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Antipeptide Antibodies Directed Against the Carboxy-Terminal Region of SV40 Structural Proteins VP2 and VP3

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Rabbits were immunized with a synthetic heptapeptide of the sequence Arg-Asn-Arg-Ser-Ser-Arg-Ser corresponding to the carboxy-terminal region of the SV40 viral proteins VP2 and VP3. The raised antibodies recognize the viral proteins in enzyme-linked immunosorbent (ELISA) and Western blot assay. Specificity of the antibodies were confirmed by competition experiments. The antibodies recognize VP2 and VP3 in infected cells by immunofluorescence and in subcellular fractions by ELISA. No interaction with virions was observed.

Key words: Western blot, ELISA, competition, immunofluorescence, immune electron microscopy, subcellular fractionation

Synthetic peptides have become one of the most powerful tools in virus research. The widespread field of application is summarized by Walter and Doolittle [1].

In the case of the well documented SV40 [2,3], one of the papovaviruses, this technique has been useful to contribute to viral organization and composition. Antisera against synthetic peptides corresponding to the carboxy- and amino-terminal regions of SV40 large tumor antigen, respectively, have been demonstrated to react specifically with the viral protein [4,5].

Our intention was to study the intracellular localization of the SV40 structural proteins VP2 and VP3. For this purpose we raised specific antibodies by immunization with a synthetic heptapeptide of the sequence Arg-Asn-Arg-Ser-Ser-Arg-Ser corresponding to the carboxy-terminus of the SV40 proteins VP2 and VP3. By tryptic peptide analysis it has been demonstrated that VP2 contains the entire VP3 chain at the carboxy-terminal region [6,7].

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MATERIALS AND METHODS

Synthesis of the Peptide

For this study, the peptide was synthesized by a solid phase method [8] using the 9-fluoromethylmethoxycarbonyl-(Fmoc)-group [9] for amino-protection. The Fmoc-group can be cleaved by mild basic conditions. The side chain protection of Ser is achieved by the acid labile tertiary butyl group, whereas Arg is used as the 4-methoxy-2,3,6-tri-methylbenzenesulfonyl (Mtr) derivative. This strategy avoids subsequent deprotection reactions under drastic conditions.

The loading of the resin [10,11] with the first amino acid was 0.46 mmol/g. Deprotection was done by the reaction with 50% piperidine in dichloromethane. After several washings, the next amino acid was introduced by coupling in three-fold excess with N,N′-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The reaction was monitored by an semiquantitative ninhydrin test. Treatment of the peptide resin at the end of the synthesis with 90% trifluoroacetic acid in thioanisole containing 0.2 M methanesulfonic acid gave complete cleavage and removal of the protecting groups.

Purification of the crude product was performed on a reversed phase column. The resulting mainproduct was homogenous by high performance liquid chromatography and thin layer chromatography. The composition of the peptide was confirmed by quantitative amino acid analysis, which was performed by Dr. H. H. Kiltz, University of Bochum.

Preparation of the Synthetic Antigen

The peptide was added in 30-fold molar excess to 0.15 μM bovine serum albumine (BSA) in 1 ml of 0.1 M sodium phosphate buffer (pH 7.5) [12].

Three hundred microliters of 21 mM glutaraldehyde was added drop-wise with stirring. After 30 min at room temperature, the conjugate was purified on a Sephadex G-15 fine column. Glutaraldehyde cross-linked peptide was prepared in an analogous way [13].

Immunization of Animals

Immunization of animals was done as described [4] with 1 mg of peptide-BSA-conjugate suspended in 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.5) and 0.5 ml of complete Freund's adjuvant. The injection was repeated after 4 wk in the same manner.

After another 4 wk, a booster injection was given with glutaraldehyde cross-linked peptide. The animals were bled 2 wk after the last injection.

Enzyme-Linked Immunosorbent Assay (ELISA)

The reactivity of the antisera was determined by ELISA.

Immulon plates (Dynatech, Plochingen, FRG) were coated with 5–10 μg peptide or 50–100 ng purified SV40 per well in 0.05 M carbonate buffer (pH 9.6).

