THE UNIVERSITY OF MANITOBA

PRODUCTION AND CLASSIFICATION OF ESH SARCOMA VIRUS

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ABSTRACT

PRODUCTION AND CLASSIFICATION OF ESH SARCOMA VIRUS

The previously unclassified avian sarcoma virus Esh sarcoma virus (ESV) has been placed within the A subgroup of the avian sarcoma-leukosis complex. Cloned stocks of ESV were able to form foci in C/O and C/B type chick embryo fibroblasts but its plating efficiency was significantly reduced in cells resistant to subgroup A viruses. Rous associated virus of subgroup A interfered with the infectivity of ESV but Rous associated viruses of subgroups B, C, and D were unable to do so. Neutralization tests indicated immunological relatedness of ESV to subgroup A avian sarcoma viruses.

Some evidence is presented that indicates that ESV is cytopathic.

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INTRODUCTION

INTRODUCTION

Esh sarcoma virus was isolated from a spontaneously occurring tumor of a White Leghorn chicken. The virus when inoculated into the wingweb of chicks, caused the formation of tumors in almost all of the birds. Subsequent passaging and enhancement procedures led to a 1.25 log increase in titre as determined by the latent period analysis test (Wallbank et al., 1966a). In addition to inducing tumor formation, Esh sarcoma virus (ESV) was found to produce foci of transformed cells in chick embryo fibroblast cultures and was used in a study of the viral inhibitory effects of 1-adamantanamine hydrochloride (Wallbank et al., 1966b), and of a-methyl-1-adamantane-methylamine hydrochloride (Wallbank, 1969). Further, bioassay of ESV was performed utilizing both focus production in vitro and tumor formation in vivo as a means of quantitatively estimating virus concentration (Wallbank et al., unpublished data). The results indicated that the focus assay is an accurate method of determining the virus titre, as demonstrated by the linearity of the regression curve calculated from the titration data in which the quantity of virus was progressively reduced by a factor of two.

The aim of this project was to propagate large quantities of ESV in order to determine its relationship to the other viruses of the avian sarcoma-leukosis complex. The basic approach used to discover this relationship was to study the cross neutralization, interference, and host range properties of ESV and compare them to those of the prototype viruses of the known subgroups within the complex.

It is of particular interest that ESV is a recent field isolate since it permits us to study a virus that has had a minimum of exposure to any of the other viruses of the avian sarcoma-leukosis complex and presumably its initial characteristics have undergone no drastic change since its isolation.

LITERATURE REVIEW

LITERATURE REVIEW

Other than the studies mentioned in the introduction there is no further literature pertaining to ESV. For this reason, any review of the literature must be directed to the avian leukosis-sarcoma group as a whole, but particularly to Rous sarcoma virus (RSV) which provides an excellent model system for the study of the virus-host interaction.

I. PROPERTIES OF THE AVIAN SARCOMA-LEUKOSIS VIRUSES

For practical purposes the viruses of the avian sarcoma-leukosis group (avian tumor viruses) can be divided into those that cause leukosis diseases (avian leukosis viruses) and those that cause fibrosarcomas (avian sarcoma viruses). The members of the group, however, are all morphologically similar and share other physiochemical properties as well as possessing certain biological and antigenic relationships.

Avian tumor virus particles are spherical in shape, with a diameter ranging from 80 nm to 120 nm (Beard et al., 1955). In thin section, electron microscope images of the particle show a central dense nucleoid of high contrast which has a ground substance of relatively low electron density (Beard et al., 1957). Virus particles are released from the infected cell by budding from the host cell plasma membrane, from which they acquire their outer lipoprotein envelope (Courington et al., 1967).

The avian tumor viruses resemble the myxoviruses in that they are sensitive to such lipid solvents as ethyl ether and chloroform (Andrewes and Horstman, 1949), to heat (Eckert et al., 1955; Dougherty, 1961), as well as to acid (Bryan et al., 1950).

Biochemical studies of Rous sarcoma virus have indicated that it contains ribonucleic acid (RNA) as the sole nucleic acid constituent (Crawford and Crawford, 1961). However the precise nature of the nucleic acid of the sarcoma virus was uncertain due to the presence of associated helper viruses. More recent results by Robinson et al., (1965) show that there is no detectable difference between the nucleic acid of the sarcoma virus and the associated leukosis virus. In addition, the nucleic acid of the recently discovered Rous sarcoma virus free of associated virus [RSV(0)] consists exclusively of RNA (Robinson, 1967). However, recent report by Levinson et al., (1970) indicated that the genome of RSV contains a small deoxyribonucleic acid (DNA) component.

Inhibition studies of RSV growth in tissue culture utilizing 5-bromodeoxyuridine, 5-iododeoxyuridine, and cytosine arabinoside demonstrated a requirement for the synthesis of DNA in its replication cycle (Bader, 1964; 1965). The requirement was found to be transitory, which implied that a viral DNA primer was synthesized in the infected cells (Bader, 1965). In addition to the production of RSV, the transformation of the host cells was dependent on at least one round of DNA synthesis. The growth of the malignant cells, however, once the transformation had been fixed, could then proceed normally, even when DNA synthesis was inhibited for a period of 12 hours after infection (Bader, 1966). Furthermore, inhibition by ultraviolet light, actinomycin D, and mitomycin D showed that an intact DNA component was necessary throughout the growth cycle of RSV, even after the requirement for DNA synthesis has passed. It was concluded that virus production was continuously dependent on the participation of DNA, although synthesis was required only during the early stages of infection (Nakata and Bader, 1968).

The requirement for DNA synthesis in a RSV infected cell suggested to Temin (1964) that a DNA component, in the form of a "provirus", was incorporated into the cell genome. Hybridization experiments performed by Temin indicated that there was a new DNA, complementary to the RNA isolated from purified RSV, in RSV infected cells. However, the presence of the postulated "provirus" was disputed when Wilson and Bauer (1967) found that RSV RNA could hybridize to DNA obtained from noninfected cells to the same extent as it could to DNA from RSV infected cells. The issue seemed to be resolved when Temin (1970) discovered that RSV virions possessed a RNA-dependent DNA polymerase (reverse transcriptase). This was confirmed by Spiegelman et al. (1970) who also detected DNA-dependent DNA polymerase activity in the virions of six oncogenic RNA viruses including RSV. The evidence suggested that in all oncogenic RNA viruses a single stranded DNA template was formed and that it was converted to a double strand in order to be incorporated into the infected cells genome. However, it has been shown that the reverse transcriptase is not unique to cancer viruses: it is present in both visna virus particles (Stone et al., 1971) and in foamy virus (Parks et al., 1971), and may occur in normal human and murine cells as well (Scolnick et al., 1971).

II. ASSAY AND PRODUCTION OF AVIAN TUMOR VIRUSES

Early attempts to quantitatively study the avian tumor viruses involved the use of the latent period analysis test (Bryan, 1946). Virus infectivity titres were calculated by observing the length of time that was required for a tumor to develop in a chicken. Alternatively, the chorioallantoic membrane of an embryonated egg could be infected and the resulting centres of infection (pocks) directly counted (Rubin, 1955; Prince, 1958a, 1958b).

The first indications that chick embryo cells in vitro could be transformed to a new morphological type was provided by Halberstaedter et al. (1941) who caused the "cancerization" of chick fibroblasts by exposing them first to radium rays and then to Rous sarcoma cells obtained from a tumor induced by RSV. Later, Sanford (1952) and Lo (1955) transformed chick embryo fibroblasts by exposing the cells directly to RSV. However in neither case was any meaningful enumeration possible. Evidence of countable foci of altered chick embryo cells was provided by Manaker and Groupé (1956) who showed that the number of foci was directly proportional to the amount of virus inoculated. An accurate tissue culture assay for RSV was later described by Temin and Rubin (1958). In this assay procedure the infecting virus converts the chick embryo cells to a morphologically new and stable cell type, which multiply under a nutrient agar overlay, and results in the formation of countable foci of transformed cells. However, it was suggested that at any time ninety per cent of the cells could not be infected even at very high input multiplicities of infection. This idea was later modified by Rubin (1960a) in an analysis of the assay by use of the infective centre technique. Infected cell cultures were transferred to a feeder layer of chick embryo fibroblasts and overlayed with nutrient agar which contained antiserum to the infecting virus. Any infected cell manifested itself by forming a focus (infective centre) after several days incubation. The results indicated that all the cells were infected, but when they were transferred to the feeder layer immediately after infection, only ten per cent were capable of registering as infective centres. However, if the time period between infection and the transfer was delayed, there

was an increased possibility that the cells would form foci.

The factors influencing the determination of the cellular morphology of cells infected with RSV were defined by Prince (1962). This study indicated that Eagles Basal medium, supplemented with sera from various species, was able to support only limited transformation in cell cultures of turkey fibroblasts infected with RSV. However the addition of ten per cent tryptose phosphate broth markedly favored the production of morphological transformation.

The first examination of the kinetic relationship between avian sarcoma virus infected cells and the infecting agent was performed by Rubin (1955) with Rous Number 1 chicken sarcoma virus (the former name for the original Rous isolate). He found that each infected, virus producing cell (Rous cell) was capable of producing virus while continuing to survive and to multiply.

Other investigators of the in vitro growth kinetics of RSV on the chorioallantoic membrane revealed that the virus was adsorbed within a hour after inoculation and that the first newly synthesized virus was detected after fourteen to sixteen hours. The final rate of virus release was two focus forming units (FFU) per cell per day, and this maximum level of production was reached after forty hours of infection (Prince, 1958c).

Vigier and Goldé (1959) reported on the growth of RSV and Rous cells in primary confluent monolayers of chick embryo fibroblasts infected at low input multiplicities. They found that the number of FFU per ml increased stepwise to a plateau of production, reached after four days of infection. The growth consisted of a phase of logarithmic

growth lasting approximately two days, followed by a brief stationary period. Virus production then would again begin to increase logarithmically, reflecting what was thought to be a second cycle of infection.

Temin and Rubin (1959) studied virus and cell growth in a similar chick embryo fibroblast system. New virus synthesis was detected after twelve hours and the maximum rate of virus synthesis of four FFU per cell per day was reached after forty hours of infection. The number of virus producing cells doubled every twenty hours and it was found that the ability of a cell to produce virus was transmitted to the progeny as a strictly intracellular event.

