Studies on Proteins of Simian Sarcoma-associated Virus with Different Growth History

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SUMMARY

Simian sarcoma-associated virus (SSaV) was repeatedly passaged on three human cell lines. The proteins of the progeny virus were analysed for the presence of variant polypeptides. Occasionally, a few variant polypeptides were observed. One-dimensional peptide maps of the major virus protein p30 revealed no modifications after 25 cycles of infection on the three cell lines studied. The peptide map of Pr65 gag of virus grown through 25 passages on a human chondrosarcoma cell line was slightly different from that of the virus stock before passaging. The relative amount of the virus protein p30 as compared to p18 and p16 (possibly the SSaV equivalents of p15E and p12E) was variable depending on the host cell. Virus grown on Daudi cells was relatively deficient in p18 and p16. These virus particles were morphologically altered and had a low infectivity.

INTRODUCTION

In the recent past, a significant effort (Gallo & Wong-Staal, 1980) has been made to detect human retroviruses, as such viruses might be involved as causative factors in human cancer. A number of investigators have described the isolation of simian sarcoma-associated virus (SSaV)-like viruses from human cells (Gallagher & Gallo, 1975; Panem et al., 1975; Nooter et al., 1977). The close similarity of these human virus isolates to SSaV, a virus isolated from a woolly monkey (Wolfe et al., 1971), has led to speculation that these human retrovirus isolates could be SSaV that was somehow present in the cells from which the human isolates were recovered, and that any differences found between SSaV and the SSaV-like human isolates (Sahagan & Haseltine, 1979, 1980) might be due to differences in the history of the isolates, in particular with regard to the cell types on which the viruses had been grown. Growing viruses on different host cells is an established procedure in preventive medicine to obtain attenuated strains suitable for vaccines. Such attenuated strains are mutants of the parent virus adapted to the new host cell.

When retroviruses are grown on cells different from the normal host cell, mutations related to adaptation to the new host may also occur. In addition, recombinants may occur between the exogenous infecting virus and endogenous virus that may exist in the host cell. The recombinants may possess selective advantages when grown on certain cells (Schindler et al., 1977). Recombinants between exogenous and endogenous viruses can have proteins with altered mol. wt.

It has been found that the mol. wt. of gp70, p30 and p15 from N- and B-tropic virus are slightly different and that recombinants can be recognized on the basis of these differences (Schindler et al., 1977). If human cells do contain endogenous retrovirus, then recombination of related genomes with the exogenous SSaV infecting virus might also occur, and if an
alteration in the peptide maps of p30 was found this would be highly suggestive of a recombination event, as the p30 of SSaV and the related gibbon ape leukaemia virus (GaLV) have identical peptide maps, demonstrating the conservation of this protein (Oroszlan et al., 1977).

The current study had two purposes: (i) to investigate the stability of SSaV grown on various human cell lines; (ii) to see whether evidence could be found, after growing SSaV on human cells, to suggest recombination events with any endogenous human retrovirus genes.

METHODS

Virus. SSaV-infected HF cells from marmosets were obtained from Dr Deinhardt, Max von Pettenkofer Institut, University of Munich, W. Germany. The virus was purified by limiting dilution: dilutions of HF medium were used to infect Daudi cells. Cells infected with the highest dilution giving evidence of virus production as measured by reverse transcriptase tests were grown, and medium from these cells was diluted once more and used to infect Daudi cells. Cells infected with the highest dilution giving rise to virus production were designated Dcl cells and medium from these cells was used to start infections on other human cell lines.

Cells and media. CF2TH cells, from dog thymus were obtained from the National Cancer Institute, Bethesda, U.S.A., through the courtesy of Dr J. Gruber. Daudi (D) cells, a human lymphoblastoid cell line containing Epstein–Barr virus (EBV), were derived from a Burkitt lymphoma (Klein et al., 1967). Heem (H) cells were established in our laboratory from a human chondrosarcoma (De Man et al., 1977). R cells were established in our laboratory from a human rhabdomyosarcoma. D cells were grown in RPMI 1640; the other cell lines were grown in minimal essential medium (MEM). All media were supplemented with 10% foetal calf serum.