Prior to adding the antibody dilutions, the plates were preincubated for 1 hr with a solution of 1% ovalbumin in sodium phosphate buffer. The unbound antibody was washed away, and a peroxidase-conjugated anti-rabbit antibody was added for 30 min. Excess antibody was again washed away, and the substrate solution of 0.04% o-phenylenediamine and 0.012% hydrogen peroxide in phosphate-citrate buffer (pH 5.0) was placed into the wells. The reaction was stopped with 1 M HCl. The absorbance of the wells was measured in a Titertek Multiscan photometer at 492 nm.

Purification of the Antipeptide Serum

The antipeptide serum was purified by affinity chromatography on 2 g of Ch-Sepharose 4B to which 18 mg of peptide was coupled. Ten milliliters of serum was adsorbed to BSA-linked Sepharose, dialyzed against 20 mM Tris-HCl (pH 8.0), 29 mM NaCl, and applied to a DEAE Affi-Gel Blue column (Bio-Rad, Muenchen, FRG).

The excluded IgG fraction was added slowly to the affinity column. The bound material could be eluted with 0.1 M glycine solution (pH 2.5). The eluted fractions were immediately neutralized by addition of Tris base.

Cells and Virus

The African green monkey cell line CV-1 was grown in Dulbecco's modified Eagle medium (DMEM) containing 10% newborn calf serum. In our experiments, the SV40 strain 777 was used. The virus stocks were grown at a low multiplicity (0.01 tissue culture infective dose $[TCID]_{50}$/cell) from single plaques.

Extraction and purification of SV40 from infected cells was done exactly as described [14].

Subcellular Fractionation

Subcellular fractionation was performed essentially as described [3] into a Nonidet P-40 soluble fraction (Sol), a sodium deoxycholate/Tween 40 soluble fraction described as cytoskeletal fraction (Csk), and an insoluble fraction (Nuc) containing cell nuclei.

Briefly, infected CV-1 cells in 100-mm Petri dishes were washed twice with TD-buffer containing 1 mM sodium phosphate, 0.14 M NaCl, 5 mM KCl, and 25 mM Tris-HCl (pH 7.4).

Cells were lysed for 3 min on ice with 0.5 ml of lysis buffer 1 mM $CaCl_2$, 1 mM $MgCl_2$, 250 mM sucrose, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mM Tris-HCl (pH 7.2). The soluble fraction was removed, cells were harvested, and the supernatant of low-speed centrifugation was combined with the soluble fraction. The pellet was resuspended in 0.25 ml RSB-buffer per Petri containing 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1% Tween 40, 0.5% sodium deoxycholate, and Tris-HCl (pH 7.2). The suspension was homogenized by ten strokes in a Dounce homogenizer and underwent low-speed centrifugation.

The supernatant was referred to as the Csk fraction, whereas the pellet was built by the Nuc fraction.

Uninfected cells were fractionated exactly in the same way. Equivalent volumes of these fractions (10 μl for Csk and Nuc fraction and 20 μl for Sol fraction) were diluted with 1 ml of 0.05 M carbonate buffer (pH 9.6) and bound to Immulon plates. The reaction of a 1:100 dilution of the affinity-purified antipeptide serum with these plates was monitored.

Protein Electroblotting

Polyacrylamide gels (15%) containing sodium dodecyl sulfate were used for the separation of proteins as described previously [15,16].

Proteins were transfered to nitrocellulose (BA 85, Schleicher & Schuell, Dassel, FRG) according to the method of Towbin et al [17] with a transfer time of 4 hr at 250 mA.

The detection of the antigen-antibody complexes on the nitrocellulose sheets was carried out with the slightly modified enzyme labeled second antibody technique described by Blake et al [18].

After transfer, the nitrocellulose was twice submerged for 30 min in 0.1 M sodium phosphate buffer containing 0.5% Tween 20 to block the remaining binding sites. The antibody solution in the above mentioned buffer was added for 3 hr at room temperature.

After three washings, an alkaline phosphatase-conjugated anti-rabbit antibody was incubated for 2 hr with the blot.

