STUDIES OF INTERRELATIONSHIPS BETWEEN GENETICALLY RESISTANT CHICKEN EMBRYO CELLS AND THE BRYAN HIGH TITER STRAIN OF ROUS SARCOMA VIRUS

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ABSTRACT

STUDIES OF INTERRELATIONSHIPS BETWEEN GENETICALLY RESISTANT CHICKEN EMBRYO CELLS AND THE BRYAN HIGH TITER STRAIN OF ROUS SARCOMA VIRUS

By Min Chung

Early steps of virus infection in animal cells are adsorption, penetration and uncoating of the virus, which are under the control of both the genetic constituents of the host cell and the virus protein coat. A Bryan high titer strain of Rous sarcoma virus (BH-RSV) is composed primarily of subgroup A viruses of avian tumor agents and an extremely small fraction of subgroup B viruses. Growth experiments with BH-RSV using mixed cell cultures of genetically resistant (C/A type) and susceptible (C/O type) chicken embryo cells, and titrations of the virus in the mixed culture system and the unadsorbed fraction of input virus all indicate that BH-RSV is adsorbed with the same efficiency by C/A type cells as by C/O type cells. In addition, a subgroup A virus separated from BH-RSV, RSV(RAV-1), is also adsorbed by C/A type cells at 37° or 5°C.

An important aspect in the field of oncogenic virology is the study of hybrids or phenotypically mixed viruses. These abnormal viruses acquire the capacity to infect previously resistant lines within a species. They also may infect unnatural host animal species. Experimental evidence for the existence of phenotypically mixed Rous associated viruses in BH-RSV

or RSV(RAV-1) stock was demonstrated. The possible origin of the phenotypically mixed viruses and the possibility of experimentally producing these oncogenic agents were discussed.

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By

Min Chung

A THESIS

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Dedicated

to

My mother, Mrs. Hee Cho Chung and wife, Sung-Sil

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TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

The genome of Rous sarcoma virus (RSV) leads to the production of malignant tumors causing the death of chickens *in vivo* and the transformation of chicken embryo cells *in vitro*. Some of the other experimental animals are affected in the same manner (Temin, 1962; Hanafusa *et al.*, 1964a). Early steps of RSV infection in chicken embryo cells, i.e., adsorption, penetration and uncoating, are responsible for the interaction between the cell and the virus protein coat. These steps are under the control of genetic constituents of the cell surface and the virus coat protein which is provided by several different Rous associated viruses (Rubin, 1965; Vogt and Ishizaki, 1965). Early steps of RSV infection between genetically resistant chicken embryo cells and Bryan high titer strain of RSV or its pseudotype will be examined in this thesis, and evidence for the adsorption of the virus by the cell will be presented.

Recent emphasis in the field of oncogenic virus study is concentrated on a search for hybrids or phenotypically mixed viruses in the light of expansion of their host range within a species and/or crossing species barriers. A phenotypically mixed virus is an abnormal virus, that is, the genomic nucleic acid and the protein coat originate from two genotypically different viruses. Evidence is demonstrated in this thesis for the existence of phenotypically mixed Rous associated viruses in Bryan high titer

strain of RSV or its pseudotype stock. The possible origin of these viruses and the possibility for making hybrids or phenotypically mixed oncogenic viruses, which may infect unnatural host animals, are discussed.

LITERATURE REVIEW

A. Tumor Viruses.

I. Introduction.

Ellerman and Bang in 1908 showed that chicken visceral lymphomatosis could be transmitted to healthy chickens by cell free filtrates. Three years later, in 1911, Rous was successful in showing that a cell free extract of a solid tumor, a chicken sarcoma, injected into a healthy chicken would induce a sarcoma of the same type at the injection site.

It was apparent from these findings that viruses were capable of initiating the neoplastic process in chickens. Investigations in succeeding years found more viruses capable of inducing tumors in other animal species including humans (e.g., wart virus). Many investigations have been designed to demonstrate the mechanism by which a normal cell is rendered neoplastic through virus infection.

Although the question is not yet satisfactorily answered, a great deal of progress has been made. Much of this progress stems from work in the last decade on the virus induction of tumors in many different animal species, and the very rapid advancements that have occurred in the knowledge of viruses, cells and virus-cell interrelationships.

II. Transformation of cells by viruses and loss of contact inhibition.

Virus infection of animal cells can give one of two apparent results. The most common is a cytocidal response

in which new viruses are made and the cell stops dividing or actually disintegrates. On the other hand, oncogenic viruses can, at a frequency which depends on the virus and cell used, change the infected cell so that it grows continuously. This transformation may, and frequently does, result in a progression to a malignant tumor cell which can grow unrestrained and kill an animal (Luria and Darnell, Jr., 1967). In other words, the tumor viruses such as Rous sarcoma virus (RSV) occupy a position which lies between the cytocidal viruses and the viruses causing inapparent infections. A chicken embryo culture shows definite cytopathic changes after infection with RSV. However, the infected cells do not die, they continue to multiply, and their daughter cells remain infected (Vogt and Rubin, 1962).

It is unfortunate that this process of cellular changes as observed in animal cell cultures has come to be known as "transformation", a word that was already in use to describe genotypic changes in bacteria mediated by uptake of DNA molecules. The term "conversion", used by phage workers to describe phage-determined changes in cellular properties, might be more appropriate. Nevertheless, the term transformation is used more commonly to describe the conversion of animal cells to a pattern of unrestricted growth accompanied in many instances by morphologic change.

The phenomenon of loss of contact inhibition is a characteristic feature of tumor-inducing tissues in many cases. There is now growing evidence that, when two normal animal cells touch one another, a "signal mechanism"

is often generated which stops both cells from further division. Normal cells have a great affinity for solid surfaces and stick to the glass rather than float freely in the nutrient medium. As long as there are relatively few cells about, division proceeds regularly at approximately once every 24 hours. The division rate often slows down when the cells have formed a confluent monolayer. It is as if these cells are able to divide as long as they are not in close contact with other cells. In contrast, when certain cancer cells are observed, growth does not cease when a monolayer has formed. Instead, the cancer cells pile on top of each other, forming masses of cells several layers thick commonly referred to as a focus which has a three dimensional effect when viewed by light microscopy. The basis of loss of contact inhibition is still not clear. Contact inhibition may be related to an adhesiveness of normal cells. When normal cells touch each other, they often remain fixed, whereas many types of cancer cells have much less affinity for each other and hence do not form regular monolayers (Watson, 1965).

III. Instances of virus-caused tumors.

The variety of naturally occurring or experimentally produced tumors in animals was described by Luria and Darnell, Jr. (1967, Table 1).

Ellerman and Bang in 1908 first successfully transmitted a tumor by serum or cell-free extracts of visceral lymphomatosis of chickens. It was not recognized that

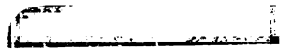

Table 1. Tumor viruses in animals*.

Date discovered	Virus designation and major host	Nucleic acid***
1908	Fowl leukemia	RNA
1911	Fowl sarcoma, Rous sarcoma virus (chicken)	RNA
1917	Human wart virus	DNA
1920	Bovine papilloma	DNA
1932	Canine oral papilloma	DNA
1932	Rabbit fibroma	DNA
1933	Rabbit papilloma	DNA
1933	Fowl lymphoma	RNA
1936	Rabbit oral papilloma	DNA
1936	Mouse mammary carcinoma	DNA
1951	Equine cutaneous papilloma	DNA
1951	Mouse leukemia, Gross	RNA
1953	Squirrel fibroma	DNA
1955	Deer fibroma	DNA
1956	Mouse leukemia, Friend	RNA
1957	Polyoma (mouse)	DNA
1962	Simian virus 40 (monkey)**	DNA
1962	Adenovirus (human)**	DNA

*Taken largely from a review by Syverton (1959).

