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ANALYSIS OF AN ADENOVIRUS TYPE 12 TEMPERATURE-SENSITIVE MUTANT DEFECTIVE IN INTERFERON INDUCTION

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Summary. — Among several temperature sensitive mutants of human adenovirus type 12 (Ad12), H12ts15 was found to be defective in induction of interferon (IFN) in chick cells. This mutant was characterized as late mutant which synthesized defective III.a and penton-base antigens and failed to assemble into infectious particles at restrictive temperature. Moreover, its defect was manifested at permissive temperature, at which defective particles were readily formed. Temperature shift experiments revealed that an early event, but neither virus adsorption nor penetration, was blocked in the process of IFN induction. Therefore, it can be assumed that conformation of the adeno virion is important for IFN induction in chick cells. Conformation alteration caused by defective structural components of the capsid at restrictive temperature prevented IFN induction. This change, however, did not affect infectivity under similar conditions.

Key words: adenovirus mutants; interferon induction; chick cells

Introduction

Human adenoviruses (Ad) have been extensively studied because they can serve as a model for eukaryotic gene regulation and because they induce tumours in certain rodents and transform nonpermissive cells in tissue culture. All human Ad types tested so far induced IFN formation in nonpermissive chick cells (Béládi and Pusztai, 1967). Among the 41 recognized serotypes of human Ad the degree of oncogenicity varies from one serotype to others and the types differ also in their ability to induce IFN (Mucsi *et al.*, 1970).

Despite of the efforts to clarify the mechanism of IFN induction by adenoviruses, this is not quite understood. It appears that the induction does not depend on viral DNA replication (Bakay and Burke, 1972; Ustacelebi, 1973; Pusztai *et al.*, 1969). There are data implicating the involvement of virus protein (Béládi and Pusztai, 1967; Béládi *et al.*, 1979). Analysis of the defect of two temperature sensitive mutants of Ad5, which failed to induce IFN at restrictive temperature, revealed that induction results from an early

interaction between a viral structural antigen and the cell. Moreover, since the defective structural polypeptide present in the mutant virions might be of some relevance to the lack of IFN production, a detailed characterization of temperature sensitive (ts) mutants H5ts18 and H5ts19 had been undertaken (Taródi *et al.*, 1977; Taródi *et al.*, 1979).

A number of ts mutants of Ad12 have been isolated (H12ts1, H12ts15, etc.) and partially characterized (Shiroki *et al.*, 1972). This serotype is a good IFN inducer and it has been already supposed that high IFN production resulted from a different induction mechanism as compared to that of less efficient inducers like Ad5. Therefore, it was tempting to undertake a study in which Ad12 temperature sensitive mutants with defective inducing ability would be sought and the nature of the defect would be analysed. By comparing the defect in ts mutants of effective and less effective IFN inducers, I aimed at better understanding of the mechanism of IFN induction in Ad-infected chick cells.

Materials and Methods

Cells and viruses. Primary chick embryo fibroblast (CEF) cells were cultured in Eagle's medium containing 5 % calf serum. HEp-2 cells were grown as monolayers in the same medium. Monolayers of CEF in 60 mm Petri dishes or 6 × 4 well tissue culture plates (Greiner) were used for IFN induction.

Adenovirus type 12 (Huie strain) was propagated in HEp-2 cells. Temperature sensitive mutants of Ad12:H12ts1, H12ts15, H12ts32, H12ts78 were obtained from Dr. Shimojo (Institute of Medical Sciences, Tokyo) and grown in HEp-2 cells at 32 °C. The viruses were purified by fluorocarbon (Arcton; Serva, Heidelberg, F.R.G.) extraction followed by a velocity and two equilibrium centrifugations in caesium chloride as described previously (Taródi *et al.*, 1977).

