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DIFFERENT INTERFERON-INDUCING ABILITY OF HUMAN ADENOVIRUS TYPES IN CHICK EMBRYO CELLS

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Summary. — Human adenovirus (Ad) types differ in their ability to induce interferon (IFN) in chick cells. Of 12 types investigated, Ad8, Ad12, Ad 18 and Ad 31 proved to be more effective IFN inducers than Ad2, Ad3, Ad4, Ad5, Ad6, Ad7, Ad15 and Ad19. Ultraviolet (UV) irradiation decreased the IFN-inducing ability of the more effective inducers only, indicating that transcription of viral DNA might play a role in IFN induction by these types. DNAs isolated from Ad2, Ad5 and Ad12 alike induced low amounts of IFN in chick cells. The IFN-inducing capacity of phage DNA was similar to that of adenovirus DNA, but induction by non-viral DNA (prokaryotic and eukaryotic) did not result in detectable IFN production. It is assumed that viral DNA and virus particles promote IFN production in different ways. Probably the viral component responsible for IFN induction by the effective Ad types differs from those having lower IFN-inducing ability.

Key words: *interferon*; *adenoviruses*; *UV inactivation*

Introduction

As described previously, human adenoviruses (Ad) induced IFN in chick cells (Béládi and Pusztai, 1967). The different degree of IFN-inducing ability of human Ad types has been soon recognized (Pusztai *et al.*, 1969). Much effort was devoted to the attempts at elucidation of these differences associated with the mechanism of IFN induction by Ad. However, neither the viral component responsible for IFN induction nor the events involved in this process have been cleared yet.

In the present communication we report on the results of systematic investigation of IFN-inducing abilities of human adenovirus types. Our results revealed that human Ad types fall into two groups according to their IFN-inducing abilities. The effective inducers were further characterized as to the sensitivity of their IFN-inducing ability to UV irradiation and low infectivity in human cells. In contrast, types that were less effective IFN inducers proved to be UV radiation-resistant in respect of IFN induction and had higher infectious titre. The involvement of viral DNA in IFN induction was studied with the representatives of both more and less efficient inducers.

Materials and Methods

Cell cultures. Ad strains were propagated in HEp-2 monolayer cells or in suspension culture of KB cells. Primary and secondary chick embryo cells (CEC) were used for IFN production and assay.

Viruses. Ad types 2, 3, 4, 5, 6. 7, 12, 15, 19 and 31 were kindly provided by Dr. H. G. Pereira, National Institute for Medical Research, London. Ad18 was obtained from Dr. Wigand, Institut für Hygiene und Mikrobiologie der Universität des Saarlandes, Homburg. Ad 8 was isolated in our laboratory (Béládi *et al.*, 1963). The crude virus material was obtained by repeated freezing and thawing of the infected cells in the original culture medium. The suspension was clarified by low-speed centrifugation. Vesicular stomatitis virus (VSV), Indiana strain, was used as challenge virus to evaluate the IFN titre.

Purification of adenoviruses. Virus was purified from infected cells by fluorocarbon treatment and by repeated cycles of equilibrium centrifugation in CsCl density gradients (Tóth *et al.*, 1982).

Cytotoxicity test. Infected and control CEC cultures were pulse-labelled at 1, 3, 9, 16, 24 and 48 hr post infection (p.i.). In methionine deprived medium 7.4 kBq/ml ^{35}S-methionine was added to cells and after one hour labelling the cell monolayers were washed and fixed with 6% trichloroacetic acid (TCA). The acid insoluble activity from the TCA-extracted cells was determined by dissolving the cells in 1 N NaOH and by counting appropriate samples in a Triton X containing scintillation liquid. The protein content of the samples was measured by the method of Lowry *et al.* (1951).

Isolation of DNA. DNA from Ad2, Ad5, Ad12 and λ-phage were prepared from purified particles as described by Pettersson and Sambrook (1973). Calf thymus and chick erythrocyte DNAs were purchased from Reanal Fine Chemicals (Hungary) and Serva GmbH (Heidelberg). DNAs were extracted from *E. coli* and KB cells according to Pettersson and Sambrook (1973).

