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Characterization of Avian Adenovirus Associated Virus

II. Replication of AAAV in Chicken Embryos

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Introduction

Avian adenovirus associated virus (AAAV) is a small, defective DNA virus found in many avian adenoviruses as a contaminant.[2,5,11,13]

The virus grows in chicken embryos and in chicken kidney cell cultures coinfected with a "helper" virus such as an adenovirus without showing any cytopathic effect[10].

It has been suggested that some avian adenoviruses cause overt diseases and a vaccine against avian adenovirus infection is needed[8]. However, before such a vaccine can be made, the role of AAAV in avian adenoviruses must be clarified.

The objective of this study is to determine the dependence of the multilplication of AAAV on avian adenovirus and the role played by each of them.

Materials and Methods

Virus

AAAV: The preparation of AAAV stock was described in the first part of this study[7]. The viral infectivity was expressed as the mean complement fixation antigen inductivity (CFI_{50}) and determined by methods described by Yates *et al*[10]. One CFI_{50} is the least amount of AAAV which can induce AAAV antigen detectable by the complement fixation test, from more than 50% of the chicken embryos which are coinfected with a proper amount of the "helper" virus.

Avian Adenoviruses: The seven avian adenovirus used in this study were from departmental stocks maintained by Dr. V.J. Yates at the University of Rhode Island. These viruses were Irish strains, 58, 75, 340, 506, 685 and 764[5] and chicken-embryo-lethalorphan (CELO) virus[9].

Source of Embryonated Eggs: Embryonated chicken eggs used in this study were obtained from the Animal Science Department, Agricultural Experiment Station, University of Rhode Island.

Assay of AAAV and Avian Adenovirus: Complement fixation (CF) and immuno-diffusion (ID) tests were used to assay the AAAV and avian adenovirus common group antigens. The testing procedures and the preparation of antiserum were described in the first part of the study[7].

Growth Kinetics of AAAV in Chicken Embryo: Constant amounts of AAAV ($10^4 CFI_{50}$) were used with 10^5 PFU of CELO virus to coinfect 11-day-old chicken embryos via the chorioallantoic sac route. Preand post-inoculation amnioallantoic fluids were harvested and pooled in equal amounts. As controls, either CELO virus or AAAV was inoculated alone intoem bryos. The viral yield of AAAV and avian adenovirus were determined by the CF test.

Effects of Doses of "Helper" Virus on AAAV Replication: Varying amounts of "helper" CELO virus were used with various concentrations of AAAV to coinfect a set of four embryos. Infected AAF was harvested from each embryo five days later and assayed for AAAV antigen by the CF test. AAAV antigen titers of the four embryos were converted to $\log_2$ scale and expressed as the geometric mean. Antigen titers less than two were regarded as 0 in this scale.

"Helper" Activities of Avian Adenovirus on AAAV Replication: Seven strains of chicken embryo idney (CEK) cell adapted avian adenovirus were

used in this study. Approximately 10^5 PFU of avian adenovirus was inoculated with constant amounts of AAAV(10^4 CFI_{50}) into a set of three embryos. Secondary egg passages followed using primary egg passaged AAF as the inoculum. As a control, avian adenovirus alone was inoculated as described above. Infected AAF was collected five days later, pooled in equal amounts, and assayed for avian adenovirus common antigen by the ID test and for AAAV antigen by the CF test.

Sensitivity of Chicken Embryo Replication System for AAAV Isolation and Identification: Sets of three embryos were dually infected with 10^5 PFU of CELO virus and varying amounts of AAAV. Infected AAF was collected and pooled in equal amounts five days later, The harvested AAF was used as the inoculum for a second egg passage. The AAAV antigen of the primary and secondary egg passaged AAF was assayed by the CF and the ID tests.

Results

Growth Kinetics of AAAV in Chicken Embryo: As presented in Fig. 1, CELO viral antigen was first detected at 72 hrs. after inoculation, however, the yield of CELO virus antigen was clearly inhibited in embryos coinfected with AAAV.

AAAV antigen was detected.24 hr after CELO viral antigen. The maximum yield of AAAV antigen appeared the fifth day postinoculation, however, it was less than that of CELO virus. No AAAV antigen was detected in embryos inoculated with AAAV alone.