Fluorescent antibody procedure. The technique of Philipson (1961) with slight modification was used for measuring infectious Ad12 by fluorescent focus formation on HEp-2 cells. Fluorescent foci were counted over an ocular grid in a Leitz fluorescent microscope. For each sample, fluorescent foci were counted in 10 fields bordered by the grid and an average number of fluorescent foci per grid was compared with the value obtained for virus sample of known infective titre and it was used as a relative measure for Ad12 infectivity.

For evaluation of tumour (T) antigen production, chick cells were fixed 24 or 48 hr after infection and stained for T antigen. The T antigen producing cells were evaluated by indirect immunofluorescence technique as described by Pusztai *et al.* (1977).

Interferon induction and assay. IFN induction by Ad12 wild type and mutant viruses in CEF was performed as previously described (Béládi and Pusztai, 1967). In usual experiments primary chick cells were infected at a multiplicity of 10^{-2} focus forming unit (FFU) per cell and incubated either at 32 °C or at 39 °C, i.e. at permissive or restrictive temperatures. IFN samples were harvested at 48 hr post-infection (p.i.).

IFN titration was carried out on secondary CEF in microtitre plates by the micromethod described previously (Taródi *et al.*, 1977).

Measurement of heat sensitivity of the virions. Crude virus stocks were diluted to 1 : 10 in PBS and then dispensed into samples of 0.1 ml per tube. The suspensions were incubated in a water bath at 50 °C. The samples were withdrawn at various intervals, immediately diluted with ice cold Eagle's medium. The residual infectivity was titrated by fluorescent focus assay at 32 °C.

Enzyme reactions. Chymotrypsin and thermolysin were obtained from Serva, trypsin from Wortington (Freehold N. J.). Virus digestion was carried out in saline buffered by 50 mmol/l Tris-HCl pH 7.5 (thermolysin), 8.2 (trypsin) and 7.8 (chymotrypsin), respectively, using the specified virus to enzyme concentration. Digestion was terminated by addition of SDS and urea and samples were immediately used for SDS-PAGE analysis.

Radioactive labelling. For pulse labelling experiments confluent monolayers of HEp-2 cells in Petri dishes were infected with wt and mutant viruses and incubated in the presence of 3 mmol/l arginine-butyrate at 39 °C. At appropriate intervals, the labelling was carried out in methionine-

-deprived medium by adding 1.85 MBq/ml of L-(35S)-methionine (Amersham) for 1 hr at 39 °C. At the end of the labelling period the cells were either harvested or the label was chased by replacing the "hot" medium with that containing a 10 times increased concentration of unlabelled methionine.

For polypeptide phosphorylation studies infected cells were labelled at 6, 24 and 48 hr p.i. with 3.7 MBq/ml ^{32}P-orthophosphate (Izinta, Hungary) for 1 hr. The labelled cells were analysed similarly as the methionine labellled ones.

To prepare ^{35}S-methionine labelled virus, cells were infected at permissive temperature and labelled in the presence of 1/10 methionine concentration. Labelling was performed from 24 hr p.i. for 10 hr; then 2 vol of normal medium were added and the cultures were further incubated until an appropriate cytopathic effect had developed when the cells were harvested and the labelled virus was purified as usual.

Polyacrylamide gel electrophoresis (PAGE) and autofluorography. SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed in a discontinous buffer system described by Russell and Blair (1977) with some modification (Taródi *et al.*, 1982). The gels were treated with 2.5-diphenyl-oxazole (PPO) as described by Bonner and Laskey (1974) or with Na-salicylate (Chamberlain, 1979) and then dried and autofluorographed at −70 °C.

Results

IFN inducing ability of mutant viruses

Several temperature sensitive mutants of Ad12 were tested for their IFN inducing ability. Appropriate multiplicity of infection and the time of maximum yield of IFN production were determined in preliminary experiments. Infection with ts mutants at restrictive temperature of 39 °C usually gave similar IFN yield as compared to that obtained at the permissive temperature of 32 °C, however, when H12ts15 was used as inducer, only negligable amount of IFN was produced in chick cells (Table 1).

