Isolation of Infectious DNA from Avian Myeloblastosis Virus Transformed Leukemic Cells

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(Z. Naturforsch. **29 c**, 437–441 [1974]; received April 22, 1974)

Avian Leukemic Myeloblasts, Infectious DNA, Avian Myeloblastosis Associated Virus, Nephroblastoma

Chick helper factor (chf) negative chick embryo cells were exposed in the presence of DEAE-dextran to chromosomal DNA isolated from avian leukemic myeloblasts which had been induced by infection with the BAI-A strain of avian myeloblastosis virus (AMV) (subgroup B). After several transfers, avian C-type virus production was observed in the cultures with electron microscopy, although no alteration in the cell morphology was apparent.

Virus interference tests revealed that this virus was infectious and belonged to subgroup B of the avian RNA toumor virus (ATV) group. The virus induced nephroblastomas 2 months after intraperitoneal inoculation of chickens; no myeloblastosis was observed, however, and the virus could neither transform chick embryo cells *in vitro* nor induce wing web tumors *in vivo*. The nephroblastomas also produced virus which was again shown to be a member of subgroup B. From these results it was concluded that the virus transmitted in these experiments was avian myeloblastosis associated virus (MAV), an avian leukosis virus known to be present as a major component in AMV stocks.

Introduction

Implicit in the provirus theory of Temin [1] is the idea that the DNA of cells infected with an RNA tumor virus might be infectious since it contains the viral genome in the form of a DNA transcript or provirus. In 1971, Hill and Hilova [2] first proved the existence of this infectious DNA by infecting permissive chick embryo cells (CEC) with DNA from Rous sarcoma virus (RSV)-transformed rat cells (XC cells). Subsequently other authors have also reported the infectivity of DNA from a variety of virus-transformed cells, including XC cells [3, 4], RSV-transformed hamster cells [3–5], RSV-transformed chick embryo cells [6], AMV-transformed myeloblasts [7], and murine sarcoma virus (MSV)-transformed mouse and hamster cells [8].

The present study was concerned with demonstrating the infectivity for CEC of chromosomal DNA isolated from AMV transformed myeloblasts with the Hirt procedure [9]. Chick helper factor (*chf*) negative CEC were exclusively used in order to exclude as much as possible a helper effect of the endogenous chicken virus.

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Materials and Methods

Virus strains

The BAI-A strain of AMV was derived from a stock supplied by Dr. J. W. Beard, Durham, North Carolina. The strain employed for these studies was composed exclusively of subgroup B viruses. The source and properties of the Schmidt-Ruppin strain of RSV (SR RSV-H), subgroup D, which had been rescued from transformed hamster cells, has been described [10]. The following viruses were obtained from Dr. P. K. Vogt, Los Angeles, California: the Prague strains of RSV (PR RSV), subgroup A, B, and C were employed as standard nondefective avian sarcoma viruses; myeloblastosis associated virus (MAV-1), subgroup A, Rous associated virus (RAV-6), subgroup B, RAV-49, subgroup C, and RAV-50, subgroup D were used as reference avian leukosis viruses.

Animals and tissue cultures

Avian leukemic myeloblasts were isolated by sedimentation of leukemic peripheral blood from acutely leukemic chickens. Chickens for tumor induction were obtained from a line 15 flock which has been maintained for several years in our laboratory and rigorously tested for the presence of avian leukosis viruses with consistently negative results [11]. This flock was also used as a source of eggs for the preparation of C/O phenotype CEC, most of which proved to be positive when tested according to the

procedure of Weiss *et al.*[12] for the presence of *chf*. A second flock of specific pathogen free (SPAFAS) chickens, maintained by Lohmann Tierzucht, Cuxhaven, W.-Germany, was the source for eggs used in the preparation of C/E phenotype CEC. These eggs were the generous gift of Dr. E. Vielitz, Lohmann Tierzucht, Cuxhaven, W.-Germany. CEC from this source were tested for the presence of *chf* as described by Weiss *et al.*[12] and according to the technique of Friis *et al.*[13], and such twice-tested *chf*-negative cells exclusively were used for the experiments described below for the detection of the infectious DNA. Japanese quail embryos were generously supplied by Dr. Wellmann, Berlin.

All tissue cultures were maintained in Dubecco's modified Eagle's medium which was obtained as a powder from Flow Laboratories, Bonn, Germany, and was supplemented with 10% tryptose phosphate broth and 2 – 5% calf serum. The medium contained in addition 150 units of penicillin and 100 μg streptomycin per ml.

DNA preparation

Marmur's method[14] supplemented by RNase A (Sigma: 100 μg/ml, 60 min, 37 °C) treatment was used to extract DNA from the pellet fraction obtained according to the procedure of Hirt[9] from AMV-myeloblasts. This procedure has been known to yield a preparation mainly composed of chromosomal DNA free from mitochondrial DNA[6]. Normal *chf* positive and SR-RSV-H transformed CEC DNA was extracted from the whole cells according to Marmur's method. The optical density 260 nm/280 nm ratio exhibited by these preparations was 1.84 – 1.89.

