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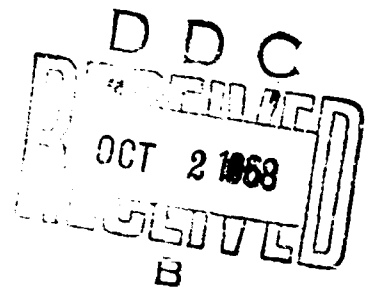
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DEPARTMENT OF THE ARMY
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ON THE SUBVIRAL AGENTS OF RABBIT MYXOMA AND
SHOPE FIBROMA VIRUSES

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ABSTRACT

An infective subviral agent (SVA) was isolated from the rabbit kidney (RK) cells infected with rabbit myxoma (South American Sanarelli strain) or Shope fibroma (OA strain) virus. When some properties of the SVA were examined briefly, they differed apparently from typical mature particles of myxoma-fibroma viruses in a few points.

Some experimental methods and results obtained are summarized as follows:

The SVA of myxoma virus was found in the virus-infected RK cell lysates after infection for 1, 4 and 6 hours. On the other hand, the SVA of fibroma virus was present in the virus-infected cell lysates after infection for 1 and 8 hours. Both myxoma and fibroma SVAs can be completely separated from the typical mature virus particles (about 200-300 m μ in diameter) by ultrafiltration through 50 m μ Millipore filters.

Both myxoma and fibroma SVAs revealed infectivity by being inoculated into the RK cell culture and injected intradermally into rabbits as well as the complete virus particles. Their infectivities were not affected by the action of DNA-ase (2 μ g/ml) at 37°C for 30 minutes.

Infectivity of the SVA from myxoma virus-infected cells was inactivated markedly more than that of myxoma virus by the effect of trypsin (100 μ g/ml) at 37°C for 30 minutes, and also neutralized completely after incubation at 37°C for 1 hour with anti-myxoma immune rabbit serum. When thermal inactivation rate of both myxoma SVA and virus was determined by incubation at 37°C for 30 minutes, surviving fraction of the SVA was less than 50% as compared with that of myxoma virus.

As speculation on these experimental results, the myxoma-fibroma subviral agents appear to contain protein and DNA, but they may be smaller particles than typical mature myxoma-fibroma virus particles. The fact that the myxoma SVA could be detected after infection for 1, 4 and 6 hours seems to be closely related to time of the growth cycle of this virus. Thus, the SVA which is detectable in earlier stage of the infection may differ from the agent found in later stage of the infection.

Although rabbit myxoma as well as Shope fibroma viruses belong to a subgroup (Myxoma-fibroma subgroup) of the Pox virus group [4], because of their tumor producing characteristics and their susceptibility to ethyl ether [3], they can be considered to have characteristics differing somewhat from other viruses within the Pox group. However, there still remain many unclarified features in the detailed characterization of the virus particles.

While investigating the reactivation of thermally inactivated myxoma virus with active fibroma virus [6, 8, 14], the author originally conducted experiments with the objective of separating the reactivating component [6] from the cells infected with both types of active viruses. The existence of a subviral agent (abbreviated SVA below) which passed through a millipore filter (50 m μ) was found in the virus infected lysate. A part of the results have been reported previously [22, 23].

In the results obtained and reported below, the author carried out a detailed study with the objectives of separating the SVA from rabbit kidney cell cultures infected with either myxoma or fibroma viruses and of elucidating its characteristics.

EXPERIMENTAL MATERIALS AND METHOD

Virus Strains

The myxoma virus used was a South American Sanarelli strain and for comparative experiments, Shope fibroma virus, OA strain was used. The material was used after 4-5 generations' passage through rabbit skin and 4-15 successive generations of rabbit kidney cell cultures. Both strains were obtained from Professor C.E. Schwerdt, Microbiology Dept., Stanford University.