IFN yields in shift up experiments

To determine the temperature sensitive step during the IFN synthesis period in the mutant-infected cells experiments were performed in which cells were transferred from the permissive to the nonpermissive temperature. Results are presented in Fig. 1. It can be seen that IFN yield became resistant to the temperature shift at around 10 hr p.i. Presumably an early event in the process of IFN synthesis in, and IFN release from the cells was inter-

Table 1. Interferon induction by Ad12 temperature sensitive mutants at permissive (32 °C) and at restrictive (39 °C) temperatures

Inducer	Interferon titres	
	32 °C	39 °C
H12ts1	256	128
H12ts115	128	2
H12ts32	128	64
H12ts78	256	64
H12wt	256	128

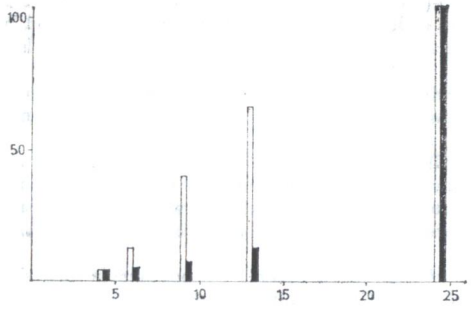

Fig. 1.

Effect of temperature shift up on the IFN yield induced by H12ts15

Infected cells were incubated at 32 °C and at indicated interval the cultures were transferred to restrictive temperature (39 °C).

Solid bars represent the amount of IFN measured at the time of temperature shift; open bars represent the final titres obtained after shift up when samples were harvested at 48 hr p.i.

Abscissa: hr p.i.; ordinate: IFN titres (IU per ml).

rupted as it can be seen from the titres of IFN samples taken at the time of shift up.

Viral polypeptides in H12ts15 infected human cells

The structural polypeptide composition of the virion of Ad12 was recently determined (Taródi *et al.*, 1982). Thus we were able to analyse the viral polypeptide synthesis in mutant-infected cells. HEp-2 cells were infected with wt and mutant viruses and labelled with ^{35}S-methionine at 39 °C. In pulse-chase experiments the label was chased for various intervals from 6 to 48 hr p.i. Preliminary experiments revealed that evaluation of SDS-PAGE analysis was complicated by the high cellular background.

It has been reported that arginine butyrate does not inhibit Ad replication in HeLa cells, in which no detectable synthesis of cellular DNA or histon occurs. By labelling of infected cells pretreated with 3 mmol/l arginine--butyrate we could achieve a dramatic reduction of the cellular background. The autoradiogram clearly showed (Fig. 2) that the major distinction between the polypeptides expressed by wt (line a) and mutant viruses (line b) was represented by two bands migrating at an apparent molecular weight of 57 and 54 kD, i.e. sizes compatible with that of III.a and penton base, respectively. It appears, therefore, that the mutant virus codes for a little smaller III.a and a slightly larger penton base polypeptide than the appropriate constituent of wt virus.

Events in infected chick cells

Infection of primary chick cells with Ad does not yield infectious virus. Analysis of infected cells revealed that most of the cells produce virus-specific antigens, the most prominent representative of these being the T antigen Staining of this early antigen was carried out in chick cells grown on coverslips 48 hr p.i. with anti-tumour sera of T antigen specificity. These sera were obtained from hamsters bearing Ad12 induced tumours (Pusztai *et al.*, 1977). The aim was to ascertain whether T antigen is produced under restrictive conditions in mutant virus-infected cells. It can be seen in Fig. 3 that no difference in T antigen production was seen between wt and mutant-infected

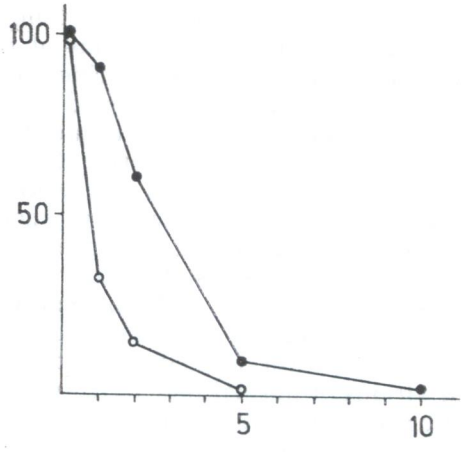

Fig. 4.
Rate of thermal inactivation of H12ts15 (○———○) and H 12 ts 15 (●———●) virions at 50 °C
Abscissa: time of heat treatment in hr; ordinate: residual infectivity in per cent of untreated control.

cells, the distribution and morphology of T-antigen being similar in both cases.