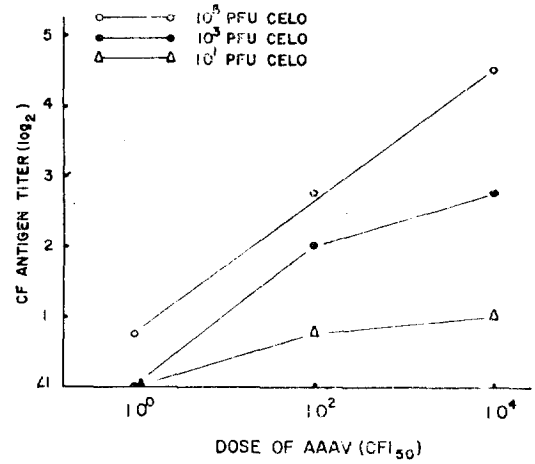
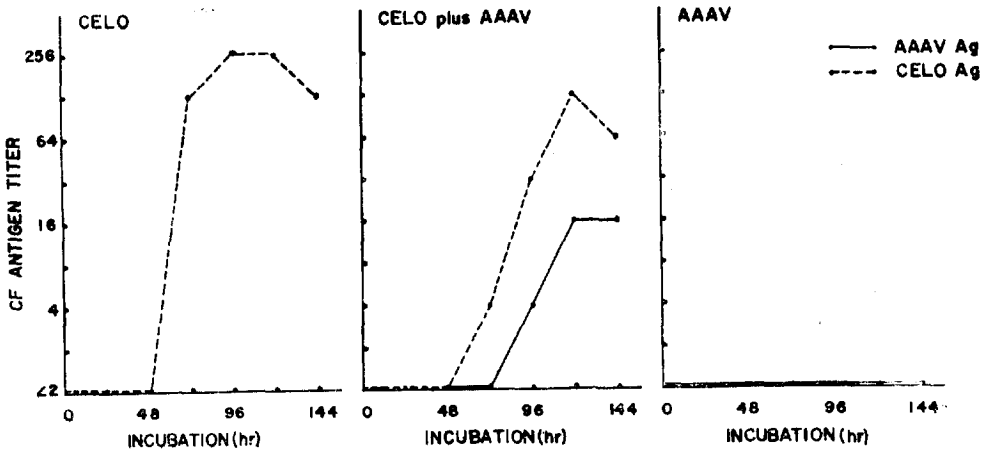


Fig. 1. Growth kinetics of AAAV in chicken embryos. Sets of 11-day-old chicken embryos were inoculated with CELO (10^5 PFU), CELO (10^5 PFU) plus AAAV (10^4 CFI) and AAAV (10^4 CFI), respectively. Equal amounts of AAF were harvested and pooled from each of 3 randomly chosen embryos at various times post-inoculation. The amount of AAAV and CELO viral antigens in AAF was determined by the CF test.

Effects of Doses of "Helper" CELO Virus for AAAV Replication: As shown in Fig. 2, the larger



Fig, 2. Effects of the Amount of "Helper CELO Virus on AAAV Replication in Chicken Embryos. Sets of four chicken embryos were inoculated with various amounts of AAAV and CELO virus. At five days post-inoculation, the AAF of 4 embryos were harvested. AAAV antigens of 4 AAF were assayed by the CF test and expressed as the geometric mean after converting CF antigen titers to log_2 value.

the amount of the "helper" virus inoculated, the larger the amount of AAAV antigen produced. With the dose of 10^5 PFU of CELO virus, AAAV CF antigen induction showed a linear relationship to the AAAV dose, and could be detected by 1 CFI unit of the virus. With the low dose of the "helper," the low level of AAAV could not be detected in the chicken embryo replication system.

"Helper" Activity of Avian Adenoviruses: Of seven strains of avian adenoviruses, only CELO, 506 and 764 multiplied satisfactorily in chicken embryos and supplied a good "helper" function for the multiplication of AAAV (Table 1).