The nitrocellulose was washed another three times with the above mentioned solution and once with 0.15 M Tris-HCl solution (pH 9.6). The substrate solution was allowed to react at 37°C with the blot until bands became visible. The substrate solution contained 20 μl 2 M magnesiumcloride, 1 ml 0.1% nitro blue tetrazolium, 100 μl 5-bromo-4-chloro-3-indolylphosphate solution (5 mg/ml DMF) in 0.15 M Tris-HCl solution (pH 9.6).

Immune Electron Microscopy (IEM)

All antisera for IEM were inactivated at 56°C for 30 min and cleared by ultracentrifugation (100,000g, 1 hr). Purified SV40 virions (20 μl) were incubated for 3 hr at room temperature and 1 hr at 4°C with 20 μl serum diluted 1:100 in phophate buffered saline (PBS). The affinity-purified antipeptide antibodies were used undiluted.

The viral particles were then deposited on copper grids coated with a formvar film and carbon. They were negatively stained with a 2% solution of phosphotungstic acid (pH 7.0). Particles were viewed with a Philips EM 300 electron microscope at 80 kV accelerating voltage.

RESULTS

Immunogenicity of the Peptide

The success of the immunization with the peptide was monitored by ELISA. Our success rate was as good on plate-fixed peptides as on plate-fixed viral proteins. While the preimmune serum did not show any reactivity, the antipeptide serum reacted with the immobilized peptide and with the viral proteins.

In order to reduce any nonspecific reaction, we purified the antipeptide serum by affinity chromatography. For this purpose, we first absorbed it to BSA-Sepharose to remove anti-BSA antibodies. The excluded IgG fraction from a DEAE Affi-Gel Blue column was further purified by its reactivity to peptide coupled to CH-Sepharose.

Purified antipeptide antibodies recognize viral proteins in a dilution of at least 1:100,000 (Fig. 1). No reactivity against extracts of uninfected CV-1 cells was observed.

Specificity of the Antipeptide Serum

The specificity of the antipeptide serum was demonstrated by protein electroblotting. Lysates from infected cells as well as proteins from purified SV40 were

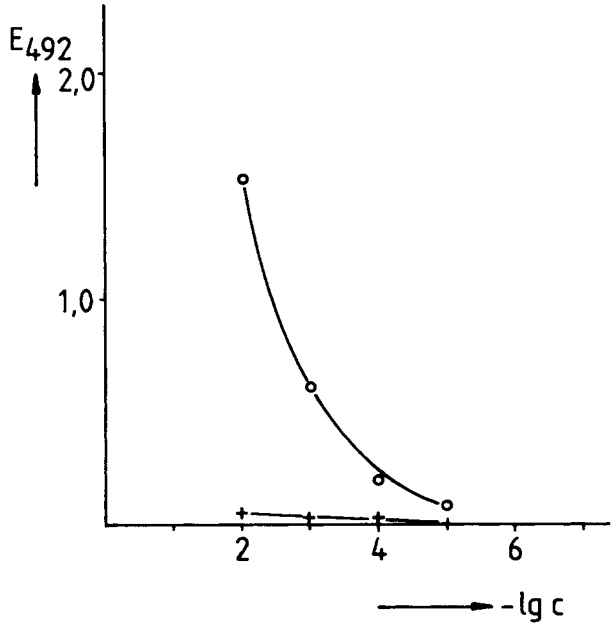

Fig. 1. Reactivity of the affinity-purified antipeptide serum (○—○—○) and preimmune serum (+-+-+) toward coated viral proteins. Coating was performed with 80 ng of SV40 viral proteins per well diluted in 0.05 M carbonate buffer (pH 9.6).

separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transfered to nitrocellulose.

The antipeptide serum was able to bind to the VP2 and VP3 proteins (Fig. 2, lane a) in lysates of SV40 infected cells. Besides VP2 and VP3, BSA with an apparent molecular weight of 68,000 daltons was recognized. This protein was not detected by affinity-purified serum (Fig. 2; lane c). The preimmune serum did not show any reactivity as should be expected from ELISA experiments (Fig. 2, lane b).