Since high input multiplicities of infection were used in the experiments of Temin and Rubin (1959), no second cycle of infection occurred. Synthesis of new virus increased exponentially for four days and then ceased. The increase in virus synthesis did not indicate a larger amount of virus released per cell, rather it reflects the increase in the number of virus releasing cells. Virus production reached a plateau when the cells, both normal and infected, ceased to multiply, and an equilibrium was established between the amount of virus being produced and the amount being inactivated by heat.

In the same cell system, Prince (1960) compared the growth of the Bryan standard strain and the Bryan high titre strain of RSV at various input multiplicities of infection. He showed that the growth of the virus in both strains was logarithmic until the virus titre reached a plateau which coincided with the arrest in cell growth. The early logarithmic growth, however, was more rapid and the eventual titre was greater with the high titre strain than with the standard strain. This

finding was related to a lower thermal inactivation rate and to a higher plating efficiency for the high titre strain. In addition, Prince pointed out that at any input multiplicity of one or above, the growth rate and the final titre were constant. However, at input multiplicities below one, biphasic growth curves occurred.

Goldé and Vigier (1961) described the growth curve of the Bryan high titre strain of RSV which was devoid of the thermal resistance marker found by Prince in his previous work with the same strain. The growth curve of the virus and of the virus producing cells were similar to those reported by Temin and Rubin (1959), Rubin (1960), and Prince (1960), except that the virus saturation of the cell cultures, i.e., when all the susceptible cells are infected, occurred at input multiplicities of 0.1 FFU per cell instead of 1.0 FFU per cell. In addition, the growth rate of the virus was slower than that found in Prince's study. The plateau of virus production was reached after four days as compared to two days. The discrepancies can be due, in part, to the fact that the virus used in this study was more susceptible to heat inactivation than was the virus used in the previous study.

The growth cycle of the other avian tumor viruses closely resembles that of Rous sarcoma virus although the growth rates may show considerable variations. In all cases the cycle is characterized by a short adsorption period of approximately thirty minutes, followed by an eclipse of twelve to eighteen hours. The virus titre then increases exponentially for a period varying between two to four days and then reaches a plateau, with a constant amount of virus being produced by the infected cells and a constant amount being heat inactivated at the same time (Vogt and Rubin, 1963; Hanafusa and Hanafusa, 1966b).

III. THE DEFECTIVENESS OF AVIAN SARCOMA VIRUSES

Both the Bryan high titre strain of RSV and the Harris strain of RSV are considered to be defective, since they are unable to produce mature progeny virus in the absence of an associated helper virus (Hanafusa et al., 1963; Reamer and Okazaki, 1970). The sarcoma viruses by themselves, are capable of infecting and transforming chick embryo fibroblasts, and are able to produce typical foci in culture under a nutrient agar overlay. When these foci are isolated and cultured, however, no infectious virus related to the original infecting stock can be recovered. The individual transformed, non virus producing cells are said to be nonproducer cells (NPC). If, however, any leukosis virus is added to the NPC, infectious virus, with the antigenic characteristics of the helper leukosis virus, will immediately be recovered.

Recent work has modified this idea and has referred to the Bryan high titre strain of RSV as being defective only in a relative sense. DiStephano and Dougherty (1965) detected virus like particles associated with the cell membrane of a culture of NPC. These particles were later isolated from the growth medium (Dougherty and DiStephano, 1965) and were shown to possess focus forming properties (Weiss, 1967; Vogt, 1967). The recovered virus, termed RSV(O) to indicate that it could multiply in the absence of any helper virus, had unusual growth properties. It was originally thought to have an extremely narrow host range; growth being restricted to C/A type chick embryo fibroblasts. It was later shown that RSV(O) could grow on some cells derived from C/O embryos and on Quail fibroblasts (Hanafusa, 1968; Weiss, 1969a). Two viruses were eventually found: one termed RSV$\propto$(O) had no known host. The other

was termed RSVβ(O), and had the host range described above. The relationship of RSVβ(O) to the other avian tumor viruses was studied by Vogt (1967), Weiss (1969b), and Ishizaki and Shimizu (1970). All of these studies indicated that leukosis viruses of subgroup B interfere with RSVβ(O). However, antisera to RSVβ(O) does not react with the viruses of subgroups A, B, or C, but it does react with the viruses of subgroup D. In addition, it was found that viruses of subgroups A and C enhance the focus forming efficiency of RSVβ(O).

PRESENCE OF AN ASSOCIATED VIRUS

When a stock of the Bryan high titre strain of RSV is diluted out beyond its endpoint of focus formation, another virus, designated Rous associated virus (RAV), can be isolated (Rubin and Vogt, 1962). RAV does not cause morphological transformation of chick embryo fibroblasts, but induces resistance to RSV infection, which enables RAV to be assayed by a quantal response interference method. RAV is indistinguishable from RSV in terms of heat sensitivity, cellular site of maturation, and growth rate. RAV differs from RSV only in its inability to cause cellular transformation of chick embryo fibroblasts *in vitro* and in its failure to induce the formation of tumors in chickens. Instead, RAV was found to cause leukemia in fowl and consequently it was determined that it is a member of the avian leukosis group.

When RAV is added to a culture of NPC, both RSV and RAV are produced as a result of the dual infection (Hanafusa *et al.*, 1964). The ability of the RAV to stimulate the production of RSV has been found to be a property of all the avian leukosis viruses so far tested (Hanafusa and Hanafusa, 1966a).

The cellular transformation but lack of viral progeny production in solitary infections by the defective RSV, indicates that the RSV genome is replicated in the NPC without the aid of an associated virus, but that the helper virus is required for the production of the mature, infective RSV particle. Since the RSV, propagated from the NPC following the helper virus infection, has the antigenic properties of the helper virus, it was concluded that the associated virus produced the protein coat for the defective RSV and thus completed the growth cycle of the sarcoma virus (Hanafusa et al., 1964). The antigenicity of all the virus resulting from the dual infection is referred to as a pseudotype (Steck and Rubin, 1966). RSV(RAV-1), which is made up of the genome of RSV and the lipoprotein coat of RAV-1, is an example of such a pseudotype.

IV. CLASSIFICATION OF AVIAN TUMOR VIRUSES

Avian tumor viruses are currently classified into four major subgroups: A, B, C, and D (Vogt and Ishizaki, 1965, 1966; Duff and Vogt, 1969). This classification is based on viral envelope protein properties as manifested by a common host range of viruses belonging to the same subgroup, viral cross interference, and by immunological cross reactivity.

HOST RANGE

The first evidence of genetic resistance to RSV was provided by Greenwood et al. (1948). They demonstrated that a specially developed line of chickens possessed a greatly reduced susceptibility to RSV. Later, Waters (1960) reported on the genetic resistance to RSV infection among five unrelated inbred lines of White Leghorn chickens. The

resistance was found to extend to the chorioallantoic membrane (Prince, 1958b), as well as to tissue cultures obtained from the embryos of resistant lines (Crittenden et al., 1963a). The genetic control of the cellular susceptibility to RSV was determined by a single autosomal gene (Prince, 1958b; Waters and Burmester, 1961), dominant for susceptibility (Crittenden et al., 1964).

Vogt (1965) described the heterogeneity of the Bryan high titre strain of RSV by using selectively resistant chick embryo fibroblasts. Two distinct variants of the strain could be differentiated: one, associated with RAV-1, was capable of infecting all of the embryos of a commercial line of chickens. The other, associated with RAV-2, could infect only forty per cent of the embryos of the same line. The difference in host range between the two viruses was reported to be controlled by the two helper viruses RAV-1 and RAV-2 and the isolated viruses were designated RSV(RAV-1) and RSV(RAV-2).

Originally two subgroups could be distinguished on the basis of their host range. The first was subgroup A, which included RAV-1, any pseudotype of RAV-1, and several other leukosis and sarcoma viruses that are related to it. The second, subgroup B, consists of RAV-2 and any antigenic relative of it (Vogt and Ishizaki, 1965).

Chick embryo fibroblasts resistant to subgroup A viruses were designated C/A cell types in imitation of phage nomenclature, while those resistant to subgroup B viruses were designated C/B cell types. Those fibroblasts resistant to both subgroups A and B were referred to as C/AB and cells susceptible to both were termed C/O cell types (Rubin, 1965; Vogt and Ishizaki, 1965).

Two new subgroups of avian tumor viruses were later discovered by Duff and Vogt (1969). Subgroup C viruses are capable of infecting C/O, C/A, C/B, and C/AB cells. Subgroup D viruses can infect C/O and C/A cells, but have a reduced ability to grow in C/B and C/AB cells.

The relationship between the various subgroups of the avian tumor viruses and the known genetically selective chick embryo fibroblasts is summarized in Table I.

The nature of the genetic resistance to the avian tumor viruses is not yet fully known. The resistance to RSV is known to be independent of the attachment of the virus to the resistant cell (Piraino, 1967). However, the ability of the insusceptible cell to synthesize virus once the viral genome has entered the cell is unaffected. The resistance, which is almost absolute, appears to occur at the stage of penetration or uncoating (Crittenden, 1968). One explanation of the selectivity, put forward by Piraino (1967), suggests that specific cell receptors must be present for the penetration of the virus. These cell receptors will attach only viruses having a protein coat of a particular type. In the case where the cells are insusceptible to a virus of a certain subgroup it is thought that the cellular receptors, specific to that subgroup are missing.

Mixed infection of susceptible chick cells with avian tumor viruses representing subgroups A and B yield progeny virus which have an expanded host range in genetically resistant cells. The ability of the viruses to overcome host range barriers in tissue culture appears to be the result of the presence of an A and B receptor in virus particles harvested from cultures which had been infected with viruses representing both subgroups

TABLE I

CELLULAR RESISTANCE TO THE AVIAN TUMOR VIRUSES

Cell type	Avian tumor virus subgroup			
	A	B	C	D
C/O	S	S	S	S
C/A	R	S	S	S
C/B	S	R	S	R-S
C/AB	R	R	S	R-S

S = susceptibility

R = resistance

R-S = reduced susceptibility

simultaneously. Phenotypic mixings of this type permit virus particles, containing a subgroup A genome but a subgroup B receptor, to enter a C/A cell type from which they are normally excluded. Similarly, viruses with a subgroup B genome may enter a C/B cell due to the presence of subgroup A receptors (Vogt and Ishizaki, 1966; Vogt, 1967).