**Not known to cause tumors in nature.

***Each viral nucleic acid has not been adequately characterized and some of those listed are by analogy with well-studied, structurally similar viruses.

this complex disease was akin to malignant tumors of the blood-forming organs in mammals. The first suggestion that virus was the possible etiology of cancer was demonstrated by Peyton Rous (1911) when he showed that a "naturally" occurring sarcoma from a Plymouth Rock chicken could be passed to other animals by inoculation with a cell-free, bacteria-free tumor filtrate.

Approximately 20 years later, new impetus was given to the tumor-virus study when Shope and his colleagues launched their studies on a virus that caused papilloma in rabbits (Shope, 1932; 1950). This virus could be readily recovered from natural and experimentally induced papillomas of the wild cotton-tail rabbit. Rarely did these papillomas become malignant. When the virus was injected into domestic rabbits two dramatic findings were made: (1) tumors resulted which regularly progressed to malignant carcinoma and (2) infectious virus which was readily recoverable from wild rabbit papillomas was seldom found in domestic rabbit tumors, although its presence could be revealed by sensitive antigenic tests. The rationale for investigating the role of "masked" viruses stems from these studies.

In 1936, Bittner demonstrated another important tumor agent. He showed that mouse mammary carcinoma arose in female mice who had been suckled by mothers carrying a milk factor. This agent was subsequently shown to possess virus-like characteristics such as the capacity to pass through bacterial filters and stimulate antibody response.

Electron micrographs of virus-infested tumor cells grown *in vitro* clearly demonstrate viral particles. This virus, however, has not been adapted to quantitative study in tissue culture. One interesting and experimentally attractive property of this system is that although an adult female mouse may carry this virus, mammary carcinoma develops only after the mammary tissue has received a hormonal stimulus either artificially or during pregnancy. Thus again the property of latency or masking was linked to oncogenic viruses. In addition, attention was drawn to the fact that a specific secondary event, in this case hormonal stimulation, is necessary to enable the virus to incite the production of the tumor.

In 1951, investigation of RSV was reemphasized when Gross demonstrated that cell-free extracts made from the leukemia tissues of a mouse strain of very high incidence of spontaneous leukemia would induce leukemia in newborn mice of another strain which had a low incidence of spontaneous leukemia. One of the most significant contributions made by Gross was to show that newborn animals were much more susceptible to the tumor inducing action of a virus. Other leukemia viruses were isolated by Friend (1958) and Moloney (1960) from transplantable mouse carcinoma and transplantable mouse sarcoma respectively.

In the last decade several other tumor viruses have been discovered as a result of the search for tumor-producing agents, stimulated by the works of Rous, Shope, Bittner and Gross. It is not the question of whether all

tumors are caused by viruses but currently in question is the mechanism of oncogenesis in those cases which are known to be virus induced. Advances in cell culture techniques have played a large part in this change of emphasis and have created the development of quantitative methods of assay for the *in vitro* transformation of cells by tumor viruses.

B. Rous Sarcoma Virus (RSV).

I. RSV as a member of myxoviruses.

Several authors indicated that avian leukosis viruses possessed certain properties similar to those of the myxoviruses (Epstein, 1958; Crawford and Crawford, 1961; Huebner *et al.*, 1964). Luria and Darnell, Jr. (1967) placed the avian leukosis viruses as a member of subgroup II myxoviruses.

The properties of myxoviruses are described as follows by Melnick and McCombs in 1966: "The members of this group are medium sized viruses, 80 to 200 mμ, containing RNA and essential lipids, and exhibiting helical symmetry. The diameter of the internal ribonucleoprotein helix is 9 mμ. The morphology varies from a true sphere to filamentous forms. The internal helical nucleoprotein of the virus is synthesized in the nucleus while the hemagglutinin is formed in the cytoplasm. The virus matures at the cell membrane. Myxoviruses are sensitive to actinomycin." However, Luria and Darnell, Jr. (1967) described the myxoviruses slightly differently. They divided the myxoviruses into two subgroups, I and II. Subgroup I viruses have a nuclear phase

in its replicative cycle and the size of RNA is not yet well defined. Subgroup II viruses, which includes the RSV, multiply only in the cytoplasm and have very large RNA in molecular weight (6 - 8 x 10^6 daltons).

Ultrastructural studies of the RSV were described by Epstein in 1957. The Rous virus particles are either round or oval. The larger round profiles of viruses have a diameter of approximately 70 and 75 mμ. The virus is surrounded by a pair of fine limiting membranes spaced approximately 3 mμ apart. This double membrane surrounds a viroplasmic mass of approximately 9 mμ in thickness. Enclosed within this membrane is a central area containing an electron-dense nucleoid.

Numerous studies of RSV infected cells with the electron microscope show complete virus forming at the cell surface and in vacuoles, but not in the cytoplasm (Gaylord, 1955; Bernhard et al., 1956; Epstein, 1957; Haguesau et al., 1958). The association of complete virus with the cell membrane was most clearly demonstrated in the myxosarcomas of the adult chicken thymus induced in vivo by an avian sarcoma virus (Nishiumi et al., 1961).

Existing evidence including electron microscopic observations favor the idea that the completion of RSV occurs at the cell membrane. Free virus predominates over cell-associated virus during the ascending part of the growth curve (Vogt and Rubin, 1961). This indicates a short release time of the infective particles and is consistent with the idea of virus maturation at the cell

surface. Further support for this view comes from the finding that between 96 and 99.7 % of the cell-associated RSV can be removed from the cell by trypsin, and neutralized by antiserum. Only about one focus-forming unit (FFU) of RSV per 10 infected cells is intracytoplasmic as judged from its inaccessibility to antibody (Vogt and Rubin, 1962).

In summary, with the information indicated above and supporting evidence to follow, RSV can be considered as a member of myxovirus group.

II. Nucleic acid of RSV.

Rous sarcoma virus appears to contain RNA, but not DNA (Epstein and Holt, 1958; Crawford and Crawford, 1961). More recently, Robinson et al. (1965) reported that the mixture of RSV plus Rous associated virus (RAV) have RNA as the only nucleic acid, and the molecular weight of the RNA was unusually high, approximately 10^7, or about five times the weight of poliovirus RNA. The nature of RSV was confirmed by inhibitor studies using actinomycin, fluorodeoxyuridine, aminopterin, and cytosine arabinoside. All inhibitors of DNA synthesis did not interrupt RSV synthesis at any time during virus production when once initiated (Temin, 1964a; Bader, 1965; 1966a and 1966b).

The replication of RSV was demonstrated to require DNA synthesis only during the early stages of the infection cycle. The necessary period for DNA synthesis is within 5 hours after infection, therefore, no further DNA synthesis is required even though virus production does not begin until about 15 hours after infection (Temin, 1964b; Force

and Stewart, 1964; Bader, 1965).

Temin (1964b) proposed a model of RSV replication to account for these findings as follows: The RNA genome of RSV is copied into a two-stranded complementary DNA replica (a provirus DNA) which is integrated into the cellular DNA. Viral RNA is then copied from the integrated viral-like DNA. The concept of a DNA provirus for a RNA virus is clearly a radical proposal (Watson, 1965). If true, it is contradictory to the belief that the flow of genetic information always goes in one direction, i.e., DNA to RNA. This unorthodox proposal may stimulate further investigation (Luria and Darnell, Jr. 1967). Luria and Darnell, Jr., however, pointed out that other explanations of the inhibitor studies are possible. For example, although actinomycin has relatively little effect on the multiplication of some RNA viruses, it does have a depressing effect on Newcastle disease virus synthesis. This myxovirus has a RNA content about the same as that of a mixture of RSV plus RAV, it multiplies in the cytoplasm, and its multiplication does not in any way depend on cellular DNA. Thus the inhibition of RSV by the inhibitors of DNA synthesis may not be caused by the drug's effect in stopping DNA-dependent RNA synthesis.

