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PARVOVIRUS CONTAMINATION OF A HUMAN TYPE 12 ADENOVIRUS STRAIN

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Summary. — A strain of human type 12 adenovirus was found contaminated with virions 27 nm in diameter. After separation by membrane filtration, adsorption on to and elution from erythrocytes and heat treatment, the contaminant was classified as a parvovirus based on its biological and physico-chemical properties and virion morphology. This parvovirus failed to produce interferon and did not affect the interferon-inducing ability of the adenovirus from which it had been separated.

Key words: *parvovirus*; *adenovirus type 12*; *heamagglutination*; *interferon induction*

Introduction

It has been established that all adenovirus types induce interferon production in chick cells (Béládi and Puzstai, 1967; Mucsi *et al.*, 1970), but the quantity of interferon has been found type-dependent. Adenovirus types 12 and 8 proved to be the most effective inducers (Pusztai *et al.*, 1969). When one of the type 12 strains was studied by electron microscopy, virus like "small particles" of 27 nm in diameter were observed among the adenovirions.

The present paper describes the experiments undertaken to isolate and characterize these small particles and to determine whether they play any role in the interferon induction by adenovirus type 12.

Materials and Methods

Cell cultures. Adenovirus strains and "small particles" were propagated in HEp-2 cells. Primary chick embryo cell (CEC) cultures and AV_3 cells were used for interferon assays. In each case the culture medium was Gey's solution with 5 % calf serum and 0.25 % lactalbumin hydrolysate. During adenovirus growth the culture medium was supplemented with 252 μg/ml arginine.

Viruses. One type 12 adenovirus strain (Ad 12K) was obtained from Dr. K. Köhler (Max-Planck Institut für Virusforschung, Tübingen), another (Ad 12P) was kindly provided by Dr. H. G. Pereira (then at the National Institute for Medical Research, Mill Hill, London), and the third strain (Ad 12 HUIE)'was obtained from the Karolinska Institute (Stockholm). Only Ad 12K strain contained the small particles. Sindbis virus and vesicular stomatitis virus (VSV) were used as challenge viruses to evaluate the titre of interferon in CEC and human cells respectively.

To separate the small particles from the adenovirions, Ad 12K virus was heated at 60 °C for 60 min and then neutralized with rabbit immune serum to 12P adenovirus. After these treatments only the small particles remained viable and the virus strain was designated PVSz. The cytopathic effect (CPE) of PVSz was studied in HEp-2 cells using 0.2 $TCID_{50}$ of virus per cell.

Haemagglutination (HA) and haemagglutination inhibition (HI) tests were done in a Takátsy's microtitrator or in WHO plates at 4 °C. Human group 0, guinea pig, rat, pig, sheep, monkey and chicken red blood cells (RBC) were used in 0.75 or 1 % suspensions. In HI tests, serial serum dilutions were incubated overnight with 8 haemagglutination units (HAU) of virus at 4 °C; then 1 % human erythrocyte suspension was added and the results were read after 2 hr incubation at 4 °C.

Immune sera to adenovirus 12K, 12P and 12 HUIE as well as to adenovirus type 5 hexon were produced in rabbits.

Interferon production in CEC was described (Béládi and Pusztai, 1967). Interferon was titrated by the plaque reduction method (Pusztai *et al.*, 1969). To determine whether PVSz induces interferon production in human leukocytes, the method described by Cantell (1974) was used. In this case the titre of interferon was evaluated by inhibition of the CPE of VSV in AV_3 cells in Limbro microplates.

Purified PVSz virus was prepared from HEp-2 cultures. The infected cells were subjected to 5 cycles of freezing and thawing, incubated at 37 °C for 20 min and subsequently the cell debris was eliminated by low speed centrifugation (2000 × g, 10 min). The supernatant was layered onto a CsCl cushion of a 1.5 g/cm³ density and centrifuged for 3 hr at 100,000 × g in a Janetzki VAC 60 ultracentrifuge. Fractions with HA activity were collected, the density adjusted to 1.38 g/cm³ with crystalline CsCl, and centrifuged for 48 hr at 120,000 × g in an MSE Superspeed 50 ultracentrifuge. Fractions collected by puncturing the bottom of the tubes were dialysed against 0.01 M Tris buffer (pH 8.1) overnight.