Treatment of chick embryo cells with DNA

A semi-confluent monolayer in 100 mm tissue culture dishes of *chf* negative CEC were exposed to DEAE-dextran (Pharmacia; 50 or 100 μg/ml) in BME buffer[15] (Eagles medium additionally buffered with 40 mM tris-HCl, pH 7.4) for 15 min at room temperature. This solution was then removed, and replaced with 2 ml of DNA solution which had been adjusted to 30 μg/ml in BME buffer, and the cultures were further incubated at 37 °C for 30 min. After this treatment, the DNA solution was removed, and cultures were maintained routinely with periodic transfers for several weeks. Controls were performed in which a DNA solution which had been predigested with DNase I (Sigma; 10 μg/ml in BME with 3 mM $MgCl_2$, 30 min, 25 °C) was employed for the treatment of cells.

Detection of the virus with electron microscopy

Samples were taken and prepared for electron microscopy as previously described[16]. They were examined with an Elmiscop type 101 (Siemens).

Virus interference and serum neutralization tests

For interference tests, C/E phenotype CEC were infected in the presence of polybrene[17] with the test virus or appropriate reference avian leukemic viruses, and the cultures were maintained for 3 passages. After the third transfer, the cells were seeded in plates, infected with individual standard avian sarcoma viruses, and overlayed on the next day in a focus assay according to the method of Rubin[18] as modified by Vogt[17]. Neutralization tests were performed using reference antisera and methods previously described[10].

Assay of viral oncogenicity in chickens

The C-type virus obtained from infectious DNA-treated cells was passaged in *chf* negative C/E phenotype CEC. Culture fluids obtained after the second passage were centrifuged (4,000 rpm for 20 min), and 1 ml of the supernatants was inoculated intraperitoneally or in the wing webs of one day old chickens. Control animals received culture fluids from uninfected C/E *chf* negative CEC. The animals were sacrified and autopsied at 60 days after inoculation.

Tumorous tissue specimens were fixed in 10% formalin and stained with hematoxylin and eosin for histopathological examination.

Results

Virus production of DNA-treated CEC

After 3 transfers of *chf* negative C/E phenotype CEC which had been exposed to DNA from avian leukemic myeloblasts, massive C-type virus (MAV-B) production was observed by electron microscopy in one trial (Table I), although no alteration in the cell morphology was apparent. No virus production was observed in three further experiments even after further 30 days cultivation of the cells. The C/O cells which had been exposed to DNA from SR-RSV-H-transformed chick cells, as a positive control for infectious DNA, showed visible transformation two or three transfers after the DNA treatment in two cases out of ten trials (Table I). The cells which were not transformed were further cultivated more than 30 days and examined for the presence of virus with electron microscopy, but no virus could be detected.

Table I. Occurrence of virus in DNA-treated normal CEC.

DNA source	No. positive experiments/No. trials: Virus production	Trans-formation	Rescued virus	Original virus
AMV-Myeloblasts	1/4	0/4	MAV-B (subgroup B) [a]	BAI-A strain of AMV (subgroup B)
AMV Myeloblasts DNase digested	0/4	0/4		
RSV-CEC	2/10	2/10	RSV (subgroup D)	SR-RSV-H (subgroup D)
RSV-CEC DNase digested	0/4	0/4		
Normal CEC chf (+)	0/6	0/6		

[a] See table II for subgroup determination.

The C/O phenotype CEC treated with the DNA from normal CEC (*chf* positive) failed to produce virus in all 6 trials (Table I). The cells which had been exposed to a DNase-digested DNA fraction showed in no case either virus production or transformation.

Infection of C/E phenotype and Q/BD phenotype Japanese quail cell with the myeloblast DNA induced virus

The virus recovered from the CEC exposed with the DNA from AMV transformed myeloblasts was examined for infectivity on C/E phenotype CEC by electron microscopy two transfers after infection. It was apparent that the virus was highly infectious for these cells indicating that the virus was not an endogenous virus of chickens (subgroup E). The Japanese quail cells which are resistant to subgroup B but susceptible to subgroup E viruses, failed to be infected by the DNA induced virus, thus confirming that no endogenous subgroup E virus was present in the induced preparation. The virus infected CEC did not show transformation although they showed some degree of cytopathic effect which is characteristic of subgroup B avian tumor viruses.

Classification of the DNA induced virus according the subgroup

The myeloblast DNA-induced virus was examined with viral interference tests and shown to be a member of subgroup B (Table II), as might be predicted from the fact that the AMV used to induce the myeloblasts, from which the DNA was orignally

Table II. Determination of the virus subgroup (virus interference tests).

