.7)
41–50	263	59 (22.4)	64 (24.3)	54 (20.5)
51–65	242	40 (16.5)	42 (17.4)	29 (12)
18–65	Total 855	187 (21.9)	187 (21.9)	154 (18)

Human sera were from healthy blood donors. Statistical analysis was performed using the χ^2 test with Yates' correction. The different prevalence of SV40 antibodies between the cohorts of individuals aged 51–65 years old was significant compared with the cohorts aged 31–40 ($p = 0.0041$), 41–50 ($p = 0.0135$), and 18–65 years old ($p = 0.04$).

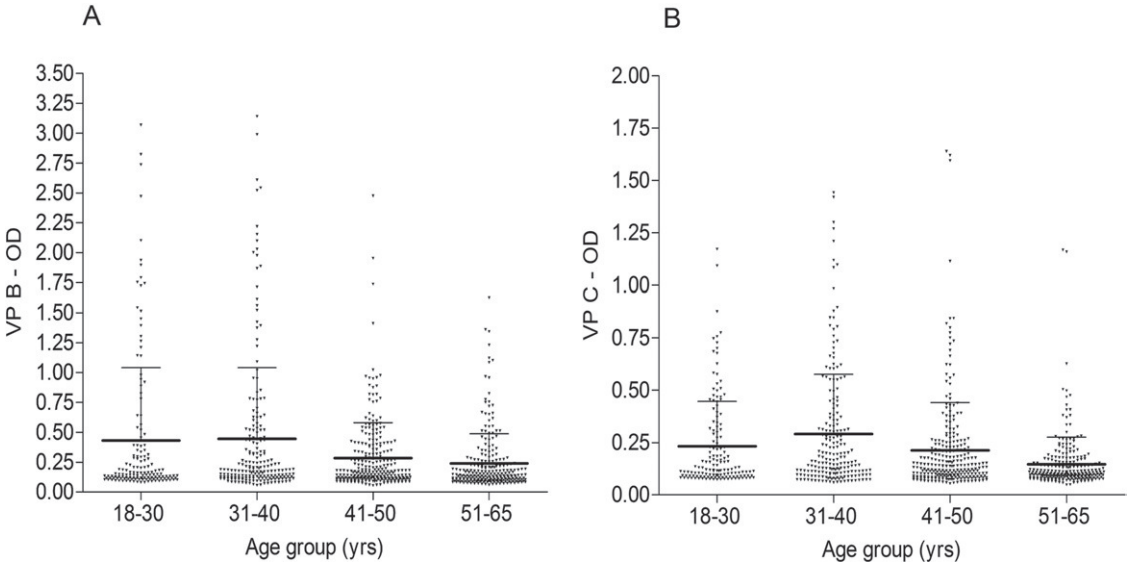

Fig. 5. Profile of serum antibody reactivity to simian virus 40 mimotopes, with data presented as values of optical density (OD) readings at λ 405 nm of serum samples diluted at 1:20 detected in indirect enzyme-linked immunosorbent assay testing. In scatter dot plotting, each plot represents the dispersion of OD values to a mean level, indicated by the line inside the box with SD (standard deviation) for each age group of subjects analyzed.

These differences are significant (Table 1). The reduced prevalence of SV40-positive sera in individuals 51–65 years old could be ascribed to natural age-dependent partial immune depression. Indeed, the immune system physiologically declines with age, rendering individuals less responsive to infections. An alternative interpretation of the result, as suggested by other studies [23,24], is that the low prevalence of SV40 antibodies in sera from the elderly could depend on the low stability of the SV40 antibody over time. Serologic profiles of serum antibody reactivity to SV40 mimotopes are presented in Fig. 5, and interrun and intrarun variability is illustrated in Fig. 6.

To verify the antibody titer, 24 sera from blood donors found to be SV40 positive for both VP1 B and VP2/3 C peptides were serially diluted from 1/20 to 1/160 and further investigated by indirect ELISA. The assay indicated that these sera carry antibodies against SV40 that remain positive at a 1/80 dilution. This result indicates that the titer of SV40 antibodies in positive sera from normal individuals does not greatly differ for the 2 different peptides. The reproducibility of the results was assessed with 3 replica experiments carried out by independent operators with no data variability.