Serial infection protocols. D, H and R cells were infected with cell-free Dcl medium. After the medium became reverse transcriptase-positive (incorporating $[^3H]TTP > 1$ pmol per ml medium per h), this medium was used to infect cells in one of the following ways. Protocol L: 0.1 ml of cell-free, virus-containing medium was used to infect $10^6$ cells (of the same cell type). The cells were grown until the medium became reverse transcriptase-positive. Then the procedure was repeated. The m.o.i. (as determined by infectivity tests) in this protocol was between 0.01 and 0.1. Protocol H: 10 ml of cell-free, virus-containing medium was used to infect $10^6$ cells. The m.o.i. in this protocol was between 1 and 10. After 24 h the medium was replaced and the cells were grown until the culture became reverse transcriptase-positive. Then the procedure was repeated. The number of repeated cycles on various cell types is designated as follows: HL15, protocol L repeated 15 times on H cells. DH6, protocol H repeated 6 times on D cells. Infection of CF2TH cells was done according to protocol H.

Virus labelling and purification. Virus was labelled by growing infected cells in the presence of $[^3H]leucine (specific activity 185 kBq/mg), 1.1 kBq (30 μCi) per ml medium in MEM without leucine in the presence of dialysed foetal calf serum. For labelling of glycoprotein $[^3H]galactose at 0.37 kBq (10 μCi) per ml medium was used. The cells were grown overnight, medium was removed and the cells were grown for an additional 24 h on complete medium. The combined cell-free media ($±5$ ml) were layered on a discontinuous sucrose gradient consisting of a bottom layer of 2 ml of 50% sucrose in 0.01 M-tris–HCl pH 7.4 + 0.15 M-NaCl (TN) and a top layer of 3 ml 20% sucrose in TN. The tubes were centrifuged in a SW41 Spinco rotor for 90 min at 35 000 rev/min at 4°C. The material in the interphase was collected, diluted with TN and layered on top of a continuous sucrose gradient ranging from 20 to 40% sucrose in TN. After overnight centrifugation at 20000 rev/min the radioactive virus peak was collected. Carrier protein was added and the proteins were
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Precipitated with trichloroacetic acid (TCA). The pellets were washed with ether and dissolved in Maizel’s sample buffer (Maizel, 1971).

Polyacrylamide gel electrophoresis (PAGE) and autoradiography. Gel electrophoresis was performed according to Maizel (1971) on SDS-acrylamide gels with 12.5% acrylamide. Peptides from enzymic digests of virus proteins were electrophoresed on 17.5% acrylamide gels. After electrophoresis the gels were treated with En3Hance (New England Nuclear), dried and autoradiographed on Kodak XR-1 films at -70 °C.

Mol. wt. estimation. The mol. wt. of proteins were estimated graphically from plots of $R_f$ against log mol. wt. Markers used were conalbumin 86K, bovine serum albumin 68K, catalase 60K, ovalbumin 43K, carbonic anhydrase 29K, horse apoferritin 18.5K, lysozyme 14.3K.

Enzymic digestion of virus proteins. Radioactive bands of virus proteins were cut from the gels. The gel pieces were rehydrated and the proteins were extracted with 0.05 M-(NH₄)₂CO₃. The extract was lyophilized, 30 µg of bovine serum albumin was added and the protein mix was digested completely by chymotrypsin A (Sigma) or Staphylococcus aureus V8 protease (Miles Laboratories) according to the procedure of Cleveland et al. (1977). These digests were analysed by gel electrophoresis and autoradiography as stated above.