Competition experiments were performed in a qualitative and semiquantitative manner.

Preincubation of purified antipeptide serum with peptide inhibited the reaction with the nitrocellulose-fixed proteins VP2 and VP3 (Fig. 2, lane d).

Figure 3 shows that preincubation of the purified antipeptide serum with increasing amounts of peptide results in increasing inhibition of binding to polystyrene-bound SV40. Related to the signal of the pure serum, inhibition of more than 75% was achieved by 10 μg/ml of peptide.

Recognition of the Viral Proteins VP2 and VP3 in Infected Cells

Figure 4 shows immunofluorescence micrographs obtained by the antipeptide serum in SV40-infected CV-1 cells. Experiments were performed at 31 hr post infection (hr p.i.) and 48 hr p.i.

Nuclear and perinuclear fluorescence was obtained as demonstrated (Fig. 4b, c). Only in a few cells could cytoplasmic staining be detected. In each case the obtained fluorescence could be inhibited by the addition of an appropriate amount of peptide (Fig. 4d). No immunofluorescence was recognized with preimmune serum

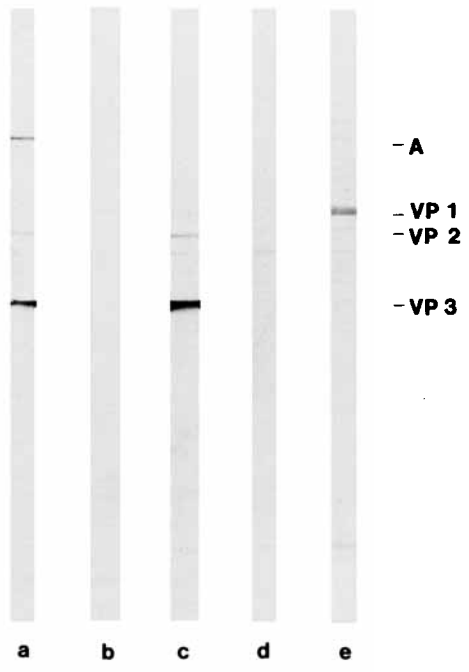

Fig. 2. Immunological detection of SV40 viral proteins on nitrocellulose strips. Lysates from SV40-infected CV-1 cells were separated by electrophoresis on a 15% polyacrylamide gel prior to electrophoretic transfer to nitrocellulose. The nitrocellulose strips were incubated with antipeptide serum (a), preimmune serum (b), antipeptide serum purified by affinity chromatography (c), and affinity-purified antipeptide serum with 20 μg/ml peptide added (d). Lanes a–d were stained by enzymatic reaction. Transfered proteins were stained with amidoblack 10B (e). Owing to the different staining procedure, the length of strip e is slightly shorter in comparison to strips a–d. (A, albumine).

(data not shown) or with the antipeptide serum applied to uninfected CV-1 cells (Fig. 4a).

Recognition of VP2 and VP3 in Subcellular Fractions

Subcellular fractions of infected cells were assayed by ELISA for the contents of VP2 and VP3. The results are summarized in Table I. ELISA was negative with fractions of uninfected control cells.

When the soluble fractions of infected cells were monitored, no VP2/VP3 was recognized by the antipeptide serum. The purified antipeptide serum showed significant reactivity against plate-bound cytoskeletal and nuclear fractions. Up to 48 hr p.i., increasing amounts of VP2/VP3 were detected in both fractions. At 72 hr p.i. the amount of the proteins in the Nuc fraction remained constant related to 48 hr p.i. In the Csk fraction, a minor amount of VP2/VP3 was found. The distribution between the Csk and Nuc fractions raised from a 1:1 ratio at 24 hr p.i. to a 1:4 ratio at 72 hr p.i.