Polypeptide phosphorylation

Previous reports have revealed that certain polypeptides of C type human Ad are phosphorylated in human cells. This is usually followed by labelling with ^{32}P-orthophosphate and analysing the cell lysate by SDS-PAGE. Ad5 can also elicit polypeptide phosphorylation in chick cells similar to that seen in human cells. It was of interest to compare the polypeptide patterns of Ad5 and Ad12 as well as of H12wt and H12ts15 to see whether the difference in IFN inducing ability of the two serotypes and the mutant virus would be perhaps related to the phosphorylation defect.

SDS-PAGE analysis of the extracts of Ad12 infected chick cells showed no virus-specific phosphorylation of polypeptides discernible either at 6, 24 or 48 hr p.i. (data not shown). This observation does not mean that there is no such viral function in this system but only that it occurs at very low rate and the result is masked by the heavy cellular background.

Characterization of mutant virion

Fig. 4 gives the results of testing the heat sensitivity of virions at 50 °C of the ts mutants and wt virus in terms of infectivity expressed as percentage of surviving fraction of the focus-forming ability after heat treatment.

The infectivity of the mutant virus was destroyed more rapidly than that of wt virus as judged from the steeper slope of the heat inactivation curves. This suggests that the mutation affected the structural component of the virion.

Polypeptide composition of the mutant virion

The foregoing experiments indicated that some structural components of the mutant viruses were defective resulting in fragile particles when cells

were infected at the permissive temperature. In an attempt to define this structural defect further, virions of the mutant and of wt virus were ^{35}S--methionine labelled and purified by CsCl equilibrium density gradient centrifugation and then analysed by SDS-PAGE electrophoresis. The autoradiogram (Fig. 5) shows certain difference between the two viruses (lanes a and b: mutants; lane c: wt virus) namely in the mobility of III.a and penton base. These two structural polypeptides of wt virus have an apparent m.w. of 57 kD and 54 kD respectively, whereas a comigrating double band (better seen in lane a) appeared on place of III.a and penton base polypeptides of mutant virus. This result is very similar to that obtained with extracts of human cells infected with the mutant virus at restrictive temperature of 39 °C. It is apparent that the defect was manifested also at permissive temperature, however, the defective structural antigens did not prevent assembly and virions of normal infectivity were produced.

Moreover, mutation has affected some other virion polypeptides as well. For example, polypeptide V which is a double band in the electrophoregram of wt Ad12, appeared as a single polypeptide though with a slightly higher m.w. Similarly, V.a was also absent from constituents of mutant virus. Noteworthy was the relative abundance of polypeptide IX, and also the presence of an additional polypeptide with m.w. of about 10 kD (Y).

Susceptibility of virions to proteolytic enzymes

Purified Ad can withstand treatment with low concentration of proteolytic enzymes without extensive degradation of the virion. This treatment, while not greatly affecting infectivity or IFN inducing ability, does produce limited cleavage of some of the virion polypeptides. Since the pattern of the resulting degradation might be characteristic, in an approach to probe the conformation of the virion, the susceptibility to various proteolytic enzymes of the mutant and of wt virion polypeptides was analysed. Preparations of purified wt and mutant viruses were digested under appropriate conditions with thermolysine, trypsin and chymotrypsin. Chymotrypsin treatment revealed specific differences between wt and mutant viruses. Most striking were the extreme sensitivity of defective III.a and penton base proteins as well as of polypeptide VI of the mutant virion (Fig. 6). Moreover, several other minor differences could also been found which might reflect the unique susceptibility of structural constituents of the mutant virion perhaps owing to its defective conformation.