Interfering viruses	Challenge viruses: PR RSV-A (subgroup A)	PR RSV-B (subgroup B)	PR RSV-C (subgroup C)	SR RSV-H (subgroup D)	Without RSV	Subgroup of interfering virus
MAV-1	−	+	+	+	−	A
RAV-6	+	−	+	−[a]	−	B
RAV-49	+	+	−	+	−	C
RAV-50	+	−[a]	+	−	−	D
AMV (BAI-A)	+	−	+	−[a]	−	B
Myeloblast DNA-induced virus	+	−	+	−[a]	−	B
Virus from nephroblastoma	+	−	+	−[a]	−	B
without virus	+	+	+	+	−	−

+: Indicates a focus formation. −: Indicates no focus formation. a: These results are in accordance with the interference between subgroup B and D viruses as observed by Duff and Vogt (1969) [27] and Bauer and Graf (1969) [11].

extracted, was of subgroup B. The titer of the myeloblast DNA-induced virus was approximately 10^6 infectious interfering units (not shown) which is comparable to other avian leukosis viruses used as standard interfering viruses.

The virus obtained from transformed CEC after treatment with the DNA from SR-RSV-H (subgroup D) infected CEC was shown to be a member of subgroup D by the antiserum neutralization tests (not shown).

Formation of nephroblastomas in chicken after inoculation of the myeloblast DNA-induced virus

The myeloblast DNA-induced virus produced nephroblastomas in 4 out of 16 chickens approximately 2 months after intraperitoneal injection, although neither myeloblastosis, lymphoid leukosis nor osteopetrosis were observed. Likewise, no tumor could be induced by injection of the virus into chick wing webs.

Intraperitoneal injection of chickens with normal C/E phenotype CEC culture fluids did not induce any tumors.

Histopathological studies showed that the features of the nephroblastomas were identical to those of AMV-induced nephroblastoma [19]. All tumors examined by electron microscopy showed C-type virus production.

Tissue culture fluids of the tumor were assayed in virus interference tests for further classification of the virus in the tumor. The virus was again found to be a member of subgroup B (Table II).

Discussion

Molecular hybridization experiments have shown that the genome of RNA tumor viruses is integrated in the form of DNA into the cellular genome [20, 21]. In support of this are the findings that the DNA extracted from RNA tumor virus-transformed cells is infectious [2–8]. It is not known why the infection of cells with such DNA is so inefficient, *i. e.* why infection of cells requires such massive amounts of DNA and even so fails to establish infection in many experiments. The possibility that infection is transmitted by RNA instead of DNA in our studies is unlikely because of the RNase treatment of the DNA before exposure to cells. This seems also to be supported by the failure of DNase treated DNA preparations to be infectious; however, due to the low rate of infection this control is not statistically highly significant.

So far the viruses produced by cells treated with infectious DNA were able to induce cellular transformation and had precisely the same biological and immunological characteristics as the parental viruses [2–8]. The AMV strain used in our studies is known to induce myeloblastosis, lymphoid leukosis, osteopetrosis and nephroblastoma in chicken [22]. It has been reported that nontransforming viruses isolated from that strain of AMV, myeloblastosis associated virus (MAV), induced lymphoid leukosis, osteopetrosis and nephroblastoma but no myeloblastosis *in vivo* [23].

The virus recovered in this study induced nephroblastomas, but no other disease after intraperitoneal inoculation of one-day-old chickens. The virus could neither transform CEC *in vitro* nor induce wing web tumors *in vivo*. The histological structure and the ultrastructure of the nephroblastomas induced in this study were exactly the same as that of the nephroblastomas induced by AMV [19, 24]. From these results it was concluded that the virus recovered was a MAV which is present as the major component in AMV stocks. The virus induced had the same antigenic properties as the parental virus and a MAV previously isolated from the same stock [10].

In our studies, care was taken that the endogenous chick helper factor could not act as a helper for a possible defective or incomplete DNA provirus which might enter a cell. Furthermore, the host range determination of the myeloblast-DNA induced MAV revealed that is was in fact not contaminated with endogenous subgroup E virus. Whether endogenous virus has influenced the results of other workers can not be decided, but it seems possible that as suggested by Hill [25], the entire genetic content of the virus must be transmitted in order to establish infection. Hence, helper effects due to *chf* or exogenous ATV infection would be limited.

Until now the CEC treated with DNA from normal *chf* positive CEC did not produce any virus, although the DNA preparation used contained virus specific sequences [26] (unpublished observation). More experiments have to be done, however, probably with more refined methods before further conclusions can be drawn.

The authors wish to thank Dr. H. Gelderblom for many advices in electron microscopic studies. We gratefully acknowledge the technical assistance of Mrs U. Hassenpflug and Mrs. U. Baum. One of the authors (H. Ogura) is a recipient of a fellowship of the Deutscher Akademischer Austauschdienst.

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