IFN induction by adenovirus particles and adenovirus DNA. IFN induction by virions in CEC was performed as previously described (Béládi and Pusztai, 1967). DNA treatment of the cells was carried out by the calcium phosphate technique of Graham and Van der Eb (1973) in minor modification. Briefly, CEC were grown in 35 mm Petri dishes. Calcium phosphate precipitation of viral DNA was performed at room temperature for 30 min in 0.5 ml HEPES-buffered saline containing different amounts of calf thymus DNA and 125 mmol/l $CaCl_2$. The precipitate was added to each cell monolayer following removal of growth medium. After 20 min at room temperature, 2 ml of fresh medium containing 5% calf serum was added, cultures were incubated at 37 °C for 4 hr. The medium was removed and the monolayers were washed twice with PBS and overlayed with growth medium. After two days the supernatants were collected and tested for antiviral activity. HEp-2 cell monolayers were used to determine the infectivity of DNA as described by Chinnadurai *et al.* (1978).

IFN assay. IFN was titrated by micromethod monitoring protection of secondary CEC from the cytopathic effect of VSV. The IFN samples, which had previously been heated at 56 °C for 30 min, were serially diluted in Linbro flat-bottomed microtitre plates (96 wells per plate) in a volume of 100 μl of Eagle's medium containing 5% calf serum and antibiotics. Then 10^5 CEC in 25 μl volumes were placed in each well and the plates were incubated for 20 hr in a CO_2 incubator. The cells were next challenged with VSV (6×10^4 PFU/well). IFN titres were compared to a reference standard chick IFN preparation (MRC Research Standard A, 62/4, Mill Hill, London), and were expressed as international units (IU) per ml.

Infectivity assay. Plaque assay in HEp-2 cells was used for quantitation of the infectivity (Williams, 1970). At the end of the incubation period the cell sheets were fixed with 10% TCA solution (5 ml per Petri dish, 20 min) and, after washing, they were stained with a 0.2% solution of crystal violet for 5 min. The plates were incubated either for 12 days in the case of Ad8, Ad12, Ad18 and Ad31, or for 10 days in the case of other types.

Detection of T antigen. An indirect immunofluorescence (IF) technique (Pusztai *et al.*, 1977) was used to examine the appearance of T antigen in CEC infected with Ad12 or transfected with Ad12 DNA.

UV irradiation. The source of UV-light was a Hanau germicide lamp. One ml portions of crude (without phenol red) or purified (diluted in 10 mmol/l Tris-HCl buffer, pH 8.1) virus samples were placed in 50 mm Petri dishes and irradiated in a dark room with 18.8 erg mm^{-2} sec^{-1} UV radiation dose. The dishes were shaken continuously (30 rev/min) during irradiation.

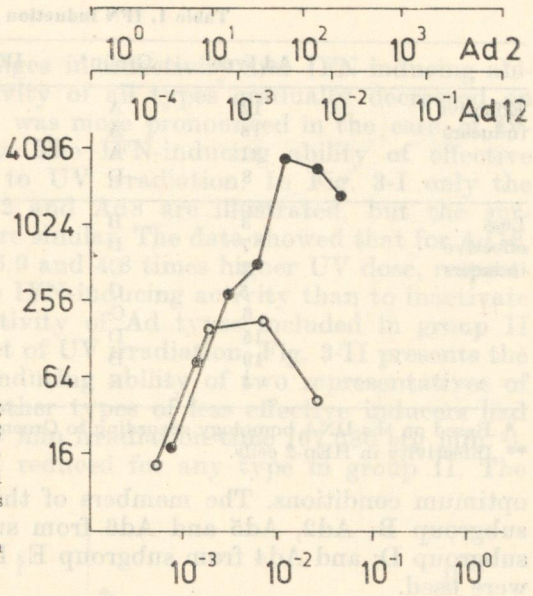

Fig. 1.
Dose-dependent response curve relating the quantity and multiplicity of Ad2 (○———○) and Ad12 (●———●) infections to the titres of IFN induced
Lower abscissa: quantity of virus/10^7 cells calculated from OD_{280} values.
Upper abscissa: MOI (PFU/cell) with each adenovirus separately.