Table 1. Multiplication of 7 Strains of Avian Adenoviruses and AAAV in Chicken Embryos

Avian[a] Adenoviruses	Dosage of AAAV (CFI)	Viral Antigen: Primary Egg Passage A. Ad Ag[b]	Primary Egg Passage AAAV Ag[c]	Secondary Egg Passage A. Ad Ag	Secondary Egg Passage AAAV Ag
CELO	0	###	—[d]	###	—
	10^4	##	16	###	64
506	0	###	—	###	—
	10^4	###	8	###	32
764	0	###	—	###	—
	10^4	###	8	###	32
685	0	+	—	##	—
	10^4	—	—	##	32
75	0	—	—	##	4
	10^4	—	—	##	8
58	0	+	—	##	2
	10^4	—	—	##	32
340	0	—	—	—	—
	10^4	—	—	—	—

a. 10^5 PFU of avian adenovirus were used.
b. The amount of avian adenovirus antigen was determined by the ID test. The concentration of antigen ranged from one (+) to four (+). One (+) indicates the least intense precipitating line. (−) in dicates no reaction.
c. The quantity of AAAV antigen was assayed by the CF test. Numbers indicate CF antigen titers.
d. No reaction at 1 : 2 antigen dilution.

The multiplication of 75, 58 and 685 was poor in the primary egg passage. In the second passage, higher adenovirus and AAAV levels were obtained. The multiplication of 340 was negative, even after two egg passages, and the "helper" function was also not detected.

CELO, 506, 764 and 685 were free from AAAV contamination, but 75 and 58 were shown to be contaminated. Contamination of 340 with AAAV was not clear in this test due to the poor adaptability of the "helper" adenovirus in chicken embryo.

Table 2. Sensitivity of Chicken Embryo Replication System for Isolation and Identification of AAAV

Dosage of AAAV (CFI)[a]	Dosage of CELO (PEU)	AAA V Antigen: Primary Egg Passage CF Test[b]	Primary Egg Passage ID Test[c]	Secondary Egg Passage CF Test	Secondary Egg Passage ID Test
10^4	10^5	32	##	32	##
10^2	10^5	16	+	32	##
10^0	10^5	2	—	32	##
10^{-2}	10^5	—[d]	—	8	+
10^{-4}	10^5	—	—	2	—
10^{-6}	10^5	—	—	—	—
10^4	0	—	—	—	—
0	10^5	—	—	—	—

a. Units of AAAV
b. AAAV antigen was determined by the CF test.
c. AAAV antigen was determined by the ID test. The concentration of antigen ranged from one (+) to fonr (+). One (+) indicates the least intense precipitation line, (−) indicates no precipitation lines.
d. No reaction at 1 : 2 antigen dilution.

Sensitivity of Chicken Embryo Replication System for the Isolation of AAAV: As shown in Table 2, AAAV antigen could be detected at a dose of 1 CFI of AAAV in the primary egg passage. AAAV antigen were detected when a second egg passage was carried out with dosage as low as 10^{-4} CFI.

Immuno-diffusion reactions were observed when CF titers were in the range of 8 to 16, but the reaction was not consistent.

Discussion

Growth kinetics studies were carried out to determine the optimum incubation time for AAAV replication, and the interaction between AAAV and the "helper" virus.

The appearance of AAAV in chicken embryos

followed that of the "helper" virus, by 24 hr. The maximum yield of AAAV occurred the fifth day after inoculation(Fig. 1). This observation was similar to that of Yates *et al.*[10] although the optimum incubation time was not the same.

It is of particular interest that AAAV infection inhibits the yield of coinfected "helper" CELO virus (Fig. 1) as observed in cells coinfected with primate adenovirus associated virus and adenovirus[1,3,6].

Considering the inhibitory effect of AAAV on the growth of avian adenovirus, the contamination of AAAV in adenovirus stocks would be a serious problem in the mass production of an adenovirus for vaccine preparation. These findings also raise questions on the contamination of AAAV in laboratories where studies are concentrated on avian adenovirus. A high incidence of AAAV contamination in avian adenovirus stocks have already been shown by Yates *et al.*[11] Contamination of AAAV in field samples should be a consideration in the isolation of avian adenoviruses in epidemiological and other studies.