Discussion

A common feature of all human Ad serotypes tested so far is that they induce IFN formation in chick cells. Preceding studies on IFN induction by ts mutants also revealed that in case of Ad5, viral protein(s) may have a crucial role in IFN induction. A number of ts mutants of human Ad12 have been isolated and classified into groups by complementation test and also partially characterized (Shiroki *et al.*, 1972). Some of these mutants belonging

to different complementation groups were analysed in respect of their IFN inducing ability in CEF cells at restrictive temperature. One of them, H12ts15 was found defective in IFN induction at 39 °C. Further study was undertaken to reveal the defect of this mutant as well as the events it elicits in chick cells in the hope to get better insight into mechanism of IFN induction by a DNA virus.

The results of temperature shift induction experiments presented in this paper suggest that the temperature sensitive step takes place at about 10 hr p.i. In this respect H12ts15 appears to be similar to H5ts18 and H5ts19 mutants which are also defective in IFN induction (Ustacelebi *et al.*, 1973; Taródi *et al.*, 1979). It appeared that the failing event affected viral DNA synthesis as well as the synthesis of a late viral structural component in chick cells. This event may not be the viral DNA replication itself, because it is not required for IFN induction (Bakay *et al.*, 1972; Taródi *et al.*, 1977). The event should be different from virus adsorption and penetration since Ad12 infected chick cells produce T antigen (Pusztai *et al.*, 1977), similarly as H12ts15 did in infected chick cells at restrictive temperature.

Supported by previous findings it was reasonable to suppose that the virion component involved in the inhibited step of IFN induction at restrictive temperature may be identical to the defect of structural antigen of the late type mutant H12ts15. Pulse labelling experiments with ts and wt infected human cell-polypeptides indicated the absence of normal III.a and penton base polypeptides on SDS-polyacrylamide gel electrophoretic analysis of samples taken at restrictive temperature. Instead, two polypeptides with m.w. compatible with III.a and penton base were observed. The presence of these two polypeptides in mutant virions produced at permissive temperature strongly suggests that H12ts15 suffers from a major pertubation: modification of polypeptide III.a and penton base. Comparison of ^{35}S-methionine labelled polypeptides of ts15 and wt virion (Fig. 5) also revealed that there are several other pertubations as well: one of the major core proteins (V) is represented by a double band in the polypeptide pattern of wt virion (42.2 kD and 36.3 kD), whereas only the 36.3 kD polypeptide is present in that of mutant virion. Similarly the lack of V.a (26.6 kD) polypeptide in mutant virion could also be observed. V.a is not a precursor to polypeptide VI (25.1 kD), since in contrast to VI, V.a is a phosphoprotein (Taródi *et al.*, 1979). It is interesting to note the overrepresentation of the polypeptide IX and the presence of as yet unidentified polypeptide of low molecular weight (Y) in the virion of ts15. The differences summarised here are characteristic for mutant virions produced at 32 °C, thus the defect is also manifested at the permissive temperature.

Several reports have confirmed that mutants with defective vertex capsomers and the lesion of which is also apparent at permissive temperature are able to form stable virions. Mutant fibres synthesized by H5ts115, H5ts116 as well as by H5ts141 at 39.5 °C are also assembled into virions to the same extent as wt fibres when temperature had been shifted down to 32 °C, even in the absence of protein synthesis (D'Halluin *et al.*, 1980;

Chee-Sheung and Ginsberg, 1982). Similarly H5ts104, which is phenotypically a fibre plus penton base defective mutant, forms "young virions" (the designation for an intermediate stage in virion maturation, Ishibashi and Maizel, 1974) at nonpermissive temperature which then evolved into infectious particles on shift down to 32 °C in the presence of cycloheximide (D'Halluin *et al.*, 1980).