Anion exchange chromatography of Ad 12K and PVSz was performed on DEAE-Sephadex A-25 (Pharmacia) columns by stepwise elution with NaCl containing 0.04 M Tris-HCl buffer according to Lengyel and Nász (1970). The presence of adenovirus antigens in the fractions was demonstrated by gel diffusion precipitation with immune sera to Ad 5 hexon and Ad 12 HUIE virus. The fractions were tested for HA activity and the extinction values at 280 nm were measured.

Electron microscopy. A JEOLCO JEM type 100 B electron microscope was used. Samples of purified virus were examined after negative staining with 2 % uranyl acetate. Thin sections of virus-infected HEp-2 cells were examined as described (Béládi *et al.*, 1974).

Results

Ad 12K was purified by density gradient centrifugation and the fractions were studied by electron microscopy. In fractions of 1.32—1.34 g/ml density, numerous small icosahedral virus particles 27 nm in diameter were observed among the adenovirions (Fig. 1). The small particles were also found in the nuclei of the Ad 12K-infected HEp-2 cells (Fig. 2).

The Ad 12K strain which contained the small particles displayed a high HA activity (HA titre 512—2048) at 4 °C with human and guinea pig RBC and a lower activity (HA titre 128—256) with pig RBC while Ad 12P showed no HA with the same RBC. No HA activity was detected with RBC of other species (chicken, rat, rhesus monkey, sheep) examined. Nor did HA occur either at room remperature or at 37 °C. When RBC agglutinated at 4 °C were exposed to temperatures between 20 and 37 °C, HA disappeared. Agglutination reoccurred when the RBC were chilled again to 4 °C.

The HA activity of Ad 12K was not affected by heating at 60 °C for 60 min. Treatment of the virus material with 10 μg/ml trypsin (Difco 1 : 250) did not influence its HA activity. Rabbit sera against Ad 12K inhibited the HA up to

dilutions from 1 : 2560 to 1 : 5120, whereas rabbit immune sera to Ad 12P and 12 HUIE had no HI effect even at a dilution of 1 : 10.

The small particles were completely separated from the adenovirions by Millipore filters (pore size 0.45, 0.22, 0.1 and 0.05 μm), but this method resulted in a considerable loss of the small particles.

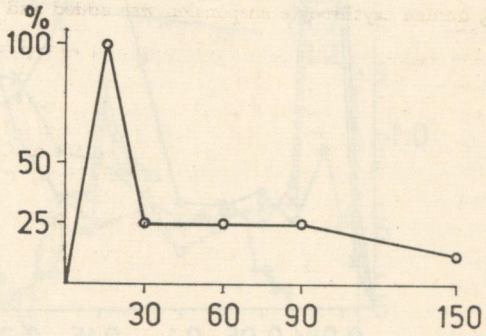

Fig. 3.
Purification of PVSz by haemadsorption-elution
Abscissa: time in min; ordinate: haemagglutination of the eluate in % of that of the original virus material

Large amounts of small particles were successfully separated from adenovirions by haemadsorption-elution. Washed human RBC were added to Ad 12K virus material to a final concentration of 20% and the suspension was incubated at 4 °C for 2 hr. The supernatant was separated by low speed centrifugation, the erythrocytes resuspended in phosphate buffered saline (PBS) pH 7.4 and incubated at 37 °C. The small particles were eluted at this temperature and after centrifugation they were found in the supernatant. The quantity of eluted particles varied with incubation time at 37 °C (Fig. 3). Complete recovery of haemagglutinin was obtained only at 15 min; prolonged incubation resulted in a considerable decrease of the HA titres. We assumed that after 15 min at 37 °C membrane debris containing virus-binding receptors was released from the RBC and that this caused the inhibition of HA. To confirm this assumption a HI test was performed at 4 °C by adding 8 HAU of Ad 12K virus to serial dilutions of the supernatnt of an RBC suspension previously incubated at 37 °C for 2 hr. The supernatnat inhibited the HA of virus up to dilutions from 1 : 64, 1 : 128. The complement fixation test was positive with the same RBC supernatant and an immune serum against MN