III. Transformation of chicken embryo fibroblasts due to RSV infection and the basis for its in vitro assay.

The cytological changes in chick fibroblasts following infection with RSV in vitro bear resemblance to the induction of sarcoma by RSV in vivo (Manaker and Groupé, 1956;

Temin and Rubin, 1958).

The chicken fibroblast, which normally grows in an elongated and flattened shape attached to the bottom of the culture dish, becomes rounded and will detach from the glass or plastic culture dish after infection with RSV. It has the tendency to grow unrestricted, which results in characteristic groups of rounded cells replicating on the culture dish. The area composed of transformed rounded cells is called a focus. Each focus can be considered as having been produced from a particle of virus if the viruses are not aggregated (Manaker and Groupé, 1956). The foci formation can be explained as a phenomenon of loss of "contact inhibition" (Vogt and Rubin, 1962; Watson, 1965). Transformed RSV infected cells are not subject to contact inhibition and, therefore, do not stop growing after the glass surface is covered with cells. Instead, they continue to proliferate and form foci, easily distinguishable from normal cells.

Temin and Rubin (1958) established a quantitative in vitro assay for RSV based on the foci formation. When chicken fibroblasts in a monolayer culture are inoculated with a small amount of virus, a countable number of foci can be observed. This foci assay is the reverse of plaque counting which measures the spreading cell destruction from a single infected cell. The foci-counting assay provided the basis for a study of the interaction of host cell with tumor virus (Rubin, 1962).

IV. Defectiveness of Bryan high titer strain of Rous sarcoma virus (BH-RSV) and non-virus-producing transformed cells (NP cells).

Until recently, RSV had been considered able to multiply in the host cells by itself. In 1962, Rubin and Vogt isolated an avian leukosis virus designated Rous associated virus (RAV) from a stock of BH-RSV. The associated virus was found to exist in different antigenic types (Hanafusa et al., 1963) as was given a specific nomenclature - Rous associated virus type 1 or RAV-1, and RAV-2. Since RAV was found invariably in stocks of BH-RSV, an attempt was made to clarify the relationship between RSV and RAV and to isolate RSV free from RAV. The study led to the discovery that this BH-RSV is a defective virus, in that it cannot produce infectious progeny in the absence of a helper virus such as RAV-1 (Hanafusa et al., 1963).

The occurrence of the non-virus-producing transformed cells or NP cells was also demonstrated by Temin (1962) with BH-RSV. The NP cells do not produce infectious virus particles. These cells can be isolated from single Rous sarcoma foci produced on chicken embryo cultures infected with 1 or 2 focus-forming units (FFU) of RSV. Even after extended periods of cultivation, NP cells retain their characteristic morphology and high proportion, if not all, produce RSV particles if they are exposed to (or activated by) a helper virus. Therefore, it would be reasonable to assume that the RSV genome, which is present in the NP cells, contains all the information necessary for its own

replication and for cell transformation. NP cells tend to acidify the culture medium much more quickly than normal cells, suggesting an increased metabolic rate of these cells. Since NP cells form an increasingly large fraction of the population with continuing transfer of cultures containing both NP cells and normal chicken cells. NP cells seem to replicate faster than normal chicken cells, although the possibility of cell-to-cell transfer of the RSV genome has not been excluded. Inoculation of NP cells into young chickens induces tumors at the site of inoculation and causes the death of the chickens (Hanafusa et al., 1964b). Frequently metastasis is found in the lung in the fatal cases. All of these tumors consisted of NP cells, which retain the capacity for RSV activation by helper virus. These results suggest that despite the absence of infectious virus production, NP cells acquire an abnormal metabolic activity and malignant behavior characteristic of Rous sarcoma cells.

If cultures containing NP cells were exposed to various concentrations of helper virus, namely RAV-1, both the number of RSV-producing cells and the yield of RSV particles at 24 hours after superinfection with RAV-1 were found to be proportional to the concentration of added RAV-1 up to that concentration where all NP cells were saturated with RAV-1 (Hanafusa, 1964). This indicates that one infectious unit of RAV-1 is enough to activate RSV from NP cells. If a high concentration of RAV-1 was added to a culture containing various numbers of NP cells, the yield of RSV would be

proportional to the number of NP cells in a culture. Therefore, the yield of RSV from a culture is a function of both the number of NP cells in the culture and the concentration of helper virus added.

In summary, the transformation of cells and replication of the RSV genome are under the control of the RSV genome and do not require the intervention of a helper virus. However, the rate of production of infectious RSV from NP cells is limited by the growth of the co-infecting helper virus which supplies a common pool of virus coat protein used for its own progeny and for that of the RSV genome.

V. BH-RSV and its pseudotypes.

The BH-RSV is defective and requires the assistance of a helper virus for the maturation of viral progeny (Hanafusa et al., 1963). This defectiveness consists, at least in part, of the inability of BH-RSV to produce a coat protein of its own. This viral component is manufactured under the control of RAV which must multiply in the same cell, and supplies a common pool of coat protein used for its progeny and for that of RSV genome. Consequently, the RSV particle is antigenically indistinguishable from its helper (Hanafusa et al., 1964b). Since all avian leukosis viruses currently tested can function as helpers, it is possible to create variants of RSV which differ from the antigenic makeup of their coats. However, coat antigenicity belongs to RSV only phenotypically by association with a distinct helper virus.

The genome of all RSV variants derived from NP cells by activation with different helpers is the same. These phenotypically different but genotypically identical variants of RSV have therefore been termed pseudotypes (Rubin, 1965). At least three antigenic types of RAV, acting as natural helper viruses of the BH-RSV have been isolated from stocks of this virus to date.

Rous associated virus type 1 or RAV-1 isolated at first from BH-RSV differs from the second Rous associated virus type 2 or RAV-2 in several ways (Vogt, 1965; Vogt and Ishizaki, 1965; Hanafusa, 1965): (1) The two helper viruses are antigenically distinct variants. (2) RAV-2 differs from RAV-1 by its growth properties in cell culture derived from individual chicken embryos. About 40% of the embryos from a commercial line of chickens give tissue cultures resistant to RAV-2, whereas all embryos tested by Vogt (1965) are sensitive to RAV-1. (3) There are also indications that the growth of RAV-2 is slower than RAV-1.

The third indigenous helper virus of BH-RSV, Rous associated virus type 3 or RAV-3, was found to be antigenically distinguishable from RAV-1 as well as from RAV-2. Cross neutralization and cross-staining with fluorescent antibody indicated, however, that RAV-3 was more closely related to RAV-1 than RAV-2 (Vogt and Ishizaki, 1965).

The Rous associated viruses isolated from BH-RSV are avian leukosis viruses which multiply in chicken embryo

cells without causing significant alterations in the cells.

In stocks of the BH-RSV, the titer of RAV-1 is about 4 times higher than that of RSV particles (Rubin and Vogt, 1962). The RSV genome coated by RAV-1 or RSV(RAV-1), prepared by NP cells with RAV-1, demonstrated that the ratio of RSV(RAV-1) to RAV-1 was about 1:10 (Hanafusa et al., 1964a). The RAV-2 and RSV(RAV-2) are extremely minor fractions when compared with RAV-1 or RSV(RAV-1) in BH-RSV. The BH-RSV used extensively in this investigation showed the ratio of RSV(RAV-1) plus RSV(RAV-2) to RSV(RAV-2) to be 1.6×10^5:1. The RAV-3 isolated lately from the BH-RSV probably exist at very low concentration in the stocks.

In summary, RAV-1 and RSV(RAV-1) are the largest fraction of viruses in BH-RSV stock followed by the minor groups of viruses, RAV-2 and RSV(RAV-2), or RAV-3 and RSV(RAV-3).