Cell Culture and Determination of Virus Titer

Cell cultures were made from trypsin treated kidneys from young rabbits weighing about one kg which were then cultured from 7-10 days at 37°C in large flasks (500 ml volume). Cells were also used from the 2d or 3d generations of successive transplants from the foregoing in trypsin-versene solutions (containing 0.05% trypsin, 0.02% versene). The culture solutions used were YLH (0.1% yeast extract and 0.5% lactobumin hydrolysate in Hanks' solution) and Medium 199 solutions (containing 10-15% of inactivated bovine serum, penicillin 100 μ /ml and streptomycin 100 γ /ml). For increasing and preparing both viruses, the 1st or 2d generation culture was used and for plaque assay of virus, the 2d or 3d generation of transplanted RK cells was used.

For the assay of virus as well as infective SVA, the cells were inoculated and cultured for 5-7 days at 37°C, were washed twice with Dulbecco's phosphate buffer saline (PBS), stained for 2-3 minutes in a methanol solution of carbolfuchsin, water washed, dried and calculated from the plaque or foci which could be obtained from weak magnification (10-20X) [19, 20]. In rabbits injected intradermally, infectivity was

recognized by the appearance of typical myxomatosis or fibroma formation.

Virus Preparation and Concentration

The 1st or 2d generation RK cell culture in large flasks (500 ml volume) was inoculated with 3 ml/bottle of Stock virus solution. After absorption for two hours at 37°C, cultures were made using 40-50 ml/bottle of the culture solution. In the case where the myxoma virus was injected after 4-5 days of culturing at 37°C, conspicuous CPE appeared, the cells were destroyed and separated from the glass wall. However, when inoculated with fibroma virus, no unusual change was recognizable either with the naked eye or under low magnification (50-100X). The entire cell culture was freeze-thawed three times, then subjected to sonic treatment (10kc) for two minutes and the cells destroyed. This was next ultracentrifuged for 30 minutes at 30,000 rpm and both viruses were concentrated (refer to Figure 1). The materials for each phase were variously set aside as the Virus Pool, SP, SPS, and SPSC. Then the virus titer of each fraction was determined by plaque assay from the RK cell culture.

Fig. 1 Method of preparation and concentration of myxoma-fibroma viruses from the infected RK cell cultures

The primary or secondary RK cell cultures

- | Inoculated with the viruses.
- | Incubated at 37°C for 2 hours to permit virus adsorption.
- | Overlaid with the medium and then incubated at 37°C for 4 to 5 days.
- | Frozed and thawed 3 times.
- | Sonicated with 10kc ultrasonic oscillator for 2 minutes.

The virus pools (Pool)

- | Ultracentrifuged at 30,000 rpm for 30 minutes.

The virus pellets (Pellet)

- | Suspended into small volume of the medium.

Suspended pellets of the viruses (SP)

- | Sonicated with 10 kc ultrasonic oscillator for 60 seconds.

Suspended pellets of the viruses after sonication (SPS)

- | Centrifuged at 3,000 rpm for 2 minutes.

Suspended pellets of the viruses after sonication clarified by low centrifugation (SPSC)

Preparation of Virus Infected Cell Lysate

The small flask (60 ml volume) of RK cells was inoculated at an MOI ratio (Multiplicity of infection)= 1-10 with either the concentrated myxoma virus (3.0×10^7 PFU/ml) or with the fibroma virus (5.0×10^6 PFU/ml) and cultured for 0, 1, 2, 4, 6, 8 hours at 37°C. After each time period, two or three culture flasks were removed, washed more than five times with

PBS, 5 ml/bottle of the culture solution (10-15% serum containing Medium 199) were added and frozen at -20°C . In order that each infected cell would be thoroughly adsorbed, for two hours the flask was periodically tilted in the absence of the culture solution so that the cell surface was moistened. The successively removed cells were freeze-thawed three times, ultrasonically treated for two minutes and centrifuged for 10 [minutes] at 15,000 rpm. The upper clear liquid was then suction filtered with extreme care through 50 m μ or 10 m μ Millipore filter (Millipore Filter Corp. U.S.A.). Each sample was stored frozen at -20°C until used.