Table 1. Purification of the PVSz by haemadsorption-elution combined with trypsin treatment

Virus	HA titre	
	Untreated	Trypsin-treated
Original Ad 12K	1024	2048
Supernatant after adsorption	0	64
Eluate at 37 °C	256	2048

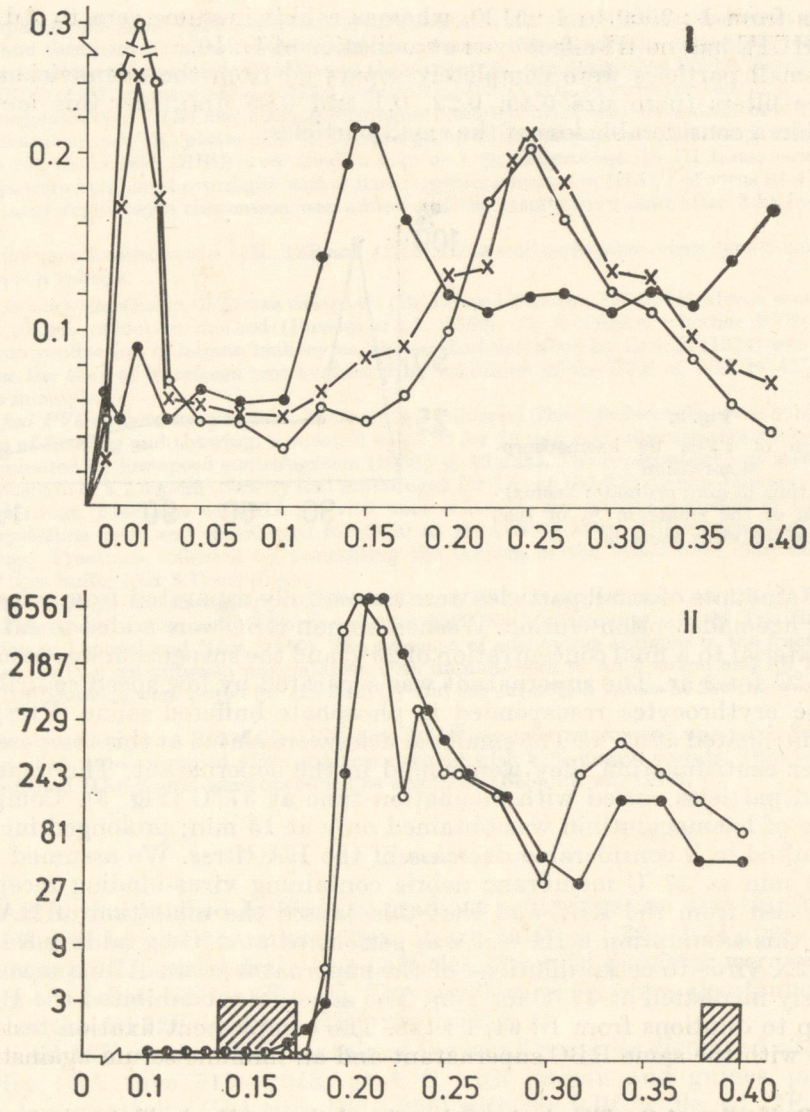

Fig. 4.