Infectivity tests. These were carried out by infecting cells with serial twofold dilutions of virus-containing medium harvested 4 h after being placed on the cells; 24 h after infection the medium was replaced by fresh medium. The infected cells were diluted twice weekly. After 3 to 4 weeks the cultures were assayed for reverse transcriptase activity.

Reverse transcriptase tests (see Van Muijen et al. 1979). Radioimmune assays for viral p30 (Herbrink et al., 1980) and electron microscopy (Mooren et al., 1980) were carried out as described previously.

Results

In the experiments shown in Fig. 1 (a) no differences in the mol. wt. of virus proteins were observed. The virus population Dcl used to start the serial infection experiments was deficient in proteins p18 and p16 when compared to virus populations that had grown on H or R cells. All virus preparations from H or R cells tested had abundant p18 and p16. The ratio of radioactivity in the gels in the p18 region as compared to the p30 region of virus from H cells varied in different experiments between 0.7 and 1.1, whereas this ratio in virus from D cells varied from 0.12 in Dcl virus to 0.31 in DH25 virus (Fig. 1 a, lanes 5 to 8).

To see whether the observed differences in relative amounts of p18 and p16 were related only to the host cell – as suggested by the fact that Dcl virus grown for one passage on H cells did contain p18 – the serially passaged virus populations from D, H and R cells were grown on CF2TH cells. As can be seen in Fig. 1 (b, lanes 1, 4) CF2TH cells infected with virus from Dcl yielded virus that was not deficient in p18 and p16. When Dcl virus was grown for one passage on H cells, the relative amount of p18 was also increased over that in Dcl virus (data not shown). Therefore, the low amount of p18 and p16 present in Dcl virus is not related to genetically altered virus but is related to some host cell factor.

Considering the large differences in relative content of p18 and p16 it was considered possible that the morphology of Dcl virus would be different from that of virus grown on H cells. By electron microscopy it was found that Dcl virus contained numerous particles with incompletely closed cores and retained immature features (data not shown). In virus from Dcl we also observed morphologically normal immature and mature virus particles. DH25 virus also contained particles with incompletely closed cores.

We tested whether infectivity of Dcl virus indicated a deficiency in this virus stock. When Dcl virus was compared to HH15 virus for infectivity on D or H cells it was found that Dcl virus with the same quantity of p30 and reverse transcriptase as HH15 virus was 40 times less infectious on both D and H cells. No systematic study of the infectivity of passaged virus
stocks was made. As stated before, we did not find major virus proteins with altered mobilities when comparing virus passaged up to 25 times in D, H or R cells. However, when these stocks were used to infect CF2TH cells we found proteins with an altered mobility in a few cases. When DH6 virus was grown on CF2TH cells a p22 protein appeared (see Fig. 1b, lane 3). In addition, small variations in mol. wt. of a protein p70 were observed (see Fig. 1b). The p70 proteins could not be labelled with [3H]galactose and were precipitable with antiserum to SSaV p30, suggesting that the p70 proteins are not the SSaV equivalent of gp70 but correspond to Pr65agg proteins. SSaV gp70 was almost completely absent from our purified virus preparations and was found mainly in the medium above virus-producing cells (data not shown).

To evaluate any qualitative alterations one-dimensional chymotryptic and V8 maps were made. No differences were observed between maps of p30 of the starting Del stock and virus passaged 25 times through H, R or D cells with protocols L and H (only a few digests are shown; see Fig. 2a, lanes 5 to 8, Fig. 2b, lanes 1 to 3). However, in one-dimensional maps of
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Fig. 2. SDS-PAGE of digests of proteins from SSaV with different growth histories. (a) Digests of *Staphylococcus* protease V8. Cells infected with virus passaged on: lanes 1 and 2, H cells according to protocol L, repeated 16 and 25 times respectively; lanes 3 to 5, D cells according to protocol cl, not repeated; lane 6, D cells according to protocol H, repeated 15 times; lane 7, D cells according to protocol L, repeated 12 times; lane 8, H cells according to protocol L, repeated 16 times. Approx. mol. wt. of virus bands digested were: lanes 1 to 4, p70; lanes 5 to 8, p30. (b) Chymotryptic digests. Cells infected with virus passaged on: lane 1, R cells according to protocol L, repeated 16 times; lane 2, R cells according to protocol H, repeated 22 times; lane 3, D cells according to protocol cl, not repeated; lanes 4 to 6, H cells according to protocol H, not repeated. Approx. mol. wt. of virus bands digested were: lanes 1 to 3, p30; lanes 4 to 6, p18, p16 and p13 respectively.