EXPERIMENTAL RESULTS

Preparation of Virus Concentrated Solution

Three typical experimental results for myxoma virus are illustrated in Tables 1 and 2. In Experiment 1, the virus was propagated on the primary

Table 1 Preparation and concentration of rabbit myxoma virus (South American Sanarelli strain) propagated in the RK cell culture (I)

Expt. 1

Myxoma R ₄ T ₁₀ *	Vol (ml)	Titer (PFU/ml)	Total (PFU)	Recovery (%)
Pool	120	4.4×10^5	5.3×10^7	100
SPSC	10	4.0×10^5	4.0×10^7	75

* The stock virus has been passaged into rabbit skins 4 generations and then into the RK cell cultures 10 generations. They were multiplied in the primary RK cell grown in the YLH medium containing 10 to 15 % bovine serum.

Expt. 2

Myxoma R ₄ T ₁₀ **	Vol (ml)	Titer (PFU/ml)	Total (PFU)	Recovery (%)
Pool	420	3.9×10^5	1.6×10^8	100
SPSC	10	2.8×10^5	7.8×10^7	490

** The virus was multiplied in the secondary RK cell grown in the M 199 medium containing 10 to 15 % bovine serum.

RK cells cultured in YLH solution containing 10-15% bovine serum. In Experiment 2 the second generation RK cell culture grown in Medium 199 solution containing 10-15% bovine serum was used. In comparing the two for the amount of virus obtained and the percent recovery, the latter was superior. The PFU/ml was concentrated 100X and recovery was 100%. The results of propagation and concentration from Experiment 3 were obtained under these same conditions. The virus titer and percent recovery from each phase are given in Table 2. Just as in Experiment 2, the PFU/ml was concentrated 100X and there was a good recovery of 100%.

In the case of the fibroma virus, as can be seen from Table 3, even though the culture conditions were the same as with the myxoma virus, the amount obtained was approximately 10 times less; the concentration as well as the percent recovery were also much less. The cause for this is one of

Table 2 Preparation and concentration of rabbit myxoma virus propagated in the RK cell culture (II)

Expt. 3

Myxoma R ₄ T ₁₉	Vol (ml)	Titer(PFU/ml)	Total (PFU)	Recovery(%)
Pool	250	2.2×10^8	5.5×10^8	100
SP	12	4.5×10^7	5.4×10^8	98
SPS	12	3.3×10^8	4.0×10^8	730
SPSC	12	3.8×10^8	4.0×10^8	820

* The virus was multiplied in the secondary RK cell grown in the M 199 medium containing 10 to 15 % bovine serum.

Table 3 Preparation and concentration of Shope fibroma virus (OA strain) propagated in the RK cell culture

Fibroma R ₄ T ₁₉	Vol (ml)	Titer(PFU/ml)	Total (PFU)	Recovery(%)
Pool	300	8.5×10^8	2.6×10^8	100
SP	15	2.0×10^8	3.0×10^7	11
SPS	15	4.2×10^8	6.3×10^7	24
SPSC	15	4.7×10^8	7.1×10^7	27

* The stock virus has been passaged into rabbit skins 5 generations and then into RK cell cultures 4 generations. They were multiplied in the secondary RK cell grown in the M 199 medium containing 10 to 15 % bovine serum.

the problems which requires future investigation.

Infectivity of Subviral Agent

When cell lysates prepared after 1, 4 and 6 hours of infection with myxoma virus were filtered through a 50 m μ Millipore filter, a small amount of infectivity was noted even in the filtrate and plaques appeared on the RK cell culture. Moreover, myxoma formation occurred upon intra-dermal injection into rabbits. Those found in the cell lysate after four and six hours of infection showed titer values of 100-400 PFU/ml while those arising after one hour of infection were far lower. The action of DNA-ase (2 μ g/ml in 0.1M MgCl₂·7H₂O solution; crystalline DNA-ase, Wash. Biochem. Corp., U.S.A.) for 30 minutes at 37°C was nearly without effect on their infectivity (refer to Table 4).

When these samples were again filtered through a 50 m μ filter, the infectivity shifted to the filtrate; however, using a 10 m μ filter, the shift was not seen. From samples prepared after 0, 2 and 8 hours of infection, the presence of infectious SVA of this type was not seen.