Anion exchange chromatography of Ad 12K virus (●) and the eluate obtained after purification by haemadsorption-elution (○)

× — Supernatant of RBC suspension after incubation at 37 °C. Shaded areas: location of type- and group-specific adenovirus antigens

Abscissae: NaCl molarity

I — E_{280} values

II — HA titres

erythrocyte antigens (Ortho). This confirmed the presence of virus-binding membrane debris in the RBC supernatant kept at 37 °C for 2 hr. HA inhibiting membrane fractions could be eliminated by centrifugation at 10000 × g for 30 min.

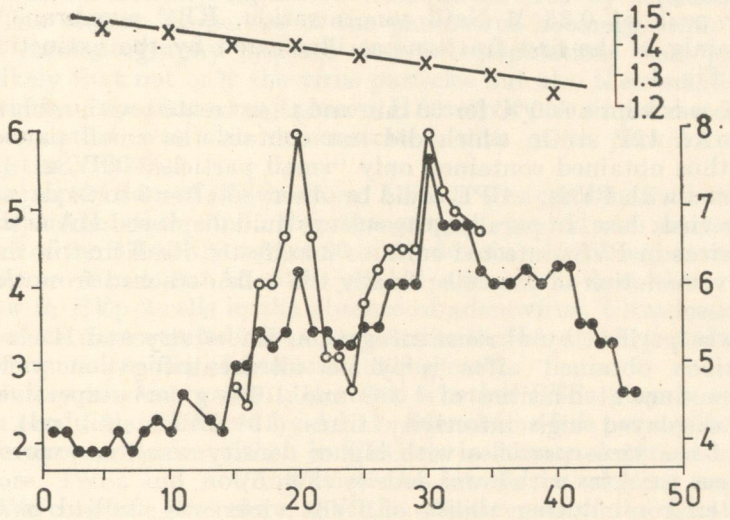

Fig. 5.

Distribution of haemagglutinating activity and infectivity of PVSz in a CsCl density gradient Abscissa: fraction number; left ordinate: HA titre ($\log_{10}$ values; ●); right ordinate: infectivity ($\log_{10}$ $TCID_{50}$/0.1 ml; ○); × — density (g/cm³)

Erythrocyte receptors which bound the small particles proved to be sensitive to the effect of trypsin and receptor destroying enzyme. Ad 12K did not agglutinate human RBC when a 20% suspension of the erythrocytes was treated with 20 mg/ml trypsin or with 400 units/ml of *Vibrio cholerae* neuraminidase (Microbiological Associates) at 37 °C for 1 hr.

As trypsin destroyed the virus-binding RBC receptors but did not influence the HA activity of Ad 12K virus, combination of the haemadsorption-elution method with trypsin treatment yielded large amounts of small particles. Trypsin treatment (5 mg/ml) of the original Ad 12 virus resulted in a further increase of the HA activity, very likely by elimination of an HA inhibiting substance from the virus material. When after 1 hr icubation the eluted material was treated with trypsin (5 mg/ml) for an additional 1 hr, the HA activity was identical to that of the trypsin-treated original virus (Table 1).

Virus material obtained by haemadsorption-elution combined with trypsin treatment contained no adenovirus. This eluate was assayed by anion exchange chromatography and compared with the elution pattern of the original Ad 12K material (Fig. 4). The sites of type-specific and group-specific adenovirus

antigens were detected by gel diffusion precipitation. The eluate contained no adenovirus antigens but the haemagglutinins showed identical elution profiles in both materials. The small particles were eluted by 0.2 M NaCl concentration following the elution of type 12 adenovirus hexon. The HA titres indicated the presence of two types of haemagglutinin in the eluate with a smaller peak at 0.35 M NaCl concentration. RBC membrane fractions eluted mainly in the first fractions as illustrated by the extinction values shown in Fig. 4—I.

Ad 12K was kept at 60 °C for 60 min and then treated with rabbit immune serum to Ad 12P strain which did not contain the small particles. The material thus obtained contained only "small particles" (PVSz). In HEp-2 cells infected with PVSz, a CPE could be observed after 3 to 24 days depending on the virus dose. In parallel, the culture fluid displayed HA activity. The effect of virus in PVSz-infected cultures manifested itself first in the elongation and vacuolation of the cells; finally the cells detached from the wall of culture vessel.