INTERFERENCE

Classification by interference makes use of the finding by Rubin (1961) that chick embryo cells, when preinfected with an avian leukosis virus, become resistant to a challenge infection by RSV. This resistance is not absolute and will be conferred on the cells only if the preinfecting dose of the leukosis virus is at high input multiplicities of infection, or if the virus is given several days to multiply in the cell culture to a high concentration before the sarcoma virus is inoculated. The interference occurs only between leukosis and sarcoma viruses having an antigenically similar or identical lipoprotein coat, i.e., the interference is subgroup specific. Thus, leukosis viruses of subgroup A will interfere with sarcoma viruses of subgroup A, but not with the sarcoma viruses of subgroups B, C, or D. Similarly, the interfering properties of the various leukosis viruses of the other subgroups is restricted to the subgroups of which they are members (Hanafusa, 1965; Vogt, 1965; Steck and Rubin, 1966; Duff and Vogt, 1969).

There is one exception to the rule that viral interference is subgroup specific. Subgroup D avian leukosis viruses interfere with subgroup B sarcoma viruses and subgroup B leukosis viruses interfere reciprocally with subgroup D sarcoma viruses (Duff and Vogt, 1969).

Bauer and Graf (1969) also observed the relatedness of the viruses of subgroups B and D and concluded that the two subgroups share an antigenic component of the viral envelope. The similarities between B and D viruses suggest that the two subgroups should be combined into a single subgroup. However, as was pointed out above, the host range of the two subgroups are distinctly different; the subgroup D viruses having a higher plating efficiency on C/B and C/AB type cells. In addition, the sarcoma viruses of subgroup D have been found to be oncogenic in newborn hamsters (Hanafusa and Hanafusa, 1966; Duff and Vogt, 1969).

The fact that leukosis viruses generally interfere only with sarcoma viruses that belong to the same subgroup suggested that envelope antigen of the interfering virus combined to a specific receptor site on the cell surface and prevented the similarly specific receptor on the challemge virus from adsorbing to the cell. Studies by Steck and Rubin (1966) indicated that this is the mechanism, since the interference can be reduced or abolished if antiserum to the preinfecting leukosis virus is added to the culture, or if the pH of the growth medium is greatly reduced. Both of these treatments can inactivate the leukosis virus only if they have not penetrated the cell. The possibility that the penetration stage of the growth cycle might be affected was ruled out, since the interference could be established at four degrees centigrade as well as it could be at thirty seven degrees centigrade. Since penetration is considered to be a temperature sensitive event (Mandel, 1958), it can be assumed that the interference occurs at the surface of the cell by the blocking of receptor sites by the preinfecting virus.

A summary of the interference patterns within the avian leukosis-sarcoma group is presented in Table II.

TABLE II

INTERFERENCE WITHIN THE AVIAN SARCOMA-LEUKOSIS GROUP

Interfering leukosis virus subgroup	Sarcoma Virus Subgroup			
	A	B	C	D
A	I	NI	NI	NI
B	NI	I	NI	I
C	NI	NI	I	NI
D	NI	I	NI	I

I = Interference

NI = No interference

IMMUNOLOGICAL CROSS REACTIVITY

The third property used in the classification of the avian tumor viruses is their immunological specificity. All the viruses of the group have two main antigens: one is group specific and the other is subgroup and type specific.

The group specific antigen is a protein, free of nucleic acid, but related to the viral RNA core (Oroszlan et al., 1970). Antiserum reactive with this antigen has been used in the tissue culture assay of the non-cytopathic avian tumor viruses, i.e. the complement-fixation test for avian leukosis viruses (COFAL test) (Sarma et al., 1964). Generally, the antiserum is produced to the Schmidt-Ruppin strain of RSV in hamsters and is then used to detect the presence of the group specific antigen in the sera of chickens.

The type specific antigen is related to the viral lipoprotein envelope and controls both the type and the subgroup specificities (Ishizaki and Vogt, 1966). The immunological classification of the avian tumor viruses is based on the cross reactivity in neutralization tests using antisera produced in fowl. The patterns of the cross reactivity indicate the existence of four antigenic subgroups, each containing several similar but distinct antigenic types (Simon and Dougherty, 1963; Ishizaki and Vogt, 1966; Duff and Vogt, 1969). These subgroups were found to correspond to the four subgroups revealed by host range specificity and by interference patterns. Sera prepared against subgroup A viruses will neutralize all the other viruses of subgroup A, but will not significantly neutralize any of the viruses of subgroups B, C, or D. Similarly, antisera prepared against subgroups B, C, and D will only neutralize the viruses of their respective subgroups.

MATERIALS AND METHODS

MATERIALS AND METHODS

SOURCE OF VIRUSES

Esh sarcoma virus (ESV) was isolated from a tumor of the thigh of a White Leghorn chicken submitted to the New Bolton Center, University of Pennsylvania, Poultry Diagnostic Laboratory. The tumor material was passed five times in Pennsylvania Farm Bureau strain one White Leghorns and the extracted virus was used as the source for all experiments (Wallbank et al., 1966).

RSV(RAV-1) and RSV(RAV-2) virus types, which were originally derived from the Bryan high titre strain of RSV, and the Schmidt-Ruppin strain of RSV (SR-RSV-2) were obtained from Dr. W. Okazaki, United states Regional Poultry Laboratory, East Lansing, Michigan.

The Bratislava strain of avian sarcoma virus (B-77) is a distinct field isolate, unrelated to the original Rous isolate. It was obtained from Dr. P. K. Vogt, Department of microbiology, University of Washington School of Medicine, Seattle, Washington.

RSV(RAV-50) is a virus type, made up of a RSV genome derived from the Bryan high titre strain of RSV and of a Rous associated virus (RAV-50) isolated from the Schmidt-Ruppin strain of RSV. It was also obtained from Dr. Vogt.

Rous associated viruses RAV-1 and RAV-2 were isolated from the Bryan high titre strain of RSV and both were supplied by Dr. Okazaki.

Rous associated viruses RAV-49 and RAV-50 were both isolated from the Schmidt-Ruppin strain of RSV and were supplied by Dr. Vogt.

PREPARATION OF VIRUS STOCKS

Most avian sarcoma stocks were prepared by inoculating C/O type chick embryo fibroblasts, seeded at 2×10^5 cells per ml in small Petri dishes (60 x 15 mm, Falcon Plastics), with the virus to be propagated. The cultures were incubated at 37°C in a CO_2 incubator adjusted to maintain a pH of 6.6 ± 0.2 and were transferred every four days through a series of passages until all the cells appeared to be transformed. Culture fluids harvested four days after the final passage, were pooled, centrifuged at 575 g for 20 minutes, and were stored at -87°C.

RSV(RAV-50) stocks were found to be contaminated with a subgroup A leukosis virus and therefore were cloned on C/A cells before working stocks were prepared. In addition, ESV was cloned on C/O type cells in order to reduce the possibility of working with a mixed virus preparation. RSV(RAV-2) was found to be cytopathic in chick embryo cell culture and so was replaced by another subgroup B virus, SR-RSV-2, whenever it was required.

Avian leukosis virus stocks were prepared according to the method of Duff (1969). Subgroup A leukosis virus (RAV-1) was produced by inoculating C/O type chick embryo fibroblasts with this virus and passaging the cultures three times. Subgroups B, C, and D leukosis viruses (RAV-2, RAV-49, and RAV-50 respectively) were similarly propagated in C/O type cells, but were passed four times before virus was harvested. All leukosis viruses were collected after a complete monolayer had formed in its final passage. The stocks were then pooled, centrifuged at 575 g for 20 minutes, and stored at -87°C.

TISSUE CULTURE PROCEDURES

COMPLETE GROWTH MEDIUM Combination medium 199 and Eagle's medium was used to make up the complete growth medium. The combination medium was made up of 100ml of 10 x medium 199, 20 ml 50 x Basal medium eagle (BME) amino acids, 10 ml of a 100 x BME vitamins solution, 10 ml L-glutamine solution containing 200 mM per ml, and 30 ml of a 2.8 per cent solution of sodium bicarbonate. Sterile double distilled water was added to make up one liter.

Complete growth medium was made of 820 ml of the combination medium, 100 ml of a 2.95 per cent solution of tryptose phosphate broth (Difco), and 80 ml of heat inactivated calf serum. The medium was adjusted to pH 7.0 with 2.8 per cent sodium bicarbonate and 100 units of ampicillin, 200μg of carbenicillin, 200μg of streptomycin, and 2.5μg of amphotericin B were added per ml.

MAINTENANCE MEDIUM This medium was made up of 97 per cent combination 199 and Eagle's medium to which three ml of inactivated calf serum was added. Final pH and antibiotic concentrations were the same as in the complete growth medium.

NUTRIENT AGAR OVERLAY The nutrient agar overlay consisted of 44 ml of 1.8 per cent agar (Difco), 8.8 ml of 10 x medium 199, 29.7 ml of sterile double distilled water, 2.2 ml of 50 x BME amino acids, 1.1 ml of 100 x BME vitamins, 1.1 ml of a L-glutamine solution containing 200 mM per ml, 13.2 ml of a 2.95 per cent solution of tryptose phosphate broth, 5.5 ml of heat inactivated calf serum, 4.4 ml of a 2.8 per cent

solution of sodium bicarbonate, and the same complement of antibiotics as used in the complete growth medium.

SUSPENSION GROWTH MEDIUM Suspension growth medium was made up of Eagle's minimum essential medium modified for suspension culture (calcium and magnesium ions removed), and 20 per cent fetal calf serum. Final antibiotic concentrations were the same as those used in the complete growth medium. Darvan No. 2 (polysodium benzoic sulfonate, R. T. Vanderbilt Co.) was added as a dispersing agent at a final concentration of 0.05 per cent.

CONDITIONED MEDIUM Condition medium was produced by incubating normal suspension growth medium over a confluent monolayer of chick embryo fibroblasts for 24 hours. The medium was then centrifuged at 250 g to remove any floating cells.

CELL FREEZING MIXTURE The freezing mixture consisted of 6.5 ml of complete growth medium supplemented with 1.5 ml of heat inactivated fetal calf serum and 2.0 ml of dimethyl sulfoxide (Crown Zellerbach Corp.).