When the highly concentrated stock solution (approximately 3.0×10^8 PFU/ml) of myxoma virus was diluted about 10^5 X with culture solution and ultrasonically treated, the infectious particle did not filter at all through the 50 m μ filter used in the previous experiment.

Such an infectious SVA was also noted during the process of propagating the fibroma virus. It was detected after one and eight hours of

Table 4 Infectivity of myxoma subviral agent (SVA) tested by the RK cell culture and rabbit

(a) Time after infection		0	1	2	4	6	8	(b) MV
(c) Tissue culture	Untreated	0	5	0	1.5×10^2	3.8×10^2	0	0
	DNA-ase treated	0	5	0	1.6×10^2	3.7×10^2	0	
(c) Rabbit skin	Untreated	-	+	-	+	+	-	-
	DNA-ase treated	-	-	-	+	+	-	

(a) Numbers show time (hours) after virus infection.

(b) The stock virus fluid was diluted about 3,000 PFU/ml with the medium, sonicated for 3 minutes and then filtered through a 50 mμ Millipore filter.

(c) Infectivity titers of the SVA were assayed by plaque forming units (PFU/ml) in the RK cell cultures and the infectivities were tested also by the intradermal inoculation into rabbits for production of typical myxomatosis (+ or -).

infection and its titer was more than 300 PFU/ml as seen in Table 5.

Table 5 Infectivity of fibroma subviral agent (SVA) tested by the RK cell culture and rabbit

(a) Time after infection		0	1	2	4	6	8	(b) FV
(c) Tissue culture	Untreated	0	2.7×10^2	0	0	0	2.5	0
	DNA-ase treated	0	2.1×10^2	0	0	0	7.5	
(c) Rabbit skin	Untreated	-	+	-	-	-	-	-
	DNA-ase treated	-	+	-	-	-	+	

(a) Numbers show time (hours) after virus infection.

(b) The stock virus fluid was diluted about 5,000 PFU/ml with the medium, sonicated for 3 minutes and then filtered through a 50 mμ Millipore filter.

(c) Infectivity titers of the SVA were assayed by plaque or focus forming units (PFU/ml) in the RK cell cultures and the infectivities were tested also by the intradermal inoculation into rabbits for production of typical fibroma (+ or -)

Treatment with DNA-ase ($2 \mu\text{g/ml}$, 37°C , 30 minutes), was ineffective and as in the case of the myxoma virus, the infective particle in the high concentration fibroma virus stock solution (approximately 5.0×10^6 PFU/ml) did not filter through the 50 mμ Millipore filter.

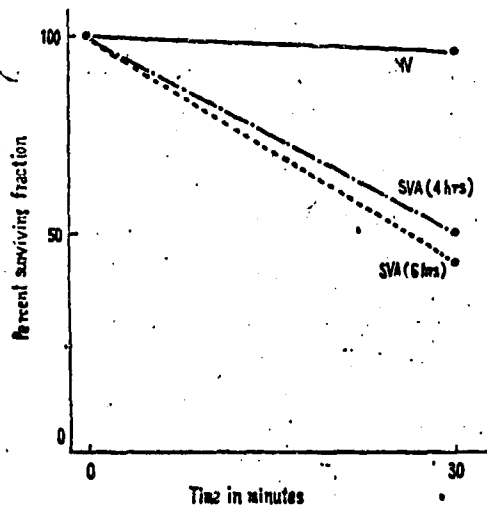
Several Characteristics of The Infective Subviral Agent

The following results were obtained from a study of several characteristics of SVA separated by filtration through a 50 mμ filter of cell lysate infected with myxoma virus (four and six hours of infection). For comparative purposes, the diluted solution of the high concentration myxoma virus (MV) stock solution was used as the mature virus particle.

By means of this experiment, it was possible to use SVA samples which were not completely diluted as well as MV samples with titers of about 1,000 PFU/ml.

First of all, it was found that SVA, when compared to mature particles, was extremely heat labile. Thus as can be seen from Figure 2, the survival rate after 30 minutes at 37°C was reduced to less than 50% according to plaque assay. Such a reduction in titer was not seen with MV.