VI. Kinetics of RSV production.

Growth kinetics of RSV in chicken embryo cells has been repeatedly studied (Rubin, 1955, 1957, 1960b; Vigier and Goldé, 1959; Temin and Rubin, 1959; Prince, 1960; Goldé and Vigier, 1961). These studies showed that RSV multiplies at a relatively slow rate. The picture of RSV infection which emerges from cytological observations corresponds well with that derived from studies of the growth and release of infectious RSV in chicken embryo cultures. Newly synthesized virus appears 12 hours after infection, increases rapidly in titer during the next

48 hours, and reaches maximum titer on day four.

Since these studies were done with BH-RSV or Bryan standard strain of Rous sarcoma virus, which are now known to be defective (Hanafusa et al., 1963), new insight has been gained about the kinetics of RSV replication in terms of the defectiveness of RSV. The yield of RSV particles is proportional to the number of cells doubly infected with RSV in combination with helper virus and is not directly related to the input multiplicity of RSV. These findings strongly support the assumption that the rate of RSV production depends on the supply of virus coat from the helper agent.

VII. RAV as resistant inducers.

All avian leukosis viruses studied to date function as resistant inducing agents if these viruses are inoculated into chicken embryo cells prior to RSV infection, and function as helper agents to produce infectious RSV particles from NP cells if these are inoculated simultaneously or after RSV infection.

Rubin in 1960(c) described a virus-induced cellular resistance against infection with RSV which is due to the infection of the chicken embryo cell cultures with a resistance inducing factor (RIF). This factor is a naturally occurring, congenitally transmitted avian leukosis virus (Rubin, 1961). It has been found that a different noncytopathic virus exists in stocks of BH-RSV (Vogt and Rubin, 1961). Like RIF, this noncytopathic virus is detected by its capacity to interfere with the growth of RSV.

To indicate its origin from stocks of BH-RSV it has been named Rous associated virus or RAV. The RAV reported by Vogt and Rubin in 1961 was renamed as RAV-1 when Hanafusa et al. (1963) isolated the second RAV, called RAV-2, in the stocks of BH-RSV. A third RAV, RAV-3 (Vogt and Ishizaki, 1965) was found in BH-RSV.

Rous associated viruses isolated from BH-RSV can infect the susceptible chicken embryo cells simultaneously with, or following, infection by RSV. If, however, the cells are infected with the avian leukosis viruses prior to RSV infection, they become resistant to a later superinfection with RSV. This interference is directed only against a pseudotype of RSV which is antigenically related to the interfering virus. A close antigenic relationship between envelope antigens and leukosis and of sarcoma virus is a prerequisite for interference or induction of resistance (Hanafusa, 1965; Vogt, 1965; Vogt and Ishizaki, 1966). For example, RAV-1 infected chicken cells become resistant to RSV(RAV-1) or RSV(RAV-3), both belong to subgroup A, but not to RSV(RAV-2) which belongs to subgroup B.

In summary, all avian leukosis viruses, when inoculated before RSV challenge function as the resistant inducing agents against the antigenically related Rous sarcoma viruses. This resistance inducing phenomenon is greater and appears more rapidly with Rous associated viruses than with the naturally occurring RIF virus.

VIII. Classification of avian tumor viruses.

Vogt and Ishizaki (1965; 1966; Ishizaki and Vogt,

1966) reported the existence of two antigenic subgroups, A and B, in the avian sarcoma and leukosis complex. The first, referred to as subgroup A, consists of RAV-1 and of viruses having envelopes antigenically related to that of RAV-1. The second, designated as subgroup B, is represented by RAV-2 and its antigenic relatives.

The subgroups of avian tumor viruses could be distinguished on the basis of their host ranges (Crittenden et al., 1963; Vogt and Ishizaki, 1965). Selective resistance of chicken embryo cultures to one or/and two subgroups should be correlated with a genetically determined lack of the corresponding cellular receptor or receptors, e.g., (1) C/A type chicken embryo cells designates a lacking receptor for subgroup A viruses - resistant against subgroup A viruses; (2) C/B type cells, a lacking receptor for subgroup B viruses; (3) C/AB type cells, lacking receptors for both subgroups A and B; (4) C/O type cells, without lacking receptors for both subgroups A and B - susceptible to both subgroups A and B.

Each subgroup consists of several distinct antigenic types. Type- and subgroup-specificity appear to be controlled by an antigen present in the virus envelope (Vogt and Ishizaki, 1966; Ishizaki and Vogt, 1966).

Vogt and Ishizaki (1965) classified the avian tumor viruses as follows (Table 2):

Table 2. Classification of avian tumor viruses.

Sub-groups	Types
A	RSV(RAV-1)*, viral envelope is controlled by Rous associated virus, type 1.
	RSV(RAV-3)*, viral envelope is controlled by Rous associated virus, type 3.
	RSV(AMV-1)*, viral envelope is controlled by avian myeloblastosis virus, type 1.
	BS-RSV, Bryan standard strain of Rous sarcoma virus.
	FSV, Fujinami sarcoma virus.
	SR-RSV-1, Schmidt-Ruppin strain of Rous sarcoma virus, type 1.
	RAV-1, Rous associated virus, type 1.
	RAV-3, Rous associated virus, type 3.
	AMV-1, Avian myeloblastosis virus, type 1.
	BS-RAV, Rous associated virus, isolated from BS-RSV.
B	RSV(RAV-2)*, viral envelope is controlled by Rous associated virus, type 2.
	RSV(AMV-2)*, viral envelope is controlled by avian myeloblastosis virus, type 2.
	HA-RSV, Harris strain of Rous sarcoma virus.
	SR-RSV-2, Schmidt-Ruppin strain of Rous sarcoma virus, type 2.
	RAV-2, Rous associated virus, type 2.
	AMV-2, avian myeloblastosis virus, type 2.
C	RSV(RAV-50), viral envelope is controlled by Rous associated virus, type 50.
	RAV-50**, Rous associated virus, type 50.

* Pseudotypes of BH-RSV.

** Isolated from SR-RSV by Hanafusa and Hanafusa (1966) and belongs to the third subgroup (c).

Vogt and Ishizaki (1965; Ishizaki and Vogt, 1966) indicated that subgroups A and B did not include all avian tumor viruses tested, namely the Carr-Zilber and Prague strains of Rous sarcoma viruses.

Recently isolated RAV-50 does not belong to either subgroup A or B. It appears to be a prototype of a third subgroup - C.

In summary, based on host range, antigenicity and interference pattern, two main classes (subgroups A and B) of avian leukosis viruses can be distinguished. However, some viruses do not belong to either one of the two subgroups A and B, e.g., Carr-Zilber and Prague strains of RSV, and RAV-50.

Media and reagents for cell cultures.

a. Balanced salt solution.

Hanks' balanced salt solution (BSS) was used throughout the experiments. Stock solution was prepared according to the method of Merchant et al. (1964). The working solution of BSS (1 x) was prepared by diluting 10 x stock 1:10 with distilled water, and autoclaved at 120°C for 15 minutes. The pH of the sterile BSS was adjusted with sterile sodium bicarbonate solution (1.4% or 5.6%) to 7.2 to 7.4.

The commercially available powdered BSS, 10 x stock solutions, or the 1 x working solutions were also used. They were purchased from Grand Island Biological Co., Grand Island, New York. The powdered BSS was rehydrated with distilled water and sterilized by filtration through a 0.22 μ Millipore filter (Millipore Filter Corp., Bedford, Massachusetts), and the pH was adjusted. These solutions were stored at room temperature.

b. Chemically defined media.

These were purchased from Microbial Associates Inc., Bethesda, Maryland, or Grand Island Biological Co., Grand Island, New York.

i. Medium 199 in Hanks' base:

Medium 199 prepared in Hanks' basal salt solution as a 10 x stock solution or 1 x working solution was purchased.