CELL CULTURE Fertile, COFAL negative, chicken eggs were obtained from several sources. SPAFAS chicken embryos, which consisted of an undefined mixture of C/O and C/B cell types, were supplied by SPAFAS Inc., Norwich, Connecticut. Genetically defined embryo types C/O, C/A, C/B, and C/AB were obtained from the United States Regional Poultry Laboratory, East Lansing, Michigan. In addition, chicken embryos of types C/O, C/A, and C/B were supplied by Heisdorf and Nelson Inc., Redmond, Washington. The latter cells were found to be superior to the others in

terms of ease of handling, and were used for the bulk of the experimental work.

All eggs were incubated for 12-14 days after receipt at 100°F dry bulb (38°C) and at 86°F wet bulb (30°C) in a Jamesway incubator which was set to rotate the eggs through 90° every two hours.

Primary chicken embryo fibroblasts were prepared by trypsinizing whole embryos in a 0.25 per cent trypsin solution consisting of 2.5 ml of concentrated trypsin (Wallis et al., 1961) in one liter of Hanks' balanced salt solution. Large particles were allowed to settle out and the cell suspension was centrifuged at 250 g for 10 minutes. The cells were washed twice with complete growth medium and then were recentrifuged at 250 g for 10 minutes. Following resuspension, the viable cells were counted in a hemocytometer and were seeded at a concentration of 1×10^6 cells per ml in five litre tissue culture bottles containing 200 ml of complete growth medium. The cultures were incubated at 37°C in a closed system until a complete monolayer was formed in three to four days. The cells were then removed from the glass surface by washing the cultures with 50 ml of a trypsin solution consisting of 2.5 ml of concentrated trypsin in one litre of a $1:5 \times 10^4$ versene-water solution. The cell suspension was decanted and centrifuged at 250 g for 10 minutes and the sediment was washed twice with complete growth medium. The concentration of the cells was adjusted to 120×10^6 cells per ml and were mixed with an equal volume of freezing mixture, sealed in one ml ampoules, and stored in the vapor phase of a liquid nitrogen freezer at -178°C.

Prior to all experiments involving the use of chick embryo fibroblasts the cells were rapidly thawed in a 37°C water bath and the contents

of one ampoule (60×10^6 cells) were inoculated into a one liter tissue culture bottle containing 60 ml of complete growth medium. After three days incubation at 37°C in a closed system the cells were removed from the glass using the trypsin-versene solution. The cell suspension was centrifuged at 250 g and the resultant cell concentrate was used as the source of any experiment.

CELL COUNTING The number of viable cells per ml was determined by adding 0.2 ml of a cell suspension to 0.2 ml of a 0.05 per cent physiological solution of trypan blue. Cells that excluded the trypan blue dye after a few minutes incubation at room temperature were considered to be viable. Counting was performed using a Spencer Bright-Line haemacytometer. The average number of viable cells counted within ten white cell chambers from duplicate samples was multiplied by a factor of 2×10^3 to give the total number of viable cells per ml.

STANDARD ASSAY PROCEDURE Secondary chick embryo fibroblasts were seeded at a concentration of 2×10^5 cells per ml in Petri plates (60 x 15 mm with 2 mm grids, Falcon Plastics) containing five ml of complete growth medium. Virus sample dilutions were made in growth medium and 0.5 ml of each of the various dilutions was inoculated into each of two replicate plates. Cultures were incubated at 37°C in a CO_2 box which maintained a pH of 6.6 ± 0.2. After overnight incubation the original medium was discarded and the cell cultures were overlayed with a nutrient agar overlay (five ml per plate). The extended incubation of the inoculum and cells was found to be practical and is possible because under conditions of low multiplicities of infection progeny virus is not detected until 18 hours after infection (Vigier and Golde, 1959). After four days further incubat-

ion three ml of maintenance medium was added to each plate and the cultures were reincubated. The plates were removed from the incubator seven days after the overlay was added, and the now fully developed foci were counted microscopically (40 X power) on the eighth day. Titres of the virus assayed were expressed as focus forming units per ml (FFU per ml) and were obtained by multiplying the total number of foci counted in ten percent of the total area of the plate times the dilution factor times ten. The titre of the virus preparation could then be calculated by multiplying by two in order to adjust for the 0.5 ml inoculum. In cases when the foci count in the representative area was less than thirty, the accuracy of the count was improved by counting a larger area. The number of foci produced in a culture plate by a given inoculum of RSV has been found to be highly reproducible (Temin and Rubin, 1958). In addition, the relationship between virus concentration and the focus count has been shown to be strictly linear. Wallbank (unpublished data) has also indicated the linear relationship between the number of Esh sarcoma virus induced foci and the concentration of the virus. Analysis of the variance of the data indicated a highly significant regression curve.

CLONING OF AVIAN SARCOMA VIRUSES Chick embryo fibroblasts, seeded at 2×10^5 cells per ml in Petri dishes were infected with an inoculum yielding three foci or less by making up serial one in two dilutions of the viruses. The cultures were treated in the same manner as that described above in the standard assay procedure. The chicken fibroblasts, which are normally flattened and elongated, are changed morphologically when they are infected with avian sarcoma viruses. As a result of infection and transformation the fibroblasts become spherical and refractile. These cells lose the property of contact inhibition and pile up to form foci. Most foci

develop to a size of approximately 0.5 mm in diameter after about eight days (estimated by reference to the 2 mm grid). However, they are first detectable after about four days. As soon as distinct foci developed, they were marked and aspirated through the nutrient agar overlay with a 23 gauge needle. Each individual focus of transformed cells was then transferred to a culture of normal chick embryo fibroblasts prepared in the same way as above. These cultures were passaged every four days until all the cells appeared to be transformed. The virus, harvested from the medium over the transformed cells, was then assayed and the procedure was repeated. After four series of clonings the virus was considered to be pure and was used as the stock virus in subsequent experiments.

Considerable variation in the morphology of foci induced by the various sarcoma viruses was noticed in these cloning procedures. Foci induced by RSV(RAV-1) and RSV(RAV-50) were compact, small and multilayered. B-77 virus caused single layered, grape cluster-like foci. ESV induced foci were quite distinctive. Most were extremely diffuse, multilayered and comparatively much larger than the foci formed by the other viruses. The diameter of the ESV foci was estimated to be approximately 1 mm.

PRODUCTION OF ANTISERA TO AVIAN SARCOMA VIRUSES

Antisera to RSV(RAV-1) and to RSV(RAV-2) was produced using a modified procedure of that described by Fink and Rauscher (1961). Prior to immunization, stocks of virus types RSV(RAV-1) and RSV(RAV-2) were prepared to a concentration of 1×10^5 FFU per ml. Formalin (37 per cent HCHO in H_2O) was then added to each virus preparation to a final concentration of 0.01 per cent in complete growth medium and the mixture was stored at $4^{\circ}C$ for seven days.

Two year old, COFAL negative, chickens were obtained from Kimber Farms Inc., Freemont, California. Two ml of formolized virus was injected into two sites in the breast muscle of each bird using a 21 gauge needle. This procedure was repeated twice in the week following the primary injection. At two weeks and at three weeks after the first injection the birds were challenged with two ml of live virus, injected in the same manner. The birds were exsanguinated by cardiac puncture six weeks after the primary inoculation.

Antisera to virus type RSV(RAV-50), as well as to virus strains B-77 and ESV, was prepared according to the procedure of Hanafusa (1965). Virus stocks, containing 10^4 - 10^5 FFU per ml, were prepared and two ml of each virus was inoculated intravenously into eight week old, C/O type, birds obtained from Heisdorf and Nelson Inc. This procedure was repeated every second day during the first week following the primary injection. Three weeks later a two ml dose of the same virus was given intravenously. The chickens were bled out by cardiac puncture eight to ten weeks after the primary injection.

Serum was collected using the method of Hirata (1963). Blood was placed in polycarbonate tubes and allowed to stand for 30 minutes at room temperature. It was then centrifuged at 2400 g for 40 minutes. The serum portion was decanted into 50 ml conical centrifuge tubes and was recentrifuged at 275 g for 20 minutes, inactivated at $56^{\circ}C$ for 30 minutes, and stored at $-20^{\circ}C$.

EXPERIMENTAL PROCEDURES AND RESULTS

EXPERIMENTAL PROCEDURES AND RESULTS

I. PRODUCTION OF ESH SARCOMA VIRUS

Since the ESV pools directly extracted from tumor material yielded virus titres of only 10^3 - 10^4 FFU per ml, experiments were performed in order to produce the large amounts of the virus that are necessary for its further study. One possible approach to this problem was suggested by Bader (1968), who propagated large amounts of RSV by growing RSV transformed chick embryo fibroblasts in liquid suspension culture. The production of ESV by transformed chick embryo cells in such a system would provide a rapid, simple method for the propagation of large amounts of ESV.

GROWTH OF AVIAN SARCOMA VIRUSES IN SUSPENSION CULTURE

RSV(RAV-1), RSV(RAV-2), and ESV were tested for their ability to confer upon chick embryo cells the capacity to grow in liquid suspension. Secondary chick embryo fibroblasts obtained from Spafas Inc., were seeded at a concentration 2×10^5 cells per ml in 100 mm Petri dishes and were inoculated with avian sarcoma viruses RSV(RAV-1), RSV(RAV-2), and ESV at an input multiplicity of 0.01. The cell cultures were placed in a 37^{o} C humidified incubator and after six days the cells were removed from the dishes with a trypsin-versene solution. Following centrifugation at 250 g and resuspension in growth medium, the cells were transferred to 100 ml spinner flasks (Bellco) at a concentration of 2×10^5 cells per ml in 50 ml of suspension growth medium. As a control, uninfected chick embryo fibroblasts were added to another flask at the same concentration.

The flasks were placed on a magnetic stirrer set for 100 revolutions per minute and incubated at 37°C for six days. Aliquots from each flask were taken daily and the number of viable cells per ml was determined. In addition, the virus titre of each sample was assayed.

The data in Table III shows that of the viruses tested, under the conditions described, only RSV(RAV-1) was capable of causing a change in the growth potential of chick cells, in such a way that they might be utilized to propagate a high concentration of virus. The data further shows the cytocidal influence of RSV(RAV-2) and of ESV on chick embryo fibroblasts in suspension culture.