Fig. 2 Thermal inactivation of the SVA (after infection for 4 and 6 hours) and myxoma virus (MV) after incubation at 37°C for 30 minutes



In general, SVA can be easily inactivated and long storage is difficult. Moreover, freezing and thawing markedly reduced this activity.

Next, the effect of trypsin is shown in Figure 3. Crystalline trypsin (Wash. Biochem. Corp., U.S.A.) was added to both SVA and MV so that its final concentration was 100 μ g/ml and incubated at 37°C for 30 minutes. Results of plaque assays indicate that the infectivity of SVA was almost completely lost. In the comparative experiment, the infectivity of the mature particles was also affected, but much less than with SVA.

The infectivity of the myxoma SVA was neutralized with anti-myxoma virus rabbit serum just as with the mature virus particles (refer to Table 6). The immune serum was prepared by repeated injections of ultraviolet inactivated virus into the veins of rabbits [24]. This was diluted 5X and mixed with an equivalent amount of the solution under examination. After treating for one hour at 37°C, its infectivity was determined from cell culture and rabbit skin [inoculation]. A solution of normal rabbit serum diluted 5X was used as the standard. The SVA was examined with the original solution as well as with solution diluted 10 times and under both conditions was always completely neutralized.

Fig. 3 Inactivation of the SVA (after infection for 4 and 6 hours) and myxoma virus (MV) after treatment with 0.01 % trypsin at 37°C for 30 minutes

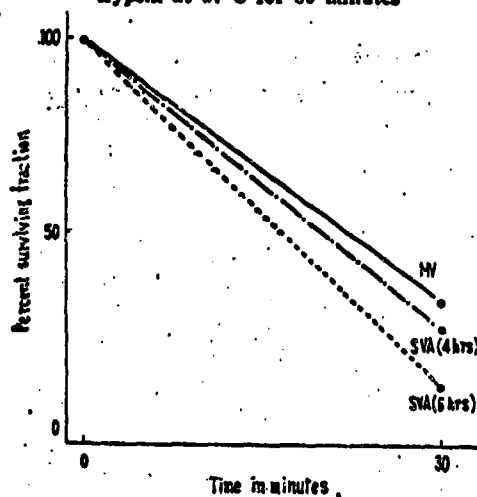


Table 6 Neutralization of myxoma SVA by anti-myxoma immune rabbit serum

Samples	Infectivity (PFU/ml)	Myxomatosis in rabbit
SVA(x1)+IRS	0	-
SVA(x10)+IRS	0	-
MV +IRS	5	+
SVA(x1)+NRS	18	##
SVA(x10)+NRS	5	##
MV +NRS	1 x 10 ⁶	##

SVA: Subviral agent

MV: Myxoma virus

IRS: Immune rabbit serum

NRS: Normal rabbit serum

DISCUSSION

Myxoma and Shope fibroma viruses belong to a subgroup of the Pox virus group and there are numerous unclarified points with regard to such things as characteristics, survival and the mechanism of tumor formation by the virus particles. The study of the physical and chemical aspects of the virus, in particular, lag behind the investigations on other pox viruses. Although the author originally had intended to study these viruses from the standpoint of the known reactivation analysis, during a specified period in the process of propagating the virus, the existence of a subviral agent which was considered to be of a far smaller size than the normal completely mature particle of 200-300 m μ was found. Thus an attempt was made to separate and investigate its characteristics.

Recently there have appeared reports on small sized particles from

other types of animal viruses resembling the SVA isolated by the author. For example, as a result of studies on the propagation and structure of Simian adenovirus grown in Green monkey kidney cell cultures, H.D. Mayor, et. al. (1965) [16, 17], observed by means of an electron microscope the presence of two types of large particles and in addition to the normal mature particles, small particles of 20 m μ size in the virus infected culture solution. These small particles were isolated from the mature virus particles by means of the Millipore filter or CsCl density gradient centrifugation. The density was 1.43 in the CsCl solution and furthermore it was said to be formed from the DNA-protein complex.

With regard to the RNA type virus group, there have recently been several reports on the nonuniformity of the virus particles. Nishimura, et. al. (1963) [18] discovered two types of infective particles in the mouse brain infected with Japanese encephalitis virus. Furthermore, Matsumura, et. al. (1963) [15] have reported the presence of two types of infective particles with differing densities obtained when the partially purified material from the mouse brain infected with dengue fever virus was profiled by means of the sucrose density gradient method.