Reactivity of the Antipeptide Serum Against SV40

As demonstrated in Figure 5A, incubation of goat anti-SV40 serum with SV40 virions resulted in aggregation of virus particles as shown by immune electron

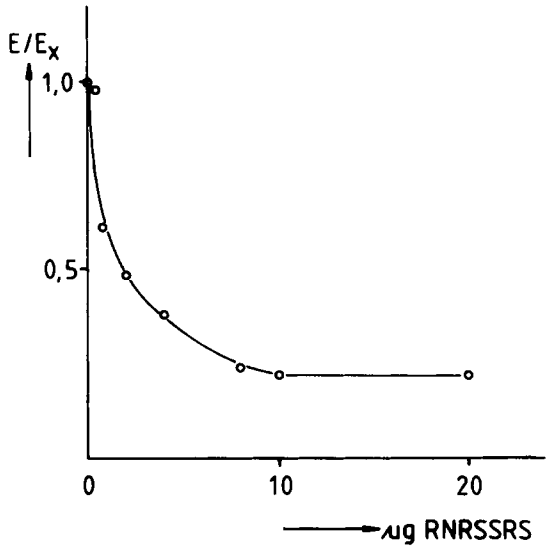

Fig. 3. Inhibition of binding of affinity-purified antipeptide serum to SV40 proteins (80 ng/well) coated on microtiter plates. Preincubation of 1 ml of antipeptide serum (dilution 1:1,000) with various amounts of peptide was performed for 1 hr. E, absorbance at 492 nm with antipeptide serum preincubated with peptide; E_x, absorbance at 492 nm with antipeptide serum without addition of peptide.

microscopy. The antipeptide serum did not show aggregation or coating of virus particles when incubated with SV40. The goat anti-SV40 serum was kindly provided by Dr. F. Mehnert, University of Bochum.

DISCUSSION

The present studies show that by immunization with a synthetic heptapeptide highly specific antibodies are obtained.

The great advantage of the antipeptide antibodies compared to normal antibodies is their predetermined specificity. This specificity is demonstrated by protein electroblotting assay and by competition experiments. The antipeptide serum recognizes the VP2 and VP3 molecules. The only undesired reactivity is caused by the carrier molecule BSA. As BSA itself is able to elicit an antibody response, the antipeptide serum reacts with BSA from cell medium. Purification of the antipeptide serum by affinity chromatography abolishes this reaction as expected.

The ease of purification of the antibodies mentioned here is a further advantage of the applied technique. Nevertheless, it should be considered that the use of a synthetic carrier might be an improvement.

Competition experiments between the heptapeptide and the viral proteins confirm the observed specificity of the antipeptide serum. In an ELISA experiment, inhibition of binding to the viral proteins can be correlated to the amount of added peptide (Fig. 3). As expected, this inhibition is equally effected by an appropriate amount of peptide-BSA-conjugate and by glutaraldehyde cross-linked peptide (data not shown). These results can be explained by the previously reported chain-end flexibility [19,20], which may enable antibodies produced against numerous confor-