Experiments using suspension growth media supplemented with various concentrations (10-40 per cent) of calf and fetal calf sera, extra supplements of amino acids and vitamins, or with conditioned media (Rubin, 1968), did not improve the growth potential of the ESV transformed cells.

GROWTH OF ESH SARCOMA VIRUS IN CHICK EMBRYO FIBROBLAST MONOLAYERS

Since it was evident that it was not possible to produce large amounts of ESV in suspension culture, consideration was given to the feasibility of producing the virus by the more conventional method of growing it in monolayers of chick embryo fibroblasts. Preliminary experiments were performed in which nonconfluent monolayers of chick embryo fibroblasts, obtained from Spafas Inc., were infected with ESV at input multiplicities of 0.01. Virus was harvested after four days when the growth of the chick fibroblasts had become confluent, and was assayed as described in Materials and Methods. In all cases the virus infectivity

TABLE III

SUMMARY OF THE RELATIVE GROWTH OF VIRUS AND VIRUS INFECTED CELLS IN SUSPENSION CULTURE

Infecting virus	Number of cells at time 0	Number of cells after six days	Factor of increase	Virus titre after one day	Virus titre after six days	Factor of increase
Control	2.0*	1.7	0.85	-	-	-
RSV(RAV-1)	2.0	3.3	1.65	1.6×10^3**	1.0×10^6	625
RSV(RAV-2)	2.0	0.1	0.05	2.5×10^2	0	<0.01
ESV	2.0	0.3	0.01	3.0×10^2	0	<0.01

* Viable cells x 10^{-5}

** FFU per ml

\- Not done

titre of the collected fluids was no higher than the titre of the input virus. This was thought to be due to the fact that the input multiplicity of infection was so low that only a small proportion of the cells become infected before growth of the monolayers stopped. Since the infection of the cells requires that the cells be actively multiplying, only those cells that were infected during the first and possibly the second cycle of infection were producing virus. In addition, since the fibroblasts consisted of an undefined mixture of genetically selective types, there was a possibility that some of the cells were resistant to infection by ESV. It was determined, therefore, firstly, to increase the total number of cells actually infected by increasing the period during which ESV was in contact with the actively multiplying cells by serially passaging the cultures; and secondly, to eliminate the possibility of resistant cells by using C/O type chick embryo fibroblasts which have been shown to be susceptible to all avian tumor viruses.

Secondary, C/O type, chick embryo fibroblasts were seeded at a concentration of 2×10^5 cells per ml in 100 mm Petri plates containing ten ml of complete growth medium. An ESV preparation containing 2×10^4 FFU per ml was inoculated into each culture. The cultures were incubated at $37^{\circ}C$ in a CO_2 box which maintained a pH of 6.6 ± 0.2. After three days the cells were removed from the plates using a trypsin-versene solution. Following centrifugation at 250 g, the cells were resuspended in complete growth medium, the concentration of the cells was adjusted, and they were reseeded on 100 mm Petri dishes at a concentration of 2×10^5 cells per ml, and were reincubated. This procedure was repeated every three days through a series of four passages. Fluids were harvested prior

to each passage in order to obtain the virus titre. Assays were performed on culture fluids as previously described in Materials and Methods.

The results of the assays are illustrated graphically in Figure 1. The growth of the virus increased exponentially for about nine days. After the third passage, however, virus growth slowed. No virus assays were attempted following the fourth passage since the cell cultures displayed obvious cytopathic effect. A one log increase in titre was obtained by this method. Several attempts were made to further increase the titre of the virus, either by increasing the number of passages before the fluids were harvested, or by increasing the input multiplicity of infection. However, final virus titres obtained never exceeded 2×10^5 FFU per ml.

II. PRESENCE OF AN ASSOCIATED VIRUS

Before any attempt could be made to classify ESV, the presence or absence of an associated virus(s) in its stocks had to be ascertained. The existence of an associated virus would suggest that ESV is defective, i.e., that it requires a helper virus in order to mature into infectious particle, as is the case with the Bryan high titre strain of RSV. The knowledge of the participation of such a helper virus would be essential to the determination of the relationship of ESV to the other viruses of the avian leukosis-sarcoma group.

END POINT DILUTION TEST

Dilution of a virus stock, which contains both a sarcoma virus and an associated leukosis virus, beyond its end point of focus formation

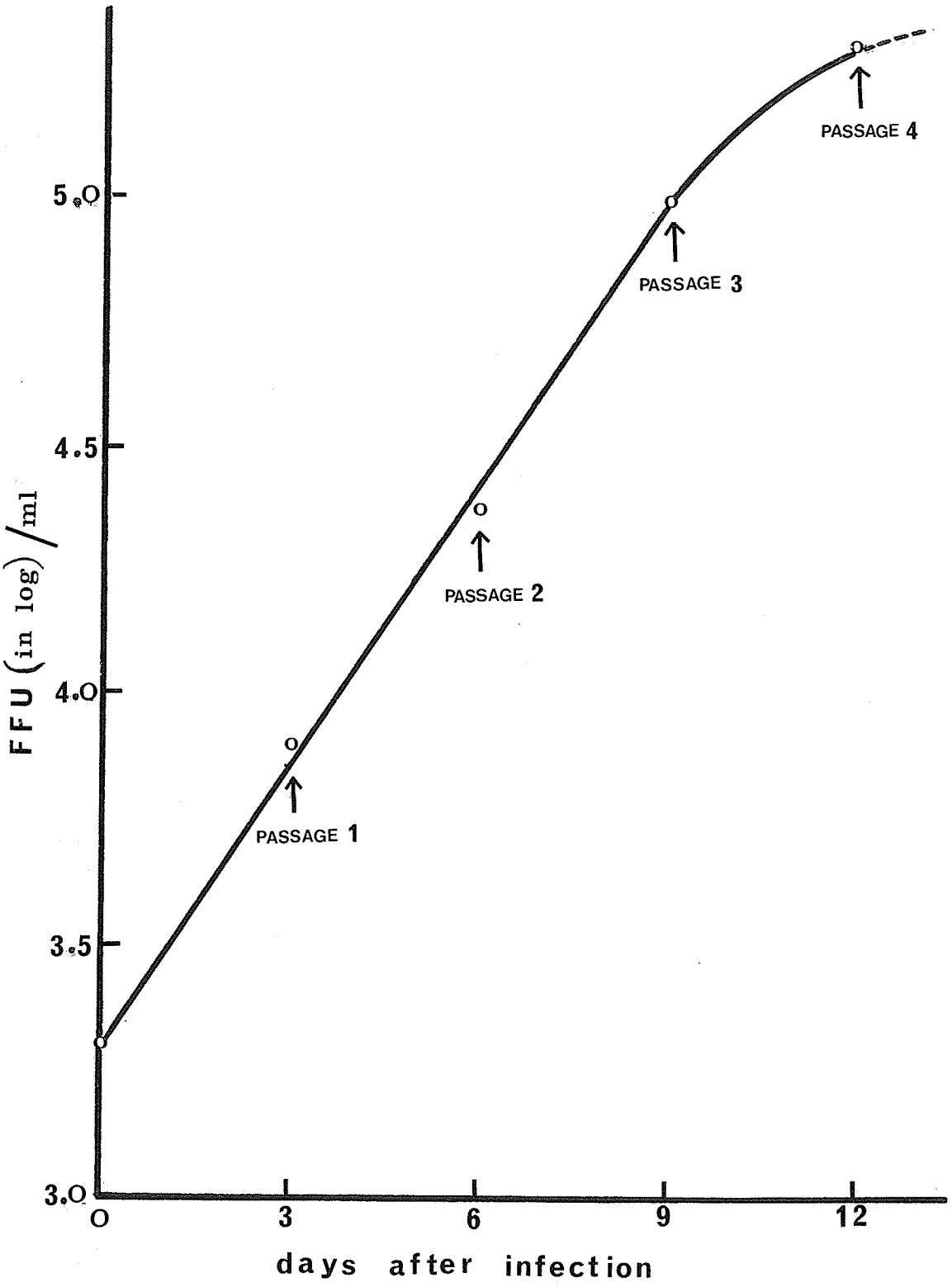

Figure 1

Growth of ESV in monolayers of chick embryo fibroblasts.

could result in the presence of a small number of associated viruses in the post end point dilutions. These dilutions are inoculated into cultures of susceptible chick embryo fibroblasts and the associated leukosis viruses are allowed to multiply for several days. The detection of the presence of the associated virus is based on the capacity of the leukosis viruses to induce interference to a related sarcoma virus. The interference is manifested by a marked reduction in the number of foci formed by a challenge sarcoma virus when it is inoculated into a culture of chick embryo cells containing high concentrations of leukosis virus. The degree of resistance induced by the leukosis viruses depends on the initial dose and on the growth rate of the virus, but no matter how small the initial dose, the cultures eventually reach a maximum level of interference to the challenge. With all avian leukosis viruses tested this maximum level of interference is reached, if the infected cultures are passed four times (Hanafusa et al., 1964). It was the purpose of the following experiment to test for the presence or the absence of an associated virus in the stocks of ESV by use of the end point dilution procedure.

A stock suspension of ESV containing approximately 5×10^4 FFU per ml was diluted 1:1000 using ten fold dilutions in complete growth medium. The last ten fold dilution was further diluted two fold out to 1:1024 in complete growth medium to obtain the post end point dilutions. All of the two fold dilutions were then each inoculated into three separate cultures of chick embryo fibroblasts seeded at 2×10^5 cells per ml in small Petri dishes containing five ml of complete growth medium. The cultures were incubated at $37^{\circ}C$ in a CO_2 atmosphere and were transferred every four days through a series of four passages. As a control, a

corresponding set of normal chick embryo fibroblasts was carried through the same procedure, but were not inoculated with the ESV stock dilutions. After the final passage, both sets of cultures were seeded at 2×10^5 cells per ml in small Petri dishes with grids and were challenges with an ESV preparation containing 5×10^2 FFU per inoculum. The cultures were then overlayed and maintained as in the standard assay procedure and eight days after being challenged, the foci were counted. In addition, the same procedure was applied concurrently to a stock of RSV(RAV-1), since it is known to carry an associated virus (Vogt, 1965), and thus can act as a procedural control.