The myxoma-fibroma SVA isolated by the author has not yet been reported from other pox group viruses and although the author's investigation is as yet incomplete, if this is a characteristic component of myxoma-fibroma virus, then it has the tumor formation characteristic and is of extreme interest from the standpoint that it exhibits several peculiar characteristics.

As is clear from the experimental results above, the infective SVA from the myxoma virus appears in the cells after 1, 4 and 6 hours of infection. When this is compared to the one-step propagation curve [21] of this virus, it is thought that there is an intimate relationship between the fact that the logarithmic period of the virus propagation begins after approximately one hour and reaches its maximum after about five hours. Thus, that which is detected during the primary infection period and that which is detected later are probably heterogeneous agents. It is conjectured that the former is related to the parent virus and the latter from the propagated progeny. However, as has been related previously, the SVA demonstrates a resistance to DNA-ase and is inactivated by trypsin. Moreover, from the fact that they are neutralized by anti-myxoma rabbit serum, these SVA can be considered to be small sized nuclear protein particles which are the partially coated myxoma antigen proteins rather than the so-called infective DNA.

Recently, successful extractions of infective DNA have been made from DNA-type viruses or from the infectious components of these viruses using phenol extraction primarily [5, 7, 9]; however, this is not necessarily simple with the pox virus group. Rather, chemical extraction has been completely unsuccessful. Recently, Abel, et. al. (1963, 1964) [1, 2] have extracted the decoating enzyme from chick embryo cells infected with rabbit pox virus and demonstrated the successful isolation of infectious DNA from thermally inactivated vaccinia virus decomposed with oxygen. Moreover, it is interesting to note that the maximum production of enzyme reaches 4-6 hours after virus infection which is in agreement with the SVA

production found by the author.

Using P^{32} and C^{14} labeled rabbit pox virus, Joklik (1964) [10-13] conducted a detailed analysis of the uncoating process and found that the uncoating of pox virus DNA occurred in two steps. In the first stage immediately after adsorption by the cell, the virus particle is decomposed even to the internal core by the essential enzyme of the cell and in the second stage the uncoating of the virus DNA is completed. It has been said that certain types of protein are unable to participate in a functional role at this point. From these experimental results it appears that the uncoating of rabbit pox virus reaches a maximum approximately 4-5 hours after infection. Thus, considering this fact, it may be that the SVA found by the author is the so-called uncoated form which appears at a certain time during the process of virus propagation. However, a detailed examination must await future investigations.

SUMMARY

The results from a study of several characteristics of infective subviral agents which can be separated from the mature particles in the cell lysate from RK cells infected with rabbit myxoma or Shope fibroma viruses are as follows:

(1) SVA from myxoma virus appeared in the sample after 1, 4 and 6 hours of infection while that from the fibroma virus was found after 1 and 8 hours of infection. Both passed through Millipore filters (50 $m\mu$) and could be separated completely from the mature particles.

(2) Just as with mature viruses, both types of SVA were infectious for RK cultured cells as well as for rabbit skin and their titers were unaffected by DNA-ase.

(3) Infectivity of myxoma SVA was markedly reduced by trypsin and was completely neutralized by anti-myxoma rabbit serum. Results from a comparison of thermal stabilities indicated that myxoma SVA was markedly heat labile compared to mature viruses and was easily deactivated upon long storage or even on freezing and thawing.

Most of the work in this report was conducted under the auspices of Professor Carlton E. Schwerdt (Dept. of Medical Microbiology, Stanford University School of Medicine, U.S.A.), to whom warm appreciation is expressed for his concern and discussions during my stay and for providing the important virus strains. To others of the laboratory also, appreciation is extended. An expression of gratitude is extended to Professor Susumu Horita for his aid in continuing the studies upon my return and for his review of this article. This investigation was partly supported by a PHS (NIH, U.S.A.) and by a special virus study financial grant from Hyogo Prefecture which are gratefully acknowledged.

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