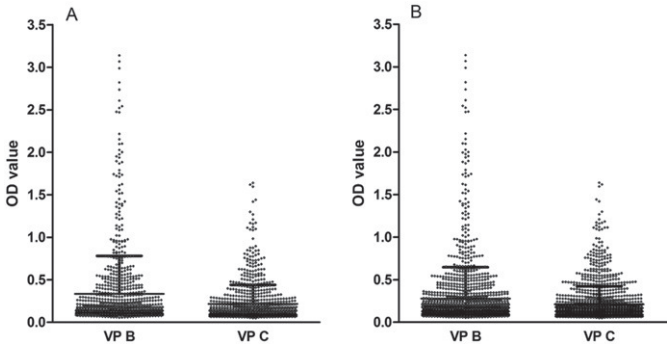

Fig. 6. Interrun and intrarun variability, optical density (OD) value of viral protein (VP) B and VP C peptides, together with standard deviations from the mean. Data are presented as scatter dot plot of OD readings at λ 405 nm, mean and standard deviations (SD) from the mean. (A) OD value variability, intrarun. VP B: mean = 0.33, SD = 0.45; VP C: mean = 0.22; SD = 0.22. (B) OD value variability interrun. VP B: mean = 0.28, SD = 0.37; VP C mean = 0.21, SD = 0.21.

3.3. Functional analysis of serum antibodies by neutralization activity testing

An inhibition test was performed to verify whether immune sera containing antibodies against SV40 carry neutralization activity against SV40 infectivity. To this purpose, 6 immune sera, with a high OD that ranged between 0.248 and 0.722, were selected from 154 SV40 seropositive samples from normal subjects to test their ability to inhibit cpe in permissive SV40-infected CV1 cells. To avoid any possible cross-reactivity, these sera were BKV and JCV negative, as determined by HAI tests. The prevalence of BKV and JCV antibodies, determined by HAI, in these 154 serum samples was 85 and 62.5%, respectively. The data are in agreement with previous reports [3]. Individuals seropositive for JCV and/or BKV exhibited no higher OD values toward the VP B and VP C peptides than those who were seronegative. The neutralization effect of SV40-immune sera is apparent in infected cells when the SV40 cpe is abolished or hampered. The experiment was carried out together with SV40 seronegative and SV40 hyperimmune sera, which were used as negative and positive controls, respectively. The cpe inhibition test was performed by mixing the serum sample with SV40 virions. Then, the mixture was employed as a viral inoculum to infect CV-1 permissive monkey kidney cells. In infection experiments, only titered SV40 viral stock was used as a positive control, whereas a SV40 hyperimmune serum with SV40 virions was employed as a negative control. Inhibition of SV40 cpe in infected cells indicated that tested sera were able to neutralize SV40 infectivity. Indeed, among the 6 SV40-positive samples, 1 (No. 58 with an OD = 0.722) inhibited SV40 cpe completely (indicated with score +++++), whereas 5 (No. 64, OD = 0.653; No. 67, OD = 0.627; No. 179, OD = 0.375; No. 68, OD = 0.355; and No. 90, OD = 0.248) only partially inhibited SV40 cpe (score ++++ or +++). A correlation existed between the OD reading and the cpe inhibition activity of the immune sera. Highly neutralizing serum samples correlated with a high OD determined in the anti-VPs ELISA. Indeed, the correlation between OD and the inhibition of cpe is significant (Spearman $r = 0.9258$, $p = 0.0167$; Fig. 7). This assay detects functional antibodies to viral structural proteins, such as VP1–3, that are capable of neutralizing viral infectivity *in vitro*. This result suggests that exposure to SV40 had occurred in those immunized individuals and that

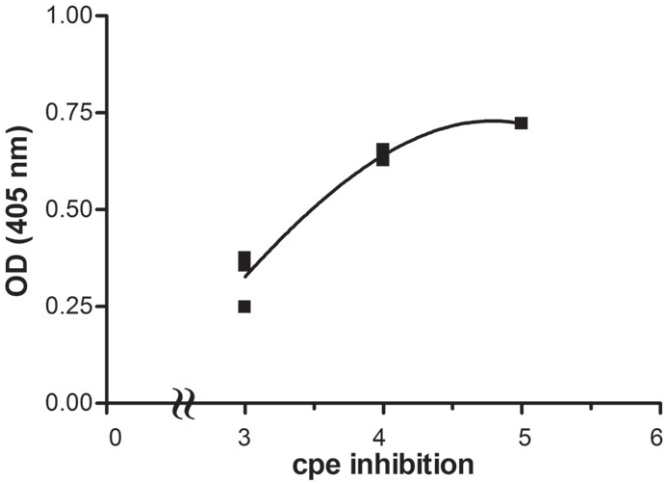

Fig. 7. Graphic representation of the functional analysis of SV40 human immune sera that display neutralization activity. Immune sera with higher OD inhibited more efficiently the SV40 cpe in CV-1-infected cells. Nonparametric correlation Spearman analyses was performed: Spearman $r = 0.9258$ and a p value of 0.0167.

the immune serum was elicited by SV40 infection and not by BKV or JCV.