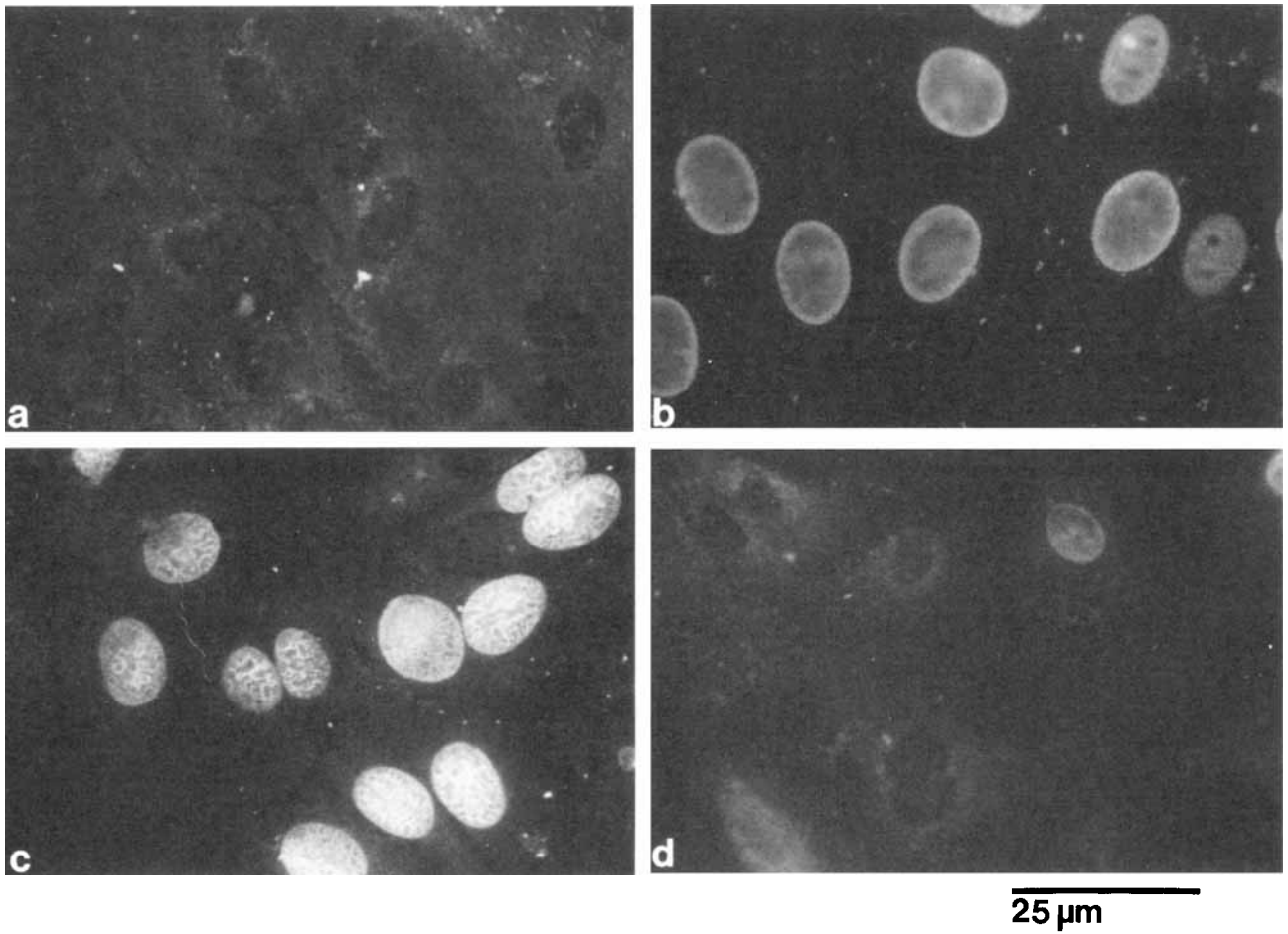

Fig. 4

TABLE I. Distribution of VP2 and VP3 Recognized by the Antipeptide Serum in Subcellular Fractions at Different Times After Infection*

	Hour (p.i.)				
Subcellular fraction	0	24	31	48	72
Sol	0	0	0	0	0
Csk	0	1.0	1.6	3.5	1.7
Nuc	0	1.0	2.9	7.0	6.8

*Equal amounts of the preparations were bound to the ELISA plate and reacted with a 1:100 dilution of the affinity-purified antipeptide serum. Absorbance of the fractions are expressed in terms of the absorbance of the Csk fraction at 24 hr p.i. (0 = not significant above the background). Listed values represent the average of three ELISA experiments. Sol, soluble fraction; Csk, cytoskeletal fraction; Nuc, nuclear fraction.