p70 from HL16 and HL25 a few bands appeared that were not present in p70 from the parent Dcl virus preparation (see lanes 1 to 4 in Fig. 2a).

The proteins p18 and p16 are related, since five out of seven bands present in a partial chymotryptic digest of p18 were also found in p16 (see Fig. 2). No similarity was found between p30-derived peptides and p18 and p16 peptides. In agreement with this observation we found that p18 and p16 were not precipitated by anti-SSaV p30 antiserum.

**DISCUSSION**

In our experiments virus was passaged through a number of different cells. Adaptations to these cells might lead to the selection of mutants. Recombinants with endogenous viruses might also occur. Two protocols for infecting the cells were followed. Although both protocols were aimed at selecting adapted variants, it was considered that using protocol H there was less chance of losing a variant with a low relative advantage as, at the end of each growth-infection cycle, the virus-containing medium was only diluted twofold.

The method of analysis permits only the detection of variant forms that have become dominant in the virus population and that have either proteins with altered mol. wt. or altered
one-dimensional peptide maps, and it will, therefore, seriously underestimate the actual number of mutated virions. In fact, we only found proteins with an altered mol. wt. when the passaged virus preparations were used to infect a totally different cell. Possibly only in this situation did certain mutants have the ability to become the dominant species. In one case, when CF2TH cells were infected with DH6 virus, we found that a new protein p22 appeared. At present we do not have data on the peptides in p22.

In a few other cases we found a slight alteration in proteins p70. The p70s presumably are the SSaV equivalent of the Pr65\textsuperscript{gag} precursor to murine virus p30 proteins, as they could be precipitated by anti-SSaV p30 antibody. Pr65\textsuperscript{gag} is often found in murine leukaemia virus preparations (Lu et al., 1979). One-dimensional peptide maps of p30 were identical in the original virus stock as compared to virus passaged 25 times through H, D and R cells, confirming the conservative nature of SSaV p30 (Oroszlan et al., 1977). The absence of altered peptides in the p30 digests indicates that no recombination has occurred with endogenous retroviral p30 genes that might be present in human cells. Five out of seven peptides from p18 were identical to those of p16 but differed from those of p30 (see Fig. 2b). The similarity of the peptides of p18 and p16 suggests that these proteins are the SSaV equivalent of p15E and p12E of Rauscher MuLV (Karshin et al., 1977).

The peptide maps of p70 of passaged virus occasionally contained an additional band not present in the parent stock. If the p70 is indeed the SSaV equivalent of the Pr65\textsuperscript{gag} precursor, the mutation in the p70 is more likely to be in the small \textit{gag} proteins, as the p30 maps of passaged SSaV are identical. The small \textit{gag} proteins have not been systematically mapped due to low recovery from the gels. Considering the limited sensitivity in detecting mutants in the present study, the fact that we obtained any mutants at all after relatively few passages shows that this virus mutated at a fairly high rate. Conceivably, therefore, the differences observed between some human retroviral isolates and SSaV can have been generated since the discovery of SSaV. We have at present no data suggesting recombination of SSaV with any retrovirus genes present in human cells. Finally, we found a relative deficiency of p18 and p16 in virus grown on Dcl cells. This virus stock also had a low infectivity. Under the electron microscope many virus particles from Dcl had incompletely closed cores. Possibly, these three observations have a common but as yet unknown cause.

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REFERENCES


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