PVSz was purified by ultracentrifugation. Infectivity and HA activity of the fractions obtained after isopycnic ultracentrifugation yielded two distinct maxima at densities of 1.398 and 1.345 g/cm^3 respectively. Both fractions displayed high infectivity titres (10^8 $TCID_{50}$/0.1 ml), but HA activity of the virus particles with higher density was less expressed than that of virus particles with lower density (Fig. 5).

The interferon-inducing ability of PVSz virus was studied in CEC and human leukocytes. PVSz (0.1 $TCID_{50}$/cell) produced no interferon and did not influence the interferon-inducing capacity of the Ad 12P strain in CEC. The titre of interferon induced by Ad 12P (0.02 $TCID_{50}$/cell) was 2048. Similarly, PVSz failed to induce interferon in human leukocytes.

Discussion

Of human adenoviruses, type 12 has been the most potent inducer of interferon production (Pusztai *et al.*, 1969; Mucsi *et al.*, 1970). We found that the type 12 strain (Ad 12K) used in interferon experiments was contaminated with icosahedral virus particles 27 nm in diameter, designated PVSz.

Considering that the multiplication site of PVSz is the cell nucleus as revealed by electron microscopy, furthermore the resistance of the strain to heat and its HA capacity, it seems likely that PVSz may belong to the parvovirus group.

As PVSz was found in association with adenovirus and showed distinct HA activity, it could be assumed that either type 4 (Ito and Mayor, 1968) or a bovine type of adeno-associated virus (Luchsinger *et al.*, 1970) has been involved. This hypothesis, however, was ruled out when PVSz has been found to grow in HEp-2 cells in the absence of adenovirus.

Based on its HA properties PVSz could not be identified with any of the known parvovirus species (Siegl, 1976). But serological examinations of PVSz (Hl, complement fixation and immune electron microscopy) by Dr. D.

Hoggan (National Institutes of Health, Bethesda) have shown a close relationship with Kirk and HS-3 viruses (Berquist *et al.*, 1972; Mircovic *et al.*, 1971) and revealed some similarities to Kilham rat virus, too (personal communication, 1978). It is very likely, therefore, that PVSz is a variant of Kirk and HS-3 viruses.

PVSz was successfully separated from adenovirus by haemadsorption-elution. The elution properties of the parvovirus haemagglutinin in anion exchange chromatography indicate that two populations were present. It appears likely that not only the virus particles but also their smaller constituents, perhaps capsomers, may possess HA activity. A similar heterogeneity of the population was also demonstrated with other parvovirus strains (Neurath *et al.*, 1969).

For the separation of PVSz from Ad 12, the combined techniques of haemadsorption-elution and chromatography proved to be successful. Incubation of the Ad 12K strain at 60 °C for 60 min and subsequent treatment with immune serum to Ad 12P yielded adenovirus-free virus material. PVSz grew in HEp-2 cells in the absence of adenovirus. Ultracentrifugation of PVSz virus demostrated virus particles with HA activity and ifectivity at two different densities (1.345 and 1.398 g/cm^3).

Our primary interest was to determine whether PVSz plays a role in the interferon production onduced by Ad 12. Adenovirus-free parvovirus induced no interferon production either in CEC or in human leukocyte cultures; furthermore, PVSz did not influence the interferon-inducing capacity of Ad 12P. We conclude, therefore, that the effectiveness of the Ad 12K strain in interferon induction was not related to the presence of the associated parvovirus.

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Explanation of Electron Micrographs (Plate II):

Fig. 1. Negatively stained preparation of Ad 12K; numerous parvovirus particles and one adenovirus particle (indicated by arrow). Length of bar = 0.1 μm.

Fig. 2. HEp-2 cell infected with Ad 12K. An aggregate of PVSz particles (thick arrow) and adenovirus particles (thin arrow) are seen. Length of bar = 0.2 μm.