Medium 199 as 2 x solution for agar overlay medium was prepared by diluting the 10 x stock 1:5 with sterile distilled water.

ii. Nutrient mixture F10:

This medium was introduced by Ham (1963) as an improved nutrient solution for diploid Chinese hamster and human cell lines. This medium alone, or a 50-50 mixture of the Medium 199 and Nutrient mixture F10 has been found superior to other media when working with chicken embryo cells in culture in this laboratory.

Nutrient mixture F10 as a 10 x stock solution or 1 x working solution was purchased.

The above chemically defined media were stored at 5°C.

c. Media for cell growth and maintenance.

i. Medium 199-Tryptose phosphate broth (Medium 199-TPB):

To the 1 x Medium 199, the following ingredients were added. Tryptose phosphate broth (Difco Laboratories, Detroit, Michigan), 10%; antibiotics, 100 units of penicillin and 100 μ gm of streptomycin per ml in final concentration.

The growth medium was supplemented with 4 to 8% of calf serum (Colorado Serum Co., Denver, Colorado).

For the maintenance of cells or the dilution of virus, 1 to 2% of the calf serum was added, and is referred to as maintenance medium.

The amount of calf serum in the growth medium or maintenance medium varied depending upon the experiments, i.e., duration of incubation, number of cells delivered for culture, or age of the cells.

ii. Medium 199-Nutrient Mixture F10 (Medium 199-F10):

The equal amounts of 1 x Medium 199 and 1 x Nutrient mixture F10 were mixed. Penicillin and streptomycin, 100 units and 100 μ gm respectively per ml in final concentration, were added routinely to the medium.

The amount of calf serum added to the growth medium or the maintenance medium was the same as for medium 199-Tryptose phosphate broth.

The above media were stored at 5°C.

d. Media for feeding the agar overlaid assay plates (Feeding medium).

When assay plates were agar overlaid for titration of RSV, 2 ml of the feeding medium was added directly on the agar layer on the 3rd or 4th day after RSV inoculation.

Medium 199-Tryptose phosphate broth, a mixture of Medium 199-Nutrient mixture F10, or Nutrient mixture F10 medium alone with 0 to 4% calf serum was used as the feeding medium. These were stored at 5°C.

e. Media for agar overlay (Hard agar).

Hard agar media for RSV assay was prepared by mixing the ingredients having the following ratios: 2 x Medium 199, 50; 1.8% agar, 50; tryptose phosphate broth, 14; calf serum, 1.2 to 4.8 (1 to 4%); 5.6% sodium bicarbonate, 2.6; stock antibiotic solution (a 100 fold concentrate), 1.2.

In some cases, the 50-50 mixture of 2 x Medium 199 and 2 x Nutrient mixture F10 medium was substituted for 2 x Medium 199.

All ingredients except 1.8% agar, called hard agar base, were mixed before use in an agar overlay and heated to 40°C. Before use the warm hard agar base was mixed with the melted agar and equilibrated at 45°C in a water bath for 10 minutes.

f. Calcium and magnesium free phosphate buffered saline (CMF-PBS).

A Mg^{++} and Ca^{++} free salt solution was needed for preparation of trypsin. It was prepared by dissolving 8 gm of NaCl, 1.15 gm of Na_2HPO_4, 0.2 gm of KCl, 0.2 gm of KH_2PO_4 in one liter of distilled water, followed by autoclaving at 120°C for 15 minutes. The pH was maintained at 7.4. This was stored at room temperature.

g. Trypsin solution.

Trypsin (1:300, Difco Laboratories, Detroit, Michigan) was prepared in CMF-PBS in concentrations of 0.2% and 0.1% and sterilized by filtration using a 0.22 μ pore size Millipore filter. This was stored at -20°C.

h. Stock antibiotic solutions.

A penicillin-streptomycin mixture was used at a concentration of 100 units of penicillin and 100 μ gm of streptomycin per ml of final volume of culture medium.

The stock solution was prepared with sterile distilled water as a 100 fold concentration and it was stored at -20°C.

i. Agar.

Difco purified agar was used as a 1.8% preparation. It was sterilized by autoclaving and stored at 5°C. The agar was melted prior to use.

j. Sodium bicarbonate solution.

A 1.4% and 5.6% $NaHCO_3$ solutions were prepared in distilled water with or without phenol red (0.002 gm per 100 ml of final volume). These solutions were autoclaved at 120°C for 15 minutes or sterilized by filtration, and stored at 5°C.

k. Phenol red solution.

Phenol red (Nutritional Biological Corp., Cleveland, Ohio) was prepared in a final concentration of 1% in distilled water and stored at room temperature.

Incubation.

Incubation for all cultures was at 37°C in a CO_2 incubator (National Appliance Co., Portland, Oregon). The concentration of CO_2 was maintained between 2.5 to 3% in a moisture saturated atmosphere.

Centrifuges.

For low speed centrifugation, an International Portable Refrigerated Centrifuge, Model PR-2 (International Equipment Co., Needham Hts., Massachusetts) was used.

For high speed centrifugation, an International Preparative Ultracentrifuge, Model B-35 was used.

Disposable tissue culture dishes.

Three sizes of plastic tissue culture dishes were used from Falcon Plastics, Los Angeles, California.

(1) 150 x 25 mm intergrid or 150 mm plastic dish.

(2) 100 x 20 mm or 100 mm plastic dish.

(3) 60 x 15 mm or 60 mm plastic dish (assay plate). This was used for the assay of RSV foci.

Inbred lines of chickens.

Eggs or young chickens of White Leghorn lines 7 and 15 isolated (15 I) were kindly provided by the United States Department of Agriculture, Regional Poultry Research Laboratory, East Lansing, Michigan. These two lines of chickens have been separately maintained at the laboratory by close inbreeding since 1939 (Waters, 1945; Waters and Burmester, 1963).

Line 15 I has been maintained in strict isolation since 1941 and has shown a high susceptibility to RSV (Waters and Pritchett, 1944; Burmester et al., 1960).

The phenotypic expression of line 15 I chicken and its embryonic cells is C/O type. Line 7 chicken is not homogeneous. Theoretically three-fourths are of the C/A type and one-fourth the C/AB type (Vogt and Ishizaki, 1965; Stone, 1967).

Preparation of chicken embryo primary culture (Rubin, 1960a; Rubin et al., 1961).

Ten-day-old chicken embryos were used. The outside of the egg shell was washed with liquid germicidal detergent (Parke, David and Co., Detroit, Michigan) and rinsed with running, warm tap water. The shell above the air sac was disinfected with tincture iodine and ethanol. The shell was removed by cutting, the embryo removed, decapitated,

and placed in a 100 mm plastic dish containing 25 ml of BSS. Three to five embryos from the same dam were pooled in one dish. With a forceps and a scissors, the extremities of the embryos were removed and the abdomens opened. The embryos were washed in fresh BSS. The washed embryos were placed in a 50 ml screw cap test tube containing 25 ml of BSS and shaken vigorously for 1 to 3 minutes using an agitator with vortex action (Vortex mixer, Fisher Scientific Co., New York, New York). The contents of the tube were poured into a plastic dish. Using these procedures, embryo skins and viscerae could be removed from the embryo body fragments. The embryo fragments were placed in a 50 ml test tube and macerated against the tube wall with a sterile stainless steel spatula until the fragments were approximately 1.5 x 1.5 mm in size. Twenty-five ml of BSS was added to the tube containing the macerated tissue and was shaken for 1 to 3 minutes with the aid of vortex mixer. The washed macerated tissue fragments were allowed to settle, and the supernatant fluid was removed and discarded. The tissue fragments were transferred into a "trypsinization flask" (Bellco Glass Inc., Vineland, New Jersey) containing a teflon-covered magnetic stirring bar and 100 ml of 0.2% trypsin solution. The flask contents were agitated slowly on a magnetic stirrer at room temperature for one hour. After this treatment with trypsin, the heavier particles were allowed to settle for 2 to 5 minutes and 75 to 80 ml of supernatant fluid removed and transferred into three 50 ml sterile screw cap test tubes. The fluids were centrifuged

at 400 x g for 10 minutes. The cell pellets were resuspended in growth medium and cells counted with a hemocytometer.