Results

Induction of IFN by purified Ad2 and Ad12

CEC were infected with different quantities of Ad2 and Ad12 purified by isopycnic CsCl gradient centrifugation and the titre of IFN produced was determined. In the experiment shown in Fig. 1, similar quantities of two virus materials (measured as OD_{280}) were necessary to obtain maximum IFN yields, but Ad12 induced 21 times more IFN than did Ad2. The optimum multiplicity of infection (MOI) for IFN production were 0.003 PFU/cell for Ad12 and 50 PFU/cell for Ad2 respectively. The viruses differed also in specific infectivity as determined by plaque assay and adjusted to $OD_{280} = 1.0$ (3×10^6 PFU and 6×10^{10} PFU for Ad12 and Ad2, respectively).

IFN induction with different Ad types

Since Ad2 and Ad12 displayed different IFN-inducing abilities, an investigation was carried out to survey this ability of 12 different types. The adenovirus types investigated could be divided into two groups, according to their capacity to induce IFN (Table 1). The first group contained the effective inducers which induced hundreds of IFN IU/ml. All types of subgroup A (Ad12, Ad18, Ad31) and Ad8 from subgroup D belonged to this group. The MOI used for IFN induction was suboptimal ($2.8—7.5 \times 10^{-4}$ PFU/cell).

The titre of IFN induced by the types belonging to the group of the less effective inducers (group II) scarcely exceeded 100 IU/ml, even under

Table 1. IFN induction by human Ad

	Ad type	Group*	IF titre (UI/ml)	MOI	PFU/ml**
Effective inducers	12	A	444	4.0×10^{-4}	4.0×10^{4}
	18	A	760	7.5×10^{-4}	7.5×10^{4}
	31	A	336	2.8×10^{-4}	2.8×10^{4}
	8	D	417	2.8×10^{-4}	2.8×10^{4}
Less effective inducers	3	B	123	0.3	3.0×10^{7}
	7	B	100	0.1	1.0×10^{7}
	2	C	140	40	4.0×10^{9}
	5	C	87	45	4.5×10^{9}
	6	C	90	46	4.6×10^{9}
	15	D	115	3.9×10^{-2}	3.9×10^{6}
	19	D	84	3.5×10^{-2}	3.5×10^{6}
	4	E	105	4.0×10^{-2}	4.0×10^{6}

* Based on the DNA homology according to Green *et al.* (1979).
** Infectivity in HEp-2 cells.

optimum conditions. The members of this group were Ad3 and Ad7 from subgroup B; Ad2, Ad5 and Ad6 from subgroup C; Ad15 and Ad19 from subgroup D; and Ad4 from subgroup E. In these experiments crude viruses were used.

Cytotoxicity of Ad2

To evaluate the cytotoxic effect of viral infection, the efficiency of protein synthesis in infected and control cells were compared. CEC cultures infected with Ad2 were pulse-labelled with ^{35}S-methionine at appropriate times p.i. Pulse-labelling of non-infected cells was done in parallel. The cpm/mg protein values of acid insoluble samples were compared. As follows from the results of a representative experiment (Fig. 2), the Ad2 infection had no significant effect on the cellular protein-synthesis.

UV irradiation sensitivity of infectivity and IFN-inducing ability of more and less effective inducers

The more and less effective inducers differed not only in IFN-inducing ability and specific infectivity, but also in the sensitivity of their IFN-inducing capacities to UV irradiation (Fig. 3). The viruses listed in Table 1

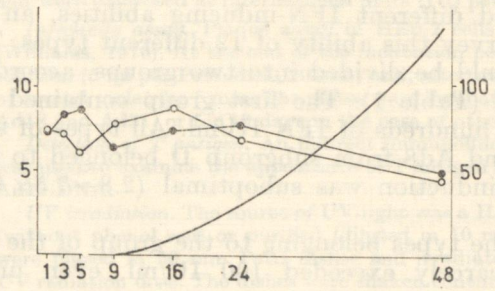

Fig. 2.
Effect of Ad2 infection on ^{35}S-methionine incorporation into acid insoluble fraction of CEC
Abscissa: time p.i. (hr)
Left ordinate: IFN titre, IU/ml
Right ordinate: counts/min × protein $mg^{-1} \times 10^{-3}$
●——●: Ad2 infected cells,
○——○: control cells.
——————: IFN titres in the culture fluids at indicated intervals p.i.