The dilutions and the results are shown in Table IV.

Cultures that were not preinoculated with either dilutions of stocks of ESV or RSV(RAV-1), but were challenged with these respective viruses after being transferred several times, show results consistent with their function as controls. Of those cultures preinoculated with dilutions of RSV(RAV-1) stock, numbers one through four, which were pre-infected with relatively low dilutions, were almost confluently transformed and no enumeration was possible. The virus dilutions inoculated into these cultures evidently contained sarcoma viruses, which multiplied to a high level during the passage procedure. Cultures five and six showed that small amounts of sarcoma virus were still present in these dilutions. However, since cultures seven, eight and nine manifested a marked reduction in the number of foci present, it was evident that these dilutions carried some leukosis virus related to RSV(RAV-1). This is indicated by the interference registered in these cultures to the RSV(RAV-1) challenge when compared to the corresponding cultures which received no preinoculation, but were challenged with an equal amount of

TABLE IV

END POINT DILUTION TEST

Culture number	Dilution of Virus stock	Number of foci per counting area*			
		Challenged with RSV(RAV-1)		Challenged with ESV	
		Control	Preinoculated	Control	Preinoculated
1	1.00×10^{-3}	46	Tntc	45	Tntc
2	0.50×10^{-3}	48	Tntc	49	Tntc
3	0.25×10^{-3}	53	Tntc	51	Tntc
4	0.12×10^{-3}	49	Tntc	54	Tntc
5	0.60×10^{-4}	45	200	60	69
6	0.30×10^{-4}	60	190	49	53
7	0.15×10^{-4}	45	6	54	57
8	0.75×10^{-5}	50	12	50	58
9	0.40×10^{-5}	48	9	48	51
10	0.20×10^{-5}	49	58	48	56
11	0.10×10^{-5}	53	48	49	49
12	0.50×10^{-6}	49	43	50	48

* Arithmetic mean of three replicate plates
Tntc Too numerous to count

RSV(RAV-1). The remaining cultures, ten, eleven and twelve, evidently were not preinoculated with either a leukosis virus or a sarcoma virus prior to being challenged. Of those cultures preinoculated with dilutions of ESV stock, cultures one through four show confluent transformation, again indicating that the preinfecting dilution contained some sarcoma virus. However, cultures five through twelve show no significantly different response to the challenge virus than do the cultures that received no preinfection of ESV stock. This indicates that none of the ESV post end point dilutions carried any leukosis virus that is related to ESV.

III. CLASSIFICATION OF ESH SARCOMA VIRUS

Several experiments were performed in order to determine the relationship of ESV to the other viruses of the avian tumor group. The first of these was the determination of the effect of polyions on the infectivity of ESV, which has been shown by Toyoshima and Vogt (1969) to be specific for each subgroup. Other experiments were done to conform with the established criteria for the placement of an unknown virus within the group. These, as defined by Duff and Vogt (1969), are host range relationships, interference patterns formed within the group, and the immunological properties of the unknown virus.

EFFECT OF POLYIONS

Vogt (1967c) and Toyoshima and Vogt (1969) found that pretreatment of chick embryo fibroblasts with the polycationic compound diethylaminoethyl dextran (DEAE-dextran, Pharmacia) enhanced the infectivity of

some of the viruses of the avian tumor group. The enhancement was restricted to subgroups B, C, and D and to RSV(O). Subgroup A viruses were either not affected or their infectivity was reduced. Solomon (1966), as well as Toyoshima and Vogt (1969), showed that the polyanionic compound heparin reduced the infectivity of the subgroup B viruses but had no effect on subgroup A viruses. These results suggested that there might be a subgroup specific electro-static charge carried by the virus particles that may be useful in the classification of an unknown virus. The following experiments were performed to show the effects of polyions on the infectivity of ESV.

EFFECTS OF DEAE-DEXTRAN Secondary, C/O type, chick embryo fibroblasts were exposed to DEAE-dextran at a final concentration of ten μg per ml for 60 minutes at 37^{o}C. The cells were then washed with complete growth medium and centrifuged at 250 g for ten minutes. This procedure was repeated twice and the cells were finally resuspended in fresh growth medium and were seeded in Petri dishes at a concentration of 2 x 10^{5} cells per ml. Virus preparations of RSV(RAV-1), SR-RSV-2, B-77, and RSV(RAV-50), representing subgroups A, B, C, and D respectively were diluted 1:5 and 1:25 in complete growth medium and then inoculated into the appropriate cultures. Treated cultures were infected with the same dilutions of an ESV preparation at the same time. Cells not exposed to DEAE-dextran, but processed in the same manner, were inoculated in parallel as a control. The cultures were then carried through the standard assay procedure as described previously in Materials and Methods. Focus forming titres of the viruses on treated and on untreated cultures were compared and the factors of enhancement were calculated. The results

are shown in Table V. The data shows the inhibitory effect of DEAE-dextran on the infectivity of both RSV(RAV-1) and ESV. It further shows the enhancement of infectivity of SR-RSV-2, B-77, and RSV(RAV-50). Enhancement was strongest with the subgroup B virus SR-RSV-2, as the titre was more than ten times higher on treated cells than on untreated cells. Subgroup C and D viruses, B-77 and RSV(RAV-50), showed a smaller degree of enhancement, but nonetheless an increase in focus formation did occur. The results obtained with four representative viruses are in general accord with the previously published reports, although the infectivity of B-77 was not enhanced in this experiment as much as has been reported.

EFFECT OF HEPARIN Complete growth medium was modified for the purposes of this experiment by the addition of heparin to a final concentration of 0.5μg per ml. Secondary, C/O type, chick embryo fibroblasts were seeded at a concentration of 2×10^5 cells per ml in five ml of this medium on Petri plates. Virus preparations of each of the representative viruses, as well as ESV, each containing approximately 10,000 FFU per ml, were diluted 1:5 and 1:25 in complete growth medium and were then assayed on these cultures as previously described in Materials and Methods. The same procedure was followed using normal complete growth medium in order to obtain a control. After eight days the foci were counted on the treated and untreated cultures and the results were compared.

The data, as shown in Table VI, further indicates the relation of ESV to RSV(RAV-1). The focus forming titres of both these viruses were not significantly changed when the viruses were assayed on cells

TABLE V

EFFECT OF DEAE-DEXTRAN ON FOCUS FORMATION BY AVIAN SARCOMA VIRUSES

Avian sarcoma virus subgroup	Representative virus	Titre without DEAE-dextran in FFU per ml	Titre with DEAE-dextran in FFU per ml	Factor of enhancement by DEAE-dextran
A	RSV(RAV-1)	6.5×10^4	7.6×10^3	0.1*
B	SR-RSV-2	2.3×10^4	2.5×10^5	10.0
C	B-77	2.7×10^4	4.6×10^4	1.7
D	RSV(RAV-50)	1.6×10^4	2.4×10^4	1.5
-	ESV	3.7×10^4	9.1×10^3	0.2

* Factor of enhancement = $\frac{\text{Virus titre with DEAE-dextran}}{\text{Virus titre without DEAE-dextran}}$

TABLE VI

EFFECT OF HEPARIN ON FOCUS FORMATION BY AVIAN SARCOMA VIRUSES

Avian sarcoma virus subgroup	Representative virus	Titre without heparin in FFU per ml	Titre with heparin in FFU per ml	Factor of enhancement by heparin
A	RSV(RAV-1)	1.2×10^4	1.5×10^4	1.20*
B	SR-RSV-2	1.7×10^4	3.7×10^3	0.15
C	B-77	1.3×10^4	2.1×10^3	0.15
D	RSV(RAV-50)	6.6×10^4	3.3×10^4	0.49
-	ESV	1.0×10^4	1.1×10^4	1.01

* Factor of enhancement = $\frac{\text{Virus titre with heparin}}{\text{Virus titre without heparin}}$

treated with heparin as compared to the titres obtained on treated cultures. On the other hand, B-77, SR-RSV-2, and RSV(RAV-50) showed a significant reduction in focus formation when they were plated on the heparin treated cells.

EFFICIENCY OF PLATING ON GENETICALLY SELECTIVE CHICK EMBRYO FIBROBLASTS

The existence of genetically selective chick embryo fibroblasts provides a convenient experimental method for the classification of an unknown avian tumor virus. The virus can be placed within the group by determining its plating efficiency on the four genetic types of chicken cells that display selectivity to the infection by avian tumor viruses. Experiments were therefore performed to discover the host range of ESV and to compare it with the host range of the viruses of the four known subgroups. Similar or identical patterns of infection with one or the other representative viruses would indicate a relationship to it.

Secondary chick embryo fibroblasts of genetically selective cell type C/O, C/A, C/B, and C/AB were seeded at a concentration of 2×10^5 cells per ml on 60 mm Petri dishes in five ml of complete growth medium. The separate sets of cultures were each inoculated with virus preparations of RSV(RAV-1), SR-RSV-2, B-77, RSV(RAV-50), and ESV, each of which contained from 500 to 1,000 FFU per inoculum. The standard assay procedure, as described in Materials and Methods, was then followed. The plating efficiency of each virus on the C/O type chick embryo fibroblast culture was considered to be equal to 1.00. The data in Table VII shows the values for the efficiencies of plating of all the viruses tested relative to the value assigned to the viruses on the C/O cells.

TABLE VII

RELATIVE EFFICIENCIES OF PLATING ON
GENETICALLY SELECTIVE CHICK EMBRYO FIBROBLASTS

Avian sarcoma virus subgroup	Avian sarcoma virus	Relative efficiencies of plating*			
		C/O	C/A	C/B	C/AB
A	RSV(RAV-1)	1.00	0.01	0.89	0.01
B	SR-RSV-2	1.00	0.99	0.04	0.02
C	B-77	1.00	0.95	1.00	1.00
D	RSV(RAV-50)	1.00	1.07	0.11	0.12
	ESV	1.00	0.02	0.97	0.00

* Mean of three experiments

The relative efficiencies of plating (REP) of the four representative viruses are in accord with the expected results. RSV(RAV-1) plated as efficiently on C/B cells as it did on C/O cells, however its REP was reduced to a negligible amount on C/A and C/AB type cells. The subgroup B virus, SR-RSV-2, plated nearly as efficiently on C/A cells as it did on C/O fibroblasts, but its efficiency of plating was sharply reduced on C/B and C/AB cells. In contrast, all four types of genetically selective cells were equally susceptible to the subgroup C virus, B-77. The fourth representative virus RSV(RAV-50) plated equally as efficiently on C/O and C/A cells, but its REP was reduced by about 90 per cent on C/B and C/AB fibroblast cultures. The four representative viruses manifested a host range that is expected in these types of genetically selective cell, so it can be concluded that the designations of the cellular genotypes are correct. Since the host range of ESV is indistinguishable from that of RSV(RAV-1), it is evident that there is a very close relationship between the two viruses.