Twenty to twenty-five million cells in a total volume of 25 ml of growth medium were then plated in 150 mm plastic tissue culture dishes. The cells in culture were incubated at 37°C in CO_2. Monolayers composed of fibroblasts were established in 3 to 5 days and were used as the cells of choice.

Preparation of later passage cultures of chicken embryo cells.

Subsequent culture (passage) of cells was prepared from fresh, actively growing primary chicken embryo cells, preferably 3 to 5 days old. Secondary cultures were used for most experiments.

The fluid of the primary chicken embryo cell culture was decanted. Cell monolayers formed in 150 mm plastic dishes were rinsed 2 times with 15 to 25 ml of BSS. To remove the monolayer of chicken fibroblasts from the 150 mm culture dishes, 10 ml of 0.1% trypsin solution was added at 37°C for 10 minutes. Those cells which unattached by this procedure were removed by forcibly pipetting the trypsin solution over the cells. Cell suspensions were transferred to 50 ml test tubes and approximately equal volume of growth medium containing 6% calf serum was added. They were then centrifuged at 400 x g for 5 to 10 minutes, and the supernatant fluid was discarded. The cell pellets were resuspended in the growth medium and pooled in a flask.

The cells were counted, and an appropriate dilution was made.

Preparation of BH-RSV.

a. Tumor propagation.

A Rous sarcoma virus preparation with a potency of approximately 10^8 FFU per ml (lot No. CT916) was used. BH-RSV lot No. CT916 was kindly furnished by Dr. W. Ray Bryan, National Cancer Institute, Bethesda, Maryland. Susceptible chickens (C/O type), 3 weeks of age, were inoculated in the pectoral muscle or the wing web with 0.2 ml of a 10^{-2} dilution of the BH-RSV. For inoculation, the virus was diluted with 0.85% saline combined with 1.0 mg % hyaluronidase and 2% RSV antibody-free calf serum. The tumors were harvested using asceptic techniques on the 8th day. Most tumors were approximately 15 to 20 mm in diameter and appeared firm and yellow. During surgery the excised tumor materials were kept at 5°C. Hemorrhagic areas in tumors were removed and discarded. All tumors harvested were pooled, minced and stored at -75°C.

b. Partial purification of the tumor material (crude extract of tumor) (Maloney, 1956).

The tumor material was thawed at room temperature and made up to 6.6% weight per volume (w/v) with 0.15 M potassium citrate containing 1 mg % hyaluronidase. The tumor material was triturated for 10 to 15 minutes intervals for one hour in a homogenizer (Macro, Vir Tis Model 45, Research Gardiner Equipment Co., New York,

New York) at 5°C. The digested material was centrifuged at 2,300 x g for 20 minutes and the supernatant fluid was removed and placed in a sterile glass container. The centrifugation was repeated. The supernatant fluid was again centrifuged at 18,000 x g for 1 hour and the supernatant fluid harvested. Penicillin and streptomycin, 500 units and 500 μ gm per ml respectively, were added to the preparation. The preparation was dispensed into 15 ml and 1 ml sterile ampules and kept at -75°C. The titer of the crude extract, designated as BH-RSV lot No. 141, was 2.8×10^7 FFU per ml in C/O type cells and 1.7×10^2 FFU per ml in C/A type cells. This stock was used in all experiments.

Preparation of pseudotypes of BH-RSV:RSV(RAV-1) and RSV(RAV-2).

Pseudotypes of BH-RSV, RSV(RAV-1) and RSV(RAV-2), were kindly supplied as solid tumors or crude extract by Dr. William Okazaki, the Department of Agriculture, Regional Poultry Research Laboratory, East Lansing, Michigan. Dr. Okazaki received these pseudotype viruses from Dr. Peter K. Vogt, University of Colorado Medical Center, Denver, Colorado. The original material was prepared by activating BH-RSV induced NP cells with RAV-1 or RAV-2. RSV(RAV-1) lot No. F522 was prepared from tumors developed in C/O type chickens. The titer was 1×10^7 FFU per ml when titrated in C/O type secondary culture cells. RSV(RAV-1) lot No. 323 was prepared by inoculating the C/O type secondary culture cells with RSV(RAV-1) F522. The culture fluid harvested on the 4th day after infection was

centrifuged twice at 400 x g for 20 minutes and 100,000 x g for 2 hours. The lower half of the fluid of second centrifugation was designated as RSV(RAV-1) lot No. 323. The titer was 1 x 10^6 FFU per ml.

RSV(RAV-2) lot Nos. F1507, F1598 and F1604 were prepared from tumors developed in C/A type chickens. The titers in the secondary culture of C/O type cells were approximately 2 x 10^5 FFU per ml.

Preparation of antisera against pseudotypes of BH-RSV: anti-RSV(RAV-1) and anti-RSV(RAV-2) sera.

Antisera prepared against the pseudotypes of BH-RSV were kindly provided by Dr. Okazaki.

Anti-RSV(RAV-1) serum lot No. F535 was produced by inoculating line 7 and 15* cross chickens with RSV(RAV-1). The serum, taken from the survivors inoculated with RSV (RAV-1) which developed tumors, was designated as anti-RSV(RAV-1) serum lot No. F535. The titer of the serum F535 was greater than 10^4 against RSV(RAV-1), i.e., the secondary culture of C/O type cells exposed to 1 ml of antiserum diluted at 10^{-4} or more showed more than 90% fewer foci than those of the control group when the cells were challenged with estimated amount of RSV(RAV-1). The titer was negative against RSV(RAV-2) and other subgroup B viruses.

* This should not be confused with line 15 I inbred chicken.

Anti-RSV(RAV-2) serum lot No. F217 was produced in C/A type chickens. The titer of the serum F217 was greater than 10^3 against RSV(RAV-2) and negative against subgroup A viruses.

Assay of RSV.

The assay of RSV in tissue culture was performed according to the method of Temin and Rubin (1958; Rubin, 1960a) using the infective center techniques, i.e., foci counting technique. Foci were counted and virus titer recorded as FFU.

a. Preparation of the assay plates.

One million cells (secondary culture) suspended in 4 ml of growth medium were plated to each 60 mm plastic dish and incubated at 37°C in CO_2.

b. Inoculation of the virus and agar overlay.

Viruses were diluted with maintenance medium using serial tenfold dilutions. Inoculation of viruses was accomplished in one of three methods.

i. On the same day the cells were prepared, 0.1 or 0.2 ml of each dilution of virus was added to each of three assay plates and the contents gently but thoroughly mixed. The following day, within 15 to 18 hours, the medium was discarded. The cells attached to the plates were rinsed with 4 ml of BSS once and replaced with 4 ml of hard agar medium.

ii. On the following day the cells were prepared, 0.2 ml of diluted virus was added to assay plates having sparsely formed cell layers. The contents were gently

but thoroughly mixed. Approximately 15 hours following virus inoculation, the medium was discarded and the cell sheet was overlaid with agar medium as described above.

iii. On the following day the cells were prepared, within 15 to 18 hours, the medium was discarded and the sparsely formed, cell monolayer was rinsed once. The virus was added for adsorption in 0.5 or 1.0 ml amount of maintenance medium to assay plates and incubated at 37°C for 90 minutes. Following the adsorption the plates were rinsed once and overlaid with 4 ml of agar medium.

C. Feeding of the agar overlaid assay plates and foci counting.

Three or four days postinfection, 2 ml of feeding medium was added directly on the agar surface. One such feeding was sufficient for any one experiment. Foci counting was done between 7 to 10 days after infection.