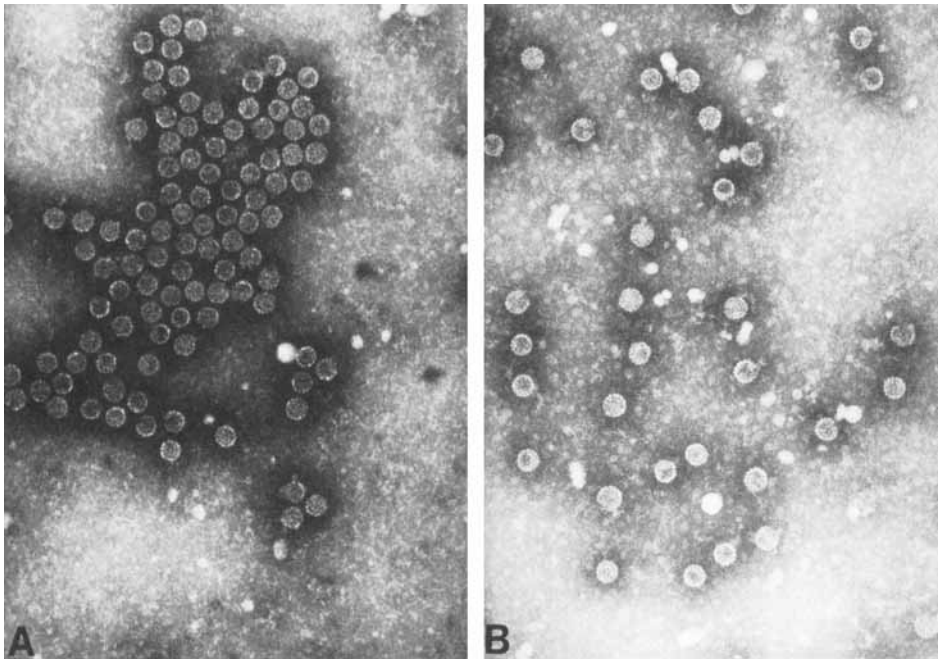

Fig. 5. Immune electron micrographs of purified SV40 at a magnification of ×110,000. In aggregation of SV40 virions by goat anti-SV40 serum is demonstrated. Twenty microliters of goat anti-SV 40 serum (diluted 1:100 in PBS) was incubated with 20 μl of purified SV40. In b, 20 μl of purified SV40 was incubated with 20 μl of affinity-purified antipeptide serum. No aggregation or coating was observed.

Fig. 4. Immunofluorescence obtained in acetone-fixed CV-1 cells grown on slide glasses. Cells were first treated with affinity-purified antipeptide serum (diluted 1:10 in PBS) and after a subsequent washing with fluorescein-conjugated goat anti-rabbit antibody (dilution 1:10 in PBS). The figures show uninfected control cells (a), SV40 infected cells (m.o.i. = 10 $TCID_{50}$/cell) 31 hr postinfection (b), and 48 hr p.i. (c). Preincubation of the antipeptide serum with 50 μg of peptide per ml of serum dilution diminishes fluorescence as demonstrated at 31 hr p.i. (d). All photographs were exposed under the same conditions. Bar = 25 μm.

mations of the immunogen to force the flexible antigen in a complementary form and in this way participate in the antigen-antibody reaction.

The high specificity of the antipeptide serum is rather useful for the localization of the viral structural proteins VP2 and VP3 in infected cells. Nuclear and perinuclear fluorescence is observed as reported previously by others [21].

Further information is available by subcellular fractionation of infected cells with detection of viral proteins by a sensitive ELISA. In the cytoskeletal and nuclear fractions, VP2/VP3 are detected in an increasing amount up to 48 hr p.i. At 72 hr p.i., when cytopatic effects become visible, the amount of VP2/VP3 is diminished in the cytoskeletal fraction. Although there are viral proteins in the soluble fraction [3], no significant amount of VP2/VP3 is detected by the antipeptide serum. It is not very probable that the content of VP2/VP3 is below the limits of detection of the applied method, although the ratio of VP1 to VP3 is reported to be 6.5 to 1 [22]. Therefore, these data support the thesis that the majority of the cytoplasmic VP2/VP3 is anchored to an insoluble structure [3,23].

The antipeptide serum is not able to detect VP2/VP3 in virions, though they were present at later stages of infection in the cytoplasm [24]. This result is confirmed by immune electron microscopy: No coating or aggregation of virus particles by the antipeptide serum could be observed, indicating that the carboxy-terminals of VP2 and VP3 are not accessible in SV40 virions.

For further investigations, a specific antibody directed against the amino-terminal region of VP2 would be helpful. This antibody would provide us with the possibility to distinguish between VP2 and VP3. Work along this line is in preparation.

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