were UV-irradiated and the changes in infectivity and IFN-inducing ability were examined. The infectivity of all types gradually decreased on UV irradiation, but the decrease was more pronounced in the case of types of higher specific infectivity. The IFN-inducing ability of effective inducers proved to be sensitive to UV irradiation. In Fig. 3-I only the results of experiments with Ad12 and Ad8 are illustrated, but the survival curves of Ad18 and Ad31 were similar. The data showed that for Ad12, Ad18, Ad31 and Ad8, a 5.0, 4.5, 5.9 and 4.8 times higher UV dose, respectively, is required to inactivate the IFN-inducing activity than to inactivate infectivity. The IFN-inducing activity of Ad types included in group II (Table 1) was resistant to the effect of UV irradiation. Fig. 3-II presents the survival of infectivity and IFN-inducing ability of two representatives of this group (Ad2 and Ad3). The other types of less effective inducers had similar survival curves. With a 60 min irradiation time (67,680 erg mm^{-2}), the IFN-inducing ability was not reduced for any type in group II. The

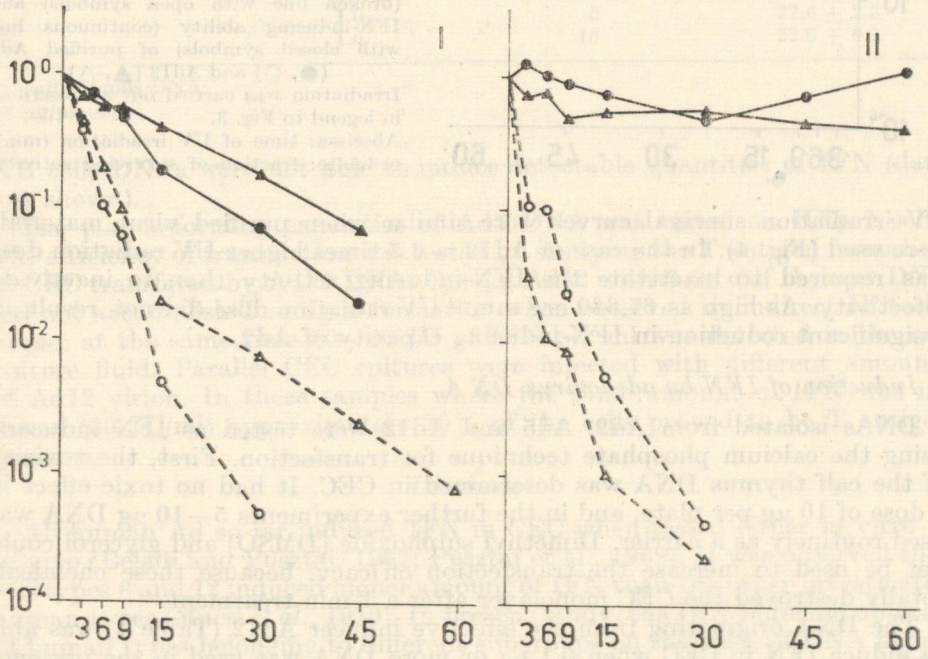

Fig. 3.
Effect of UV irradiation on the infectivity and IFN-inducing ability of effective (I) and less effective (II) inducers
Virus samples were irradiated for different periods and then tested for infectivity (broken line) in HEp-2 cells, and for IFN inducing ability (continuous line) in CEC.
I — Ad12 (●, ○) and Ad8 (▲, △) belong to subgroups A and D, respectively; II — Ad3 (●, ○) and Ad2 (▲, △) are members of subgroup B and C, respectively.
Abscissa: time of UV irradiation (min); ordinate: fraction of surviving activity.