VIRAL INTERFERENCE TEST

The ability of avian leukosis viruses to interfere with the efficiency of plating of avian sarcoma viruses is subgroup specific. If ESV is a member of one of the currently known subgroups, this relationship would be manifested by a reduction in the efficiency of focus formation on cells genetically susceptible to infection by ESV, when these cells were preinfected by a leukosis virus of the same subgroup. Hence, experiments were performed using cells preinfected with avian leukosis viruses representing the four known subgroups, followed by a

challenge dose of ESV. A reduction in its relative efficiency of plating (REP) of at least 90 per cent was required for it, or any other sarcoma virus, to be considered to be interfered with by any leukosis virus.

Stocks of avian leukosis viruses RAV-1, RAV-2, RAV-49, and RAV-50, representing subgroups A, B, C, and D respectively, were prepared as described in Materials and Methods. Secondary, C/O type, chick embryo fibroblasts were seeded on 60 mm Falcon Petri plates at a concentration of 2×10^5 cells per ml. Individual sets were each separately inoculated with one ml of each of the representative avian leukosis viruses. The cultures were trypsinized after four days incubation at $37^{\circ}C$ and the cells were reseeded at 2×10^5 cells per ml. The cultures were carried in this manner through a series of three passages. Immediately following the third passage, each set of cultures was challenged with 5,000-10,000 FFU of either RSV(RAV-1), SR-RSV-2, B-77, or RSV(RAV-50). In addition, all of the preinfected cultures were challenged with the same dose of ESV.

The experiment included two sets of control cultures. One set, the positive control, was not infected with any avian leukosis virus but was challenged with all the above sarcoma viruses. The other set was left uninfected but was carried in the same way that the infected cultures were and constituted the negative control.

After being challenged, the cultures were incubated overnight at $37^{\circ}C$ and the next day the medium was discarded and five ml of nutrient agar overlay was added. Eight days later the number of foci in the leukosis infected cultures were compared to the number in the positive control cultures. The plating efficiency of each sarcoma virus in the absence of a preinfecting leukosis virus was considered to be 1.00. The

values obtained for the sarcoma viruses in the presence of the various leukosis viruses was therefore expressed relative to the value obtained in the absence of leukosis virus.

The results of the experiment are shown in Table VIII. The REP of ESV was significantly reduced only in the cultures preinfected with the subgroup A leukosis virus RAV-1. In cells preinfected with the leukosis viruses of the other three subgroups, the REP of ESV was either not affected or was not significantly reduced. The data further shows that the interference formed shows close agreement with the previously reported patterns of interference within the group. Thus, RAV-1 and RAV-49 show solid interference with RSV(RAV-1) and B-77 respectively but did not significantly alter the plating efficiencies of any of the other representative viruses. RAV-2 reduced the amount of focus formation by SR-RSV-2 and RSV(RAV-50) almost equally, but a significant amount of focus formation still occurred. Similarly, RAV-50 interfered with RSV(RAV-50) and SR-RSV-2 to about the same degree.

SERUM NEUTRALIZATION TEST

The following experiments describe the immunological relationships of the four viruses that are representative of the known subgroups of the avian tumor group. The relationships can be detected by the serum neutralization test, since cross neutralization can only occur between viruses that belong to the same subgroup. Thus, neutralization of ESV by antiserum prepared to any of the representative viruses would indicate a relationship to the viruses which share the antigenic properties of the prototype virus. Before cross neutralization could be performed, however,

TABLE VIII

INTERFERENCE TO ESV BY AVIAN LEUKOSIS VIRUSES

Subgroup of interfering virus	Interfering virus	Relative efficiencies of plating				
		ESV	RSV(RAV-1)	SR-RSV-2	B-77	RSV(RAV-50)
-	Absent	1.00	1.00	1.00	1.00	1.00
A	RAV-1*	0.03	0.03	1.14	0.95	1.03
B	RAV-2	0.85	1.04	0.26	1.45	0.26
C	RAV-49	1.00	1.02	0.95	0.08	1.04
D	RAV-50	0.96	0.52	0.18	1.03	0.03

* Mean of two experiments

it was essential to show the specificity of the sera used. This required that the preinoculation sera show no neutralization against any of the representative viruses, and that the eight week post inoculation sera are able to neutralize at least 90 per cent of the infectivity of their homologous virus.

NEUTRALIZATION OF AVIAN SARCOMA VIRUSES BY HOMOLOGOUS SERUM

Presera and antisera to all the avian sarcoma viruses was prepared as described in Materials and Methods. One ml of each preinoculation serum was mixed with an equal volume of virus preparations RSV(RAV-1), SR-RSV-2, B-77, and RSV(RAV-50) containing approximately 1,000 FFU per volume. These mixtures were incubated at 37°C for 40 minutes and then one ml sample of each was tested for infectivity by the standard assay method on C/O type chick embryo fibroblasts. Equal volumes of the same virus preparations were mixed with complete growth medium and were incubated and assayed in parallel to obtain a positive control. At the same time post inoculation sera were diluted 1:10 and 1:100 and one ml of each of the dilutions was mixed with an equal volume of its homologous virus containing 1,000 FFU per volume. These mixtures were incubated as above and assayed for infectivity in order to measure the potencies of the antisera before they were cross reacted with ESV. The data in Table IX shows the results of the neutralization by the pre- and post inoculation sera of their homologous virus preparations. The results are presented as fractions of virus survival as compared to the positive control which is equal to 1.00. It is evident that the preinoculation sera show no neutralizing ability against any of the sarcoma viruses tested. In most cases there is a slight enhancement of the efficiencies of plating of the

TABLE IX

NEUTRALIZATION OF VIRUS BY HOMOLOGOUS ANTISERA

Antiserum prepared against	Antiserum dilution	Fraction virus survival				
		ESV	RSV(RAV-1)	SR-RSV-2	B-77	RSV(RAV-50)
ESV	Control	1.00	1.00	1.00	1.00	1.00
	Preserum	-	1.11	1.60	1.25	1.11
	Test 1:10	0.01	-	-	-	-
	Test 1:100	0.18	-	-	-	-
RSV(RAV-1)	Control	1.00	1.00	1.00	1.00	1.00
	Preserum	-	1.00	1.19	1.17	1.07
	Test 1:10	-	0.01	-	-	-
	Test 1:100	-	0.15	-	-	-
RSV(RAV-2)	Control	1.00	1.00	1.00	1.00	1.00
	Preserum	-	1.23	1.22	1.03	1.17
	Test 1:10	-	-	0.01	-	-
	Test 1:100	-	-	0.14	-	-
B-77	Control	1.00	1.00	1.00	1.00	1.00
	Preserum	-	1.03	1.08	0.94	0.93
	Test 1:10	-	-	-	0.05	-
	Test 1:100	-	-	-	0.76	-
RSV(RAV-50)	Control	1.00	1.00	1.00	1.00	1.00
	Preserum	-	1.23	1.31	1.06	1.16
	Test 1:10	-	-	-	-	0.00
	Test 1:100	-	-	-	-	0.27

- Not done

various test viruses. However, all of the post inoculation sera show neutralization of at least 90 per cent of the infectivity of their homologous viruses. This figure is arbitrarily selected as the level of neutralization that indicates antigenic relativity. Since the presera show no neutralization of any of the four representative viruses, or ESV, and since neutralization of 90 per cent of the infectivity of the homologous viruses was obtained, cross neutralization experiments were now performed.

CROSS NEUTRALIZATION OF ESV BY HETEROLOGOUS ANTISERA Each of the post inoculation sera was diluted 1:5 and 1:50 in complete growth medium and one ml samples were mixed with equal volumes of virus preparations of either RSV(RAV-1), SR-RSV-2, B-77, or RSV(RAV-50). In addition, each serum was mixed with an equal volume of ESV. After 40 minutes incubation at 37°C, the mixtures were assayed for infectivity on C/O type chick embryo fibroblasts as described in Materials and Methods. Equal volumes of each of the same viruses and complete growth medium were incubated at 37°C for the same time and were assayed in order to provide a positive control.

The data in Table X shows the neutralization patterns of antisera prepared to the representative viruses against ESV. The results show that ESV is not neutralized when the virus is incubated with sera prepared against viruses of subgroups B, C, or D. Neutralization of ESV occurred, however, when the virus was incubated with antiserum prepared against the subgroup A virus, RSV(RAV-1). However, the antiserum prepared against RSV(RAV-1) completely neutralized preparations of RSV(RAV-1), but there was a residual survival of ESV equal to about 15

TABLE X

NEUTRALIZATION OF VIRUS BY HETEROLOGOUS ANTISERA

Antiserum prepared against	Antiserum dilution	Fraction virus survival				
		ESV	RSV(RAV-1)	SR-RSV-2	B-77	RSV(RAV-50)
RSV(RAV-1)	Control	1.00	1.00	-	-	-
	Test 1:5	0.15	0.00	-	-	-
	Test 1:50	0.28	0.09	-	-	-
RSV(RAV-2)	Control	1.00	-	1.00	-	-
	Test 1:5	1.02	-	0.12	-	-
	Test 1:50	1.09	-	0.16	-	-
B-77	Control	1.00	-	-	1.00	-
	Test 1:5	1.06	-	-	0.06	-
	Test 1:50	1.05	-	-	1.03	-
RSV(RAV-50)	Control	1.00	-	-	-	1.00
	Test 1:5	1.09	-	-	-	0.00
	Test 1:50	1.10	-	-	-	0.06

- Not done

per cent of the infectivity of the original virus preparation.