4. Discussion

Computational analysis of the linear peptide (VP1 B and VP2/3 C) secondary structure and viral capsid protein (VP1, VP2, and VP3) tertiary structure revealed that under experimental conditions there is a good statistical confidence level (see prediction algorithms details) regarding VP1 B folding differences toward its conformation in the native viral protein. However, within VP1 in its pentameric form (PDB ID: 3BWQ), VP1 B forms a stable α helix domain (aa 75–78). Peptide VP2/3 C has a secondary structure that is similar to that found in the native proteins VP2/VP3, except for the 3 aa residual α helix domains found in the inferred tertiary structure. The dimensional surface visualization (Fig. 4, right column) of the inferred protein tertiary structure in which the linear peptides are mapped indicated that analyzed aa chains have partial surface localization in the considered structures, exposing several aa residues to the reaction environment. These results may suggest that the linear peptides VP1 B and VP2/3 C resemble natural SV40 linear epitopes, constituting a docking site for serum antibody repertoire directed against both linear and conformational antigens.

On these grounds, serum samples from blood donors were analyzed for their reactivity to SV40 epitopes from viral capsid proteins using indirect ELISA testing. The data indicate that the overall prevalence of SV40-VPs antibodies in humans aged 18–65 years old is at 18% in healthy individuals from Italy. No positive results were obtained with the human peptide used as a control.

It is important to note that SV40 prevalence in human sera, as detected by this immunologic study, does not differ substantially from that reported in a previous study carried out in the United States using neutralization testing against SV40 infectivity, which is considered the gold standard for measuring the presence of the SV40 antibody in terms of neutralization activity [11]. A similar prevalence in terms of SV40 sequences revealed by PCR assays in peripheral blood mononuclear cell/buffy coat samples from normal individuals was reported in earlier investigations [4,5,8].

Taken together, the data from the ELISA indicate that in normal individuals natural SV40 infection occurs at a lower prevalence when compared with the high prevalence of infection spread over the 2 well-characterized human BK and JC polyomaviruses, which is in the range of 60–90% [3]. The prevalence of anti-SV40 serum antibodies increases with age, reaching 22.7% in the cohort aged 31–40 years old, with an increase of SV40 antibody level determined by OD readings.

It has been demonstrated that SV40 is present in the urine, stool, tonsil, and blood specimens of children and adults, suggesting that different methods of transmission, such as urine, orofecal, respiratory, and hematic routes, are responsible for SV40 infection [10–18]. These data seem to confirm the results obtained in earlier studies on subjects administered SV40-contaminated vaccines by different routes. Indeed, in these vaccinees, SV40 was detected/isolated after a number of days or weeks, either in stools or from throats as a result of oral or nasal spray administration of contaminated vaccines [46,47]. However, these data differ from those of other immunological studies where the prevalence of reported SV40 antibodies is either very low or absent. It is possible that previous data were affected by technical artifacts. Indeed, earlier results were mainly obtained with recombinant VLPs or VP1 as antigens, which contain many common epitopes with SV40, BK, and JC polyomaviruses. In these investigations, the immune sera were preabsorbed with BKV and JCV antigens [22–24] in an attempt to give SV40 specificity to the assay. This procedure may eliminate or drastically reduce the presence of SV40 antibodies in analyzed sera [4,20].

Functional results on the inhibition of SV40 infectivity obtained with immune sera from normal subjects are of interest. There is a correlation between the grade of SV40 infection inhibitory effect by immune sera and their level of antibodies as determined by OD readings. The higher concentration of serum antibodies gave a stronger inhibition effect on SV40 infection in tissue cultures. Because the selected SV40 immune sera were BKV and JCV negative, there is no possible cross-inhibition effect caused by the presence of antibodies against these human polyomaviruses. In addition, it has been demonstrated that BKV and JCV immune sera are not able to neutralize SV40 infectivity [25].

To examine further the serology of SV40-positive samples, endpoint titers were determined using an indirect ELISA. The highest endpoint titer was observed at a 1/80 dilution for both VP 1 B and VP2/3 C peptides.

In this study, indirect ELISAs, using SV40 peptides from VP antigens, were set up for the detection of SV40 antibodies in human sera. ELISA gave reliable results, which can be obtained for many samples in a short period of time with affordable costs. This ELISA may provide the scientific community with a standardized assay for the study of SV40 infection in human populations and its association with human cancers. Immunological data from this study suggest that SV40 is also a human virus. Alternatively, it may be that other as yet undiscovered polyomaviruses infect humans. In this case, positive immunologic data could be the result of a new, closely related SV40 polyomavirus.

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