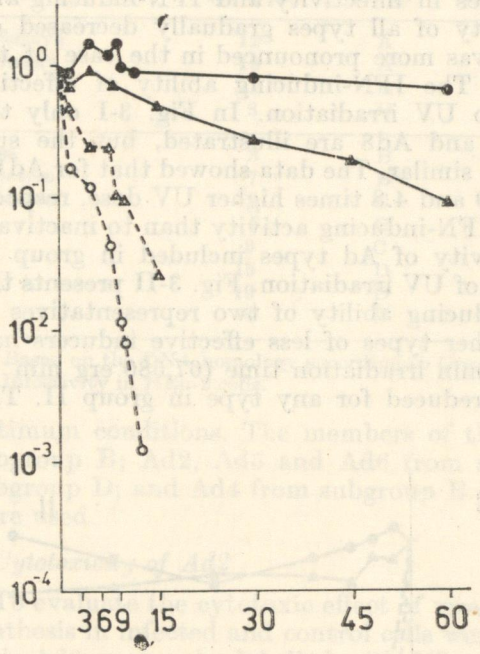

Fig. 4.

Effect of UV irradiation on the infectivity (broken line with open symbols) and IFN-inducing ability (continuous line with closed symbols) of purified Ad2 (●, ○) and Ad12 (▲, △). Irradiation was carried out as described in legend to Fig. 3. Abscissa: time of UV irradiation (min); ordinate: fraction of surviving activity.

UV irradiation survival curves were similar when purified virus materials were used (Fig. 4). In the case of Ad12, a 6.5 times higher UV radiation dose was required to inactivate the IFN-inducing activity than to inactivate infectivity. As high as 67,680 erg mm^{-2} UV radiation dose did not result in a significant reduction in IFN-inducing capacity of Ad2.

Induction of IFN by adenovirus DNA

DNAs isolated from Ad2, Ad5 and Ad12 were tested as IFN inducers, using the calcium phosphate technique for transfection. First, the toxicity of the calf thymus DNA was determined in CEC. It had no toxic effect in a dose of 10 μg per plate, and in the further experiments 5—10 μg DNA was used routinely as a carrier. Dimethyl sulphoxide (DMSO) and glycerol could not be used to increase the transfection efficacy, because these chemicals totally destroyed the CEC monolayer after a 1 min treatment.

The DNA originating from the effective inducer Ad12 (Table 1) was able to induce IFN in CEC when 0.1 μg or more DNA was used in the presence of 5 or 10 μg carrier DNA (Table 2). IFN was never detected in control cultures transfected with calf thymus DNA alone. The adenovirus DNA-induced material which inhibited the cytopathic effect of the challenge virus proved to be IFN, because it did not sediment on centrifugation at 100,000 × g for 90 min, it was resistant to pH 2 and it was destroyed by trypsin (1 mg/ml, 60 min). Similarly to Ad DNAs the phage DNA also induced IFN in CEC, whereas prokaryotic (*E. coli*) and eukaryotic (calf thymus, chick erythrocyte,

Table 2. IFN induction by Ad DNA

Inducer	Inducer DNA	Carrier* DNA	IFN titre (IU/ml) mean ± SD
Ad12 DNA	0.1**	5**	9.5 ± 4.2
	0.1	10	9.4 ± 3.8
	1.0	5	23.3 ± 6.3
	1.0	10	11.3 ± 3.2
Ad5 DNA	0.1	10	6.4 ± 2.0
	1.0	5	28.5 ± 2.1
	1.0	10	17.4 ± 0.6
Ad2 DNA	0.1	5	10.4 ± 5.8
	0.1	10	12.5 ± 0.5
	1.0	5	16.4 ± 4.6
	1.0	10	17.2 ± 3.4
λ-phage DNA	0.1	5	12.5 ± 2.1
	0.1	10	7.2 ± 3.1
	1.0	5	37.6 ± 2.3
	1.0	10	22.6 ± 6.1

* calf thymus DNA
** μg/plate

KB cell) DNAs were not able to induce detectable quantities of IFN (data not shown).

The plaque-forming capacities of Ad5 DNA was tested on HEp-2 cells. The efficiency of transfection was tested by studying the T antigen formation in CEC transfered by Ad12 DNA. One μg Ad12 with 5 μg calf thymus DNA did not accomplish T antigen formation when observed by indirect IF technique; at the same time 20—30 IU of IFN per ml was detected in the cell culture fluid. Parallel CEC cultures were infected with different amounts of Ad12 virion. In those samples where the same amount of IFN was induced (30 IU/ml) approximately 20% of the cells proved to be T antigen positive.