RECIPROCAL CROSS NEUTRALIZATION OF REPRESENTATIVE AVIAN SARCOMA VIRUSES Reciprocal cross neutralization experiments, employing antiserum prepared against ESV, were now performed. ESV antiserum was diluted 1:5 and 1:50 in complete growth medium and a one ml sample was mixed with an equal volume of virus preparations of RSV(RAV-1), SR-RSV-2, B-77, RSV(RAV-50) and ESV, all containing approximately 1,000 FFU per volume. The mixtures were incubated at 37°C for 40 minutes and were then tested for infectivity by assaying on C/O type chick embryo fibroblasts.

The results, as shown in Table XI, confirms the relationship of ESV to RSV(RAV-1). Antiserum to ESV showed no significant neutralization of any of the representative viruses except RSV(RAV-1).

TABLE XI

RECIPROCAL CROSS NEUTRALIZATION BY ANTISERUM TO ESV

Antiserum prepared against	Antiserum dilution	Fraction virus survival				
		ESV	RSV(RAV-1)	SR-RSV-2	B-77	RSV(RAV-50)
ESV	Control	1.00	1.00	1.00	1.00	1.00
	Test 1:5	0.00	0.11	0.80	0.97	1.03
	Test 1:50	0.33	0.49	0.95	1.05	1.06

DISCUSSION

DISCUSSION

Normal chick embryo fibroblasts are incapable of growing in liquid suspension culture. However, Bader (1967) demonstrated that cells transformed by RSV acquire the ability to grow in this manner. This property was suggested to be characteristic of all tumor causing cells. The results obtained in this study are in agreement with Bader's to the extent that cells transformed with RSV(RAV-1) were able to multiply in suspension culture, while normal cells were not. However, attempts to induce the growth of ESV transformed cells in suspension culture were unsuccessful. It was considered that the ability to confer upon cells the potential to multiply in the absence of contact with a physical substrate was a subgroup specific property. Bader found that only cells transformed by subgroup A avian sarcoma viruses were able to grow in suspension culture. When cells were infected with the subgroup B virus RSV(RAV-2) no multiplication occurred. However, the fact that ESV is now considered to be a member of the A subgroup indicates that this property is not a universal characteristic of these viruses.

The failure of the RSV(RAV-2) transformed cells to grow was thought to be due to the fact that these cells were killed by the infecting virus. The lack of growth of ESV transformed cells may also be due to the fact that ESV is cytopathic. The data in Table III showed that while most normal, uninfected cells remained viable over the period of the test, cells infected with ESV were destroyed to the same degree as those infected with RSV(RAV-2). The cytopathic effect of ESV was observed

again when it was being propagated in monolayers of chick embryo cells. In contrast to most avian sarcoma viruses, permanently infected, virus producing cell lines could not be established by infecting cultures with ESV. Like RSV(RAV-2), no more than three passages of the infected cells was ever possible. As a result, the titres of both of these viruses never exceeded 2×10^5 FFU per ml.

The cytopathicity of ESV is noteworthy. Till now, all oncogenic RNA viruses, with the exception of RSV(RAV-2), were considered to be non-cytopathic. Nowinski (1970), in defining the properties of the "oncornaviruses", states that this is a universal property of these viruses. The cytopathic effect of RSV(RAV-2) has long been attributed to the helper virus RAV-2 (Hanafusa et al., 1964). Since no associated virus was observed in the stocks of ESV, this may be the first indication that the transforming particle as such can be cytopathic.

Evidence from this study suggests that cloned stocks of ESV do not contain any associated leukosis viruses. However, the failure to detect any leukosis virus in the post end point dilutions of ESV does not completely rule out the possibility that one or more might be present. The end point dilution procedure is somewhat limited in that associated viruses can only be detected if they are present in some multiple number compared to the sarcoma virus. This is the case with the Bryan high titre strain of RSV, where RAV has been reported to be present in a ratio of anywhere from ten to one to four to one to the sarcoma virus (Hanafusa, 1968). As a result the associated viruses have been readily detectable by this procedure. If however, an associated virus was present in the stocks of ESV in a ratio much less than this, it would

be extremely difficult to detect it.

The discovery of an associated virus in the stocks of avian sarcoma viruses would provide evidence, though not conclusive, that the sarcoma virus was defective. Associated viruses have been found in the stocks of both the Fujinami strain of RSV and in the Schmidt-Ruppin strain of RSV, but neither have been shown to be defective (Vogt, 1965; Hanafusa, 1968). The data here, therefore, suggests only that ESV is not defective in a way similar to the Bryan high titre strain of RSV.

The infectivity of the avian sarcoma viruses tested was markedly affected by the addition of polyions to the culture medium. The polycation DEAE-dextran clearly inhibited the infectivity of RSV(RAV-1). On the other hand, focus formation of SR-RSV-2, B-77, and RSV(RAV-50) was increased when cells were pretreated with this compound. These results are in accord with those of Toyoshima and Vogt (1969), who found that various cations inhibited subgroup A viruses, but enhanced subgroup B, C, and D viruses. Thus, the reduction in the amount of focus formation by ESV in cells pretreated with DEAE-dextran provided strong preliminary evidence that ESV was a subgroup A virus. Further evidence of this relationship was demonstrated by the fact that the polyanionic compound heparin reduced the infectivity of all the avian sarcoma viruses tested except RSV(RAV-1) and ESV.

The mechanism of the polyion effect on the infectivity of the avian sarcoma viruses is not totally clear. The increase in focus formation by some of the viruses can be explained by assuming that these viruses possess a net negative charge. As a result, they would adsorb better to the likewise negatively charged cell surface in the presence

of cations. The inhibition, conversely, can be explained by assuming that these viruses have a net positive charge, and thus, cations interfere with adsorption. In the presence of polyanions, on the other hand, negatively charged viruses would be less easily adsorbed, while positively charged viruses would be more easily adsorbed. This explanation would result in the assignment of a positive charge to all subgroup A avian tumor viruses, and a negative charge to all the viruses of subgroups B, C, and D. However, Vigier (1970) has presented evidence which suggests that the enhancement of avian tumor viruses by DEAE-dextran is not due solely to an increase in the uptake of the virus, since the effect was still active even if the cation was added as much as four days after adsorption was complete. Whatever the actual mechanism of the action, it is clear that ESV and RSV(RAV-1) share a common reaction to the presence of polyions.

The relationship of ESV to subgroup A became more obvious when its host range was considered. Several experiments were performed that repeatedly demonstrated the subgroup specific selectivity of the cells used. The resistance to the appropriately excluded virus was uniformly effective on C/A, C/B, and C/AB cell types. In most cases at least a two log drop in the efficiency of plating was observed. The host range of cloned stocks of ESV on these cells was indistinguishable from that of RSV(RAV-1). Both viruses were completely excluded from C/A type cells, which are only insusceptible to subgroup A viruses. However, many unclassified avian tumor viruses were eventually shown to be mixtures of several different viruses. These mixtures consisted of viruses belonging to the same subgroup; viruses belonging to two or more different

subgroups; or were in the form of phenotypic mixings. The possibility that working stocks of ESV contained viruses of more than one subgroup was reduced by cloning the virus several times, and the results of the testing on genetically selective cells indicate that only one subgroup is represented in these stocks. The possibility of a phenotypic mixing being present was eliminated, since such viruses would be genetically unstable, and thus would not be able to exist in this form following the cloning procedure. However, despite the fact that efforts were made to isolate clones caused by single virus particles, there is no absolute assurance that the stocks of ESV do not consist of a mixture of subgroup A viruses. Indications are, however, that they do not, since the morphology of the foci induced by ESV are quite unique and readily recognizable, and appear to be the only type present in cultures infected with these stocks.

Further supportive evidence for the association of ESV with the subgroup A viruses was available from the interference tests performed. The subgroup A leukosis virus RAV-1 strongly interfered with a challenge infection by ESV. Since no leukosis virus could be isolated from stocks of ESV, reciprocal cross interference could not be performed. However, negative proof of the same relationship was provided by the fact that no other representative avian leukosis virus demonstrated any significant interference to ESV.

Immunological studies further confirmed the evidence that ESV consisted only of subgroup A virus. Antisera prepared to the viruses representing subgroups B, C, and D, which had been demonstrated to cause at least a one log loss in infectivity when reacted with their homologous

viruses, failed completely to neutralize ESV. On the other hand, the antiserum prepared to RSV(RAV-1) significantly reduced the focus formation of ESV. In addition, reciprocal cross neutralization tests, employing antiserum prepared to ESV, showed that ESV can be easily distinguished from the other representative viruses. However, the fact that the heterologous reactions between ESV and RSV(RAV-1) were not as complete as the homologous reactions indicates that, although ESV is related to RSV(RAV-1), it is not identical to it.

In conclusion, Esh sarcoma virus has been placed within the A subgroup of the avian sarcoma-leukosis complex based on the observations that it shares with the known subgroup A virus RSV(RAV-1) the following properties; 1) a common reaction in the presence of polyions, 2) a common host range, 3) a common interference by RAV-1, and 4) a similar antigenic makeup.

That ESV is a subgroup A virus is not surprising. Duff and Vogt (1969) pointed out that most subgroup A and B viruses have been propagated or isolated in North America, while most subgroup C and D viruses are of European origin. This pattern of virus occurrence is probably due to the geographical distribution of the various genetically resistant chicken lines.

SUMMARY

SUMMARY

1. Infection and transformation of chick embryo fibroblasts by ESV altered the growth potential of these cells, but not in such a way that they became capable of growing in liquid suspension culture. This was thought to be due to the fact that ESV was cytopathic in tissue culture.

2. No associated viruses were detected in cloned stocks of ESV.

3. Pretreatment of cell cultures with the polycation DEAE-dextran inhibited the infectivity of ESV, while the continuous presence of the polyanion heparin exhibited no effect on its focus forming abilities. This was considered to be presumptive evidence of a relationship of ESV to the A subgroup of the avian tumor viruses.

4. The host range of ESV was that typical of a subgroup A avian tumor virus; infectivity was restricted to C/O and C/B type fibroblasts.

5. Of the leukosis viruses representing the four known subgroups within the avian tumor group, only the subgroup A virus RAV-1 interfered with focus formation by ESV.

6. Antiserum prepared to the subgroup A avian sarcoma virus RSV(RAV-1) neutralized the infectivity of ESV and antiserum prepared to ESV reacted reciprocally with RSV(RAV-1). No other heterologous reactions were observed.

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