AAAV contamination can be removed by treating the field samples or adenovirus stocks with specific anti-AAAV serum.

The growth of AAAV was completely dependent on the "helper" virus and showed a linear relationship with the dose of the "helper" (Fig. 1, Fig. 2). This relationship was also observed when other avian adenoviruses were used as "helpers." The degree of "helper" activities of any strains of avian adenovirus depended on its ability to multiply in chicken embryos. As shown in Table 1, strain 340 adenovirus was not able to multiply in chicken embryos even after the second egg passage. It therefore could not serve as a "helper" for AAAV multiplication.

These observations also suggested that if greater amounts of "helper" virus were used, a larger yield of AAAV will be obtained. However, a dose of "helper" CELO virus larger than 10^5 PFU kills the embryos within three or four days, which was prior to reaching the maximum yield of AAAV. In this study, 10^5 PFU of CELO virus was chosen as the optimum does of "helper" in chiken embryos considering all the above mentioned factors.

When 10^5 PFU of CELO virus was used as the "helper", an inoculum of 1 CFI of AAAV was dected in the primary egg passaged AAF. Further low level of AAAV (10^{-4} CFI) could be detected at the secondary egg passage (Table 2). This observation suggests that the chicken embryo replication system is a sensitive method, and applicable for the routine diagnostic purposes of AAAV.

The yield of AAAV in CEK cell cultures was not enough to be detectable by ID and CF tests. Therefore, concentration of infected cell culture fluid was necessary. However, this process may render the virus materials anti-complementary, due to the increased concentration of tissue culture fluid.[2] However, concentration of infected cell culture fluid does not effect the ID test. The chicken embryo replication system provides higher concentrations of AAAV and does not require further cencentration. It is the preferred system in preparation of AAAV for ID and CF tests. However, the effect of maternal antibodies in eggs must be considered.

The ID reaction was method of choice in the identification of AAAV, but the sensitivity of the reaction is low and should be considered. The CF test was sensitive enough to detect low levels of AAAV antigen, however, careful interpretation is important to eliminanate false positive reaction.

Considering the above mentioned problems, it is suggestive that both the ID and CF tests be applied for the identification of AAAV.

Conclusion

Avian adenovirus associated virus (AAAV) was propagated in 11-day-old chicken embryos coinfected with "helper" CELO virus. AAAV was identified by immuno-diffusion and complement fixation tests using specific anti-AAAV rabbit serum.

The complete dependency of AAAV on "helper" virus for replication was observed. The growth of AAAV showed a linear relationship with the dose of the "helper." It was also observed that an AAAV infection inhibited the yield of "helper" virus.

Of seven strains of avian adenoviruses, six supplied "helper" activities, however, the degree was directly related to the multiplication of the "helper" virus in chicken embryos.

The growth kinetics of AAAV were determined in embryonating chicken eggs using CELO virus as the "helper." The maximum yield of AAAV was on the fifth day post inoculation when 10^5 PFU of CELO virus was used as "helper."

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Avian Adenovirus Associated Virus (AAAV)의 특성에 관한 연구

II. 계태아에서의 AAAV의 증식

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抄 錄

순수하게 분리 정제된 AAAV를 "helper"인 가금 adenovirus와 공동으로 11일령의 계태아에 감염시켰으며 토끼 유래 특히항혈청을 사용하여 AAAV의 증식성을 추시하였다.

AAAV의 증식은 "helper"와의 공동감염인 경우에만 인정되었으며 "helper"의 양과 AAAV의 증식은 서로 일치하였으나, AAAV와의 공동감염인 경우 "helper"의 증식은 억제되는 경향을 보였다.

이 시험에 공여된 7주의 가금 adenovirus중 6주는 AAAV의 증식을 위한 "helper"능을 부여하였으며 "helper"로써의 기능은 계태아에서의 증식성과 일치하였다. 계태아에 순화된 (CELO) 바이러스를 "helper"로 하여 계태아에서의 AAAV의 증식을 조사한바, 10^5 PFU의 CELO 바이러스를 "helper"로 하였을 경우 접종후 5일에 최고에 달하였다.