Discussion

All human Ad so far induced IFN in CEC when tested either in vitro or in vivo (Béládi and Pusztai, 1967; Pusztai et al., 1969). It was observed that serotypes 8 and 12 induced higher amount of IFN than the other investigated human types (Mucsi *et al.*, 1970). In present study, the IFN-inducing abilities of human types belonging to different subgroups on the basis of DNA homology were compared. It was found that types 18 and 31 were as good IFN inducers as type 12. Besides the types of subgroup A, only type 8 of subgroup D proved to be an effective inducer (Table 1). Interestingly enough, type 19 of subgroup D, which has a similar pathogenicity as type 8 (Hierholzer *et al.*, 1974; Green *et al.*, 1979) differed in its IFN-inducing ability from type 8. On the basis of IFN-inducing capacity, the human types could be divided into two groups: the first group consisting of effective inducers, i.e.

the types of subgroup A and type 8, another one containing all other types belonging to subgroup B, C, D and E (Table 1). Low infectivity in human cells is a striking common charasteristic of the effective inducers, while the types having higher infective titres are less effective inducers. In the light of this observation one would consider the possibility that higher infectious serotypes induce lower levels of IFN in CEC simply because they effectively shut off protein synthesis in these cells. This possibility, however, can be ruled out since Ad2 infection did not influence the protein synthesis in CEC during the period of IFN induction (Fig. 2).

Another common characteristic of effective inducers is the sensitivity of their IFN-inducing ability to UV irradiation. On the other hand, types which are less effective inducers were extremely resistant as inducers to UV irradiation, whereas their infectivity decreased markedly after UV treatment (Figs 3-I, 3-II). Similar results were obtained using purified adenoviruses which were free of the possible disturbing effects of incomplete and empty particles (Fig. 4). As UV irradiation exerts its primary effect on nucleic acid, it seems that functional viral DNA is required for IFN induction with effective inducers. Infectivity of the effective inducers is more UV sensitive that their IFN-inducing ability, thus it also seems that not the whole genome, but only a relatively small segment of DNA is responsible for IFN induction. This assumption is corroborated by the finding that incomplete particles of Ad12 carrying fragments of viral DNA displayed IFN-inducing capacity, while empty particles did not (Taródi *et al.*, 1979).

The IFN-stimulating capacity of less effective inducers as it is suggested by the present experimental data cannot be attributed to the functional viral nucleic acid, but to some other UV-resistant virus component(s). Similar results were obtained with certain RNA virus-inducers. The IFN--inducing capacity remained unaffected, whereas the infectivity of Sendai virus was completely abolished by UV irradiation (Ito *et al.*, 1978*b*). Likewise, the early IFN response was triggered by a UV-resistant virion component of Newcastle disease virus in FS-4 cells (Kohase and Vilcek, 1979). It was suggested that not the nucleic acid, but some other viral component was involved in the induction of IFN by these viruses (Ito *et al.*, 1978*a*, *b*; Kohase and Vilcek, 1979).

Since functional viral DNA might be involved in IFN induction at least in case of effective inducers, the IFN-inducing ability of adenovirus DNA was studied. DNAs induced IFN production in CEC independently of whether they originated from effective or less effective virus inducer types. Of interest was the fact that in spite of the seven magnitude difference between the infectivity of Ad5 virion and DNA there was no great difference in their IFN-inducing capacity (Table 2 and 3). It is noteworthy that we failed to detect T antigen production in CEC transfected with Ad12 DNA. Upon induction by Ad12 virus, however, T antigen formation paralleled the IFN production. Therefore, it seems likely that IFN is induced by different mechanisms upon action of deproteinized viral DNA and virus particles. Only DNAs of viral origin (Ad or phage) but not the prokaryotic and eukaryo-

tic DNAs are suitable for IFN induction. Similarly, reduction in VSV plaque formation was obtained only with λ phage DNA but not with calf thymus DNA (De Clerq *et al.*, 1970).

Our results indicate the existence of two distinct mechanisms of IFN induction by different types of adenoviruses and, moreover, two independent pathways of IFN induction by adenovirus nucleic acid and adenovirus particles. Obviously, further efforts are needed to throw more light on events involved in these processes of IFN induction.

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