Foci counting was done with an inverted microscope (Inverted Standard Microscope, Carl Zeiss and Co., German) using a magnification of 80 x. The center piece of the stage was removed and a counting grid installed. A representative area on each plate, usually one-tenth or one-fifth of the total area, was counted and the total number of foci per plate calculated. The entire plate was counted when only a few foci were present, for example, 20 or less foci in the representative area. Since each focus is presumably initiated by a single infectious unit, a count of

the foci gave a presumptive titer of the RSV preparation. Rubin (1960a) has suggested the addition of one log to the titer to compensate for his finding that only one of ten infected cells actually proceedes to initiate a focus. For this dissertation, however, Rubin's adjustment of foci counting was not employed.

Test for resistance inducing activity of RAV (RIF test).

The presence of RAV in test materials was detected by interference with foci formation by BH-RSV, RSV(RAV-1) or RSV(RAV-2) infection. If susceptible chicken embryonic fibroblasts are infected with RAV prior to RSV infection, they become resistant to a later superinfection with RSV. This interference is directed only against a pseudotype of RSV which is antigenically related to the interfering virus (Hanafusa, 1965; Vogt, 1965; Vogt and Ishizaki, 1966). The RIF test method of Rubin and Vogt (1962) was modified as follows: Secondary culture of C/O type cells (1.5×10^6 cells) suspended in 10 ml of growth medium was inoculated with 0.5 to 1 ml of test material. After 3 days incubation, the culture fluid was discarded. The cell sheet was rinsed once with BSS and replaced with fresh maintenance medium. On the 5th day after inoculation the cells were removed by trypsinization and transferred to assay plates (1×10^6 cells per plate). Assay plates having the tertiary cell culture were challenged with measured amount of BH-RSV, RSV(RAV-1) or RSV(RAV-2). The control group was prepared by using chicken embryonic cell metabolite. Foci were counted 7 to 10 days later and the fraction (ratio) of foci

on the test material inoculated cells to those on control plates was recorded. The fraction was referred to as "relative sensitivity" of the cells.

EXPERIMENTAL

BH-RSV Grown in Mixed Cell Cultures.

Methods

Primary culture cells used were prepared from a pool of 3 to 5 ten-day-old embryos of line 15 I (C/O type) or line 7 (C/A type) chickens. Three groups of chicken embryo cells were prepared by using the secondary culture of each line. The first group of cells (C/O type) were plated in 100 mm plastic dishes in a concentration of 1×10^6 per 10 ml of growth medium; the second group, C/O type cells, 1×10^6, plus 2×10^5 cells of C/A type, and the third group: C/A type cells in a concentration of 1×10^6.

The three groups of chicken embryo cells were inoculated with 1×10^5 FFU of BH-RSV on the first day. After 4 days of incubation at 37°C in CO_2, the culture fluids were discarded, the cell sheets were rinsed with BSS once, and replaced with 10 ml of fresh maintenance medium. The following day, the culture fluids were harvested and centrifuged at 400 x g for 15 minutes. The supernatant fluids having free viruses from each group were titrated by means of FFU per ml in the secondary culture of C/O type cells.

Results

The titers of the free viruses from mixed or pure cells of C/O and C/A type cells inoculated with BH-RSV are shown in Table 3.

Table 3. Titration of free viruses from C/O type, C/A type or mixed chicken embryo cells infected with BH-RSV.

Cells used for harvesting BH-RSV	FFU per ml titrated in C/O cells	
	Experiment 1	Experiment 2
C/O cells, 1×10^6 (control)	1.22×10^6 (1.0000)[a]	1.78×10^5 (1.0000)
C/O cells, 1×10^6 plus C/A cells, 2×10^5 (test)	3.33×10^5 (0.2732)	8.17×10^4 (0.4590)
C/A cells, 1×10^6	0 (0.0000)	0 (0.0000)

( )[a] : Fraction of FFU (Test/Control).

Table 3, experiment 1 demonstrates that the free viruses harvested from the mixture of C/O and C/A type cells inoculated with BH-RSV revealed 27.3 % of the foci found in the control group (pure C/O type cell group). Experiment 2 showed 45.9 % of the foci of the control. Both experiments 1 and 2 did not show any foci in pure C/A type cell group.

These results indicated that the small number of BH-RSV resistant chicken cells, C/A type, mixed with BH-RSV susceptible chicken cells, C/O type, were effective in reducing the production of FFU in C/O type cells by BH-RSV infection.

Titration of BH-RSV in Mixed Cell Cultures.

Methods

Five groups of secondary cultures were prepared in assay plates by using the primary cells derived from a pool of 3 to

5 ten-day-old embryos of line 7 (C/A type) or line 15 I (C/O type) chickens.

Group I, mixed cells - C/A type cells in suspension, 2×10^5 in 4 ml of growth medium, were inoculated with 0.1 ml of each tenfold dilution of BH-RSV. Following one hour incubation at 37°C, 1×10^6 of C/O type cells was added.

Group II, mixed cells - simultaneously mixed cell group: C/A type cells, 2×10^5, and 1×10^6 of C/O type cells were plated simultaneously in assay plates containing 4 ml of growth medium. Immediately after the plating 0.1 ml of each tenfold dilution of the virus was inoculated.

Group III, mixed cells - C/O type cells, 1×10^6 in 4 ml of growth medium, were inoculated with the virus. Following one hour incubation at 37°C, 2×10^5 of C/A type cells was added.

Group IV, pure C/O type cell group - C/O type cells, 1×10^6 in 4 ml of growth medium, were inoculated with the virus.

Group V, pure C/A type cell group - C/A type cells, 1×10^6 in 4 ml of growth medium, were inoculated with the virus.

These assay plates were incubated at 37°C in CO_2 overnight. The medium was discarded and all sparsely formed cell sheets were rinsed with BSS once followed by agar overlay with 4 ml of hard agar medium.

These plates were titrated by FFU counts.

Results

Titers (FFU/ml) of BH-RSV titrated in five groups are shown in Table 4.

Table 4. Titration of BH-RSV in mixed or pure cell cultures of C/A and C/O type cells.

Test groups	Descriptions	FFU per ml
I. Mixed cells - C/A cell first group	C/A cells, 2×10^5, inoculated with the virus; 1 hour later 1 $\times 10^6$ of C/O cells were added.	2.03×10^4 (0.2900)[a]
II. Mixed cells - simultaneous group	C/A cells, 2×10^5, mixed with 1×10^6 of C/O cells were inoculated with the virus.	5.97×10^4 (0.8529)
III. Mixed cells - C/O cell first group	C/O cells, 1×10^6, inoculated with the virus; 1 hour later 2 $\times 10^5$ of C/A cells were added.	7.10×10^4 (1.0143)
IV. Pure C/O cell - control group	C/O cells, 1×10^6, inoculated with the virus.	7.00×10^4 (1.0000)
V. Pure C/A cell group	C/A cells, 1×10^6, inoculated with the virus.	0 (0.0000)

( )[a] : Fraction of FFU (Test/Control: group IV).

Table 4 shows that only group I revealed a significant lowering of the titer, i.e., group I has 29% of the foci found in the control.

These results indicated that the FFU of BH-RSV examined was reduced significantly (71% reduction) by exposing BH-RSV

to the genetically resistant C/A type cells before adding BH-RSV susceptible C/O type cells.

Adsorption of BH-RSV or RSV(RAV-1) by C/A Type Chicken Embryo Cells.

A. Adsorption of BH-RSV or RSV(RAV-1) by C/A type chicken embryo cells at 37°C - Titration of unadsorbed fraction.

Methods

The cells used were prepared from a pool of 3 to 5 ten-day-old embryos of each line 7 (C/A type) or line 15 I (C/O type) chickens.

Six groups of assay plates were prepared as follows: group I consisted of 6 assay plates of secondary culture of C/A type cells, 1 x 10^6 cells per plate; group II consisted of 6 plates of C/O type cells, 1 x 10^6 cells per plate; in group III 6 plates were devoid of cells but 4 ml of growth medium was used; groups A, B and C consisted of 5 plates for each seeded with 1 x 10^6 of C/O type cells per plate.