A number of data have confirmed the importance of certain virion components for structural stability of the virion. Especially the function of two of the smaller capsid proteins VIII and IX has been clearly defined (Colby and Shenk, 1981; Liu *et al.*, 1985). Other still unmapped mutations which confer alteration to the stability of the virion have also been described previously (Young and Williams, 1975).

It is noteworthy that IFN noninducing Ad mutants tested so far were characterized as having thermolabile virions (Taródi *et al.*, 1979), and possess an additional low m.w. polypeptide (Béládi, 1979; Taródi *et al.*, 1979). Phenotypically, however, they were referred to as III.a or polypeptide VI mutants.

In the experiments described here we found that H12ts15 showed much greater heat lability than wt virus. Moreover, in another approach to probe the conformation of the virion, difference was sought in substrate availability of polypeptides for proteolytic enzymes. Digestion of the virions with chymotrypsin showed that polypeptide VI of the mutant is much more susceptible to degradation than that of wild type virion implying that conformation of this polypeptide is different. It again bears resemblance to other IFN negative mutants of Ad5 (Taródi *et al.*, 1979).

Previous results supported the hypothesis of a virus coded or virus induced cellular endopeptidase which would cleave the precursors of virion proteins VI, VII, VIII (Bhatti, 1979). Another studies reported similar processing of polypeptide III.a in the "young virion" (Taródi *et al.*, 1979; Weber *et al.*, 1977; Boudin *et al.*, 1980). The processing of these polypeptides as well as the appearance of cleavage product were shown to take place during development of "young virions" into mature ones. One cannot exclude the not very likely possibility that a single mutation in H12ts15 caused such a modification in configuration which might have created new preferential cleavage sites for the endopeptidase.

It has already been reported that there are Ad mutants which proved to be defective in the synthesis of a whole group of antigens (Shiroki *et al.*, 1972; Suzuki *et al.*, 1972; Weber *et al.*, 1977). Assuming a single mutation, it is conceivable that regulatory functions are involved in the appearance of these phenotypes. Similarly to that, data obtained argue for the role of some regulatory function which links these apparently independent pertubations in H12ts15 we described in this report.

Evaluating results on temperature sensitive mutants of Ad defective in IFN induction which were reported in previous communications, it has appeared that H5ts18, H5ts19, H5ts58, as well as H12ts15 share some common characteristics. They all seem to have defects which affect the process

of virion assembly and maturation and it results in the formation of thermolabile virion with altered conformation. This defect is also manifested at the permissive temperature. Whether the presence of additional small polypeptides together with altered substrate availability of polypeptide VI for enzymes are the primary cause of the conformational change as well as of the defect in inducer function remains to be studied.

Data available are certainly compatible with the likelihood that either on account of defective uncoating the synthesis of certain viral product(s) does not take place, or it fails to be combined with cellular inducer-receptor. As a consequence of this derepression of IFN gene does not take place.

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Legend to Figures (Plate XXV—XXVIII)

Fig. 2. Autoradiogram after SDS-PAGE analysis of extracts of H12ts15 infected (lane b) and of H12wt (lane a) HEp-2 cells. Infected cells were incubated at 39 °C, at 24 hr p.i. they were labelled with ^{35}S-methionine for 1 hr.

Fig. 3. T antigen detected in H12ts15-(I) and H12wt-infected (II) CEF. Cells grown on coverslips were infected and stained at 48 hr p.i. with T antiserum by the indirect immunofluorescent technique.

Fig. 5. Autoradiograms after SDS-PAGE analysis of purified virions produced in HEp-2 cells at 32 °C. Infected cells were labelled with ^{35}S-methionine for 10 hr from 24 hr p.i. (Lines a and b: mutants; line c: wt virus).

Fig. 6. Autoradiogram after SDS-PAGE of purified ts mutant and wt viruses which were digested with chymotrypsin at virus/enzyme protein ratio of 15 to 1 (mg/mg). Labelling procedure was the same as described in legend to Fig. 5. CH+ treated, CH— untreated samples.