When cell monolayers were sparsely formed, the culture fluids of groups I, II and III plates were discarded and the cell sheets were rinsed with BSS once. To each plate of group I, II and III a measured amount of BH-RSV in 1 ml of maintenance medium was inoculated and incubated at 37°C in CO_2 for 90 minutes for the adsorption of the virus. The fluids of group I, II and III were removed after the adsorption period and the fluid of each group was pooled. The pooled fluid was transferred to assay plates of groups A, B and C, as follows: the fluids from 6 plates of group I to 5 plates of group A; from group II to group B; from group

III to group C. The culture fluids of the assay plates of groups A, B and C were discarded and the cell sheets rinsed with BSS once before 1 ml of test fluid was added to each plate. Group A, B and C plates which received the unadsorbed fractions of BH-RSV by C/A, C/O or no cell plates respectively were incubated at 37°C in CO_2 for 90 minutes. Following the adsorption period the fluids were poured off, the cell sheets were rinsed, agar overlaid and the foci counted (Table 5).

The same procedure was repeated with RSV(RAV-1) (Table 6).

Results

The FFU of the unadsorbed fraction of BH-RSV at 37°C by the C/A or C/O type chicken embryo cells are compared in Table 5.

Table 5. Titration of unadsorbed fraction of BH-RSV at 37°C by C/A or C/O type cells.

Group	Descriptions	FFU/ml of unadsorbed fraction	
		Experiment 1	Experiment 2
A: Test	Unadsorbed fraction of BH-RSV by C/A cells during 90 minute adsorption period at 37°C.	294 (0.7136)[a]	62 (0.4844)
B: C/O cell control	Unadsorbed fraction of BH-RSV by C/O cells during 90 minute adsorption period at 37°C.	228 (0.5534)	58 (0.4531)
C: No cell control	BH-RSV stayed in plastic plates devoid of cells for 90 minutes at 37°C.	412 (1.0000)	128 (1.0000)

( )[a] : Fraction of FFU (Test or C/O cell control/No cell control).

The foci counted in group C were taken arbitrarily as a control. Table 5, experiment 1 shows the unadsorbed fraction of BH-RSV at 37°C by C/A type cells (group A) and C/O type cells (group B) was 71.4% and 55.3% of the foci in the control (group C) respectively. Experiment 2 showed 48.4% and 45.3% of the control in group A and B respectively.

Though there were some fluctuations between the two experiments, there was a reasonable indication that C/A type cells adsorbed some fractions of BH-RSV during the 90 minute adsorption period at 37°C.

The FFU of the unadsorbed fraction of RSV(RAV-1) at 37°C by C/A or C/O type chicken embryo cells are compared in Table 6.

Table 6. Titration of unadsorbed fraction of RSV(RAV-1) at 37°C by C/A or C/O type cells.

Groups	Descriptions	FFU/ml of unadsorbed fraction
A: Test	Unadsorbed fraction of RSV(RAV-1) by C/A cells during 90 minute adsorption period at 37°C.	570 (0.5975)[a]
B: C/O cell control	Unadsorbed fraction of RSV(RAV-1) by C/O cells during 90 minute adsorption period at 37°C.	500 (0.5241)
C: No cell control	RSV(RAV-1) stayed in plastic plates devoid of cells for 90 minutes at 37°C.	954 (1.0000)

( )[a] : Fraction of FFU (Test or C/O cell control/No cell control).

Table 6 shows that the unadsorbed fraction of RSV(RAV-1) at 37°C by C/A (group A) and C/O type cells (group B) were

59.8% and 52.4% of the foci found in the control (group C) respectively.

This result indicated that C/A and C/O type cells adsorbed the RSV(RAV-1) with a similar efficiency at 37°C.

B. Adsorption of RSV(RAV-1) by C/A type chicken embryo cells at 5°C - Titration of unadsorbed fraction.

Methods

Cells used were prepared from a pool of 3 to 5 ten-day-old embryos of each line 7 (C/A type) or line 15 I (C/O type) chickens.

Six groups of assay plates were prepared as follows: group I consisted of 4 assay plates of the secondary culture of C/A type cells, 1×10^6 cells per plate; group II, 4 plates of C/O type cells, 1×10^6 cells per plate. One plate from group I or II was used as a cell control. Group III contained no cells but 4 ml of growth medium; groups A, B and C consisted of 5 plates for each group (1×10^6 C/O type cells per plate). The following day the cells were prepared, culture fluids of the plates in groups I, II and III were discarded and the cell sheets were rinsed. To each of 3 plates of group I, II and III a measured amount of RSV(RAV-1) in 1 ml of maintenance medium was inoculated and all plates were placed at 5°C for 60 minutes. Following adsorption the fluids were removed, and the fluid from each group was pooled to give approximately 3 ml of unadsorbed fraction of the virus. Assay plates of groups I, II and III were washed separately with two 3 ml portions of maintenance medium (total 6 ml). The wash solutions were pooled with

the original unadsorbed fraction (3 ml) so that each group contained approximately 9 ml of fluid with a dilution factor of 3. The culture fluids of groups A, B and C plates were discarded. The cell sheets then were rinsed once. Each received 1 ml of the fluids removed from groups I, II and III as follows: the fluid from group I to 5 plates of group A; from group II to group B; from group III to group C. The plates of groups A, B and C (5 plates of each) were incubated at 37°C for 60 minutes. Following adsorption fluids were discarded, the cell sheets were rinsed and agar was overlaid. The foci were counted and FFU per ml were calculated by multiplying the number of foci by the dilution factor 3.

Results

The FFU per ml of the unadsorbed fraction of RSV(RAV-1) at 5°C by C/A and C/O type cells are compared in Table 7.

Table 7. Titration of unadsorbed fraction of RSV(RAV-1) at 5°C by C/A or C/O type cells.

Groups	Descriptions	FFU/ml of unadsorbed fraction
A: Test	Unadsorbed fraction of RSV(RAV-1) by C/A cells during 60 minute adsorption period at 5°C.	444 (0.2960)[a]
B: C/O cell control	Unadsorbed fraction of RSV(RAV-1) by C/O cells during 60 minute adsorption period at 5°C.	396 (0.2640)
C: No cell control	RSV(RAV-1) stayed in plastic plates devoid of cells for 60 minutes at 5°C.	1500 (1.0000)

( )[a] : Fraction of FFU (Test or C/O cell control/No cell control).

Table 7 shows that the unadsorbed fraction of RSV(RAV-1) by C/A cells (group A) and C/O cells (group B) at 5°C were 29.6 and 26.4% of the foci found in the control (group C) respectively.

Phenotypically Mixed Rous Associated Viruses Existing in BH-RSV or RSV(RAV-1) Stock.

A. Experiments with the free viruses from C/A type cells (a pool of line 7 chicken embryo cells) inoculated with BH-RSV stock.

I. Preparation of test material - agent X.

The primary cells used were prepared from a pool of 3 to 5 ten-day-old embryos of line 7 (C/A type) chickens. The secondary culture of the cells at a concentration of 1.5×10^6 per 10 ml of growth medium were inoculated on the first day with 2×10^5 FFU of BH-RSV while the cells were in suspension. On the 2nd, 3rd and 4th days of postinoculation, the cell sheets were rinsed and replaced with maintenance medium. On the 5th day postinoculation, the culture fluid was harvested and centrifuged at 400 x g for 15 minutes. The supernatant fluid having free viruses was referred to as agent X. Agent X and subsequent passage of the agent X are differentiated by subscripts where X i j indicates the culture fluids obtained after passage of the agent from BH-RSV i and j times in C/A and C/O type cells respectively. The agent X derived from one passage in C/A followed by one passage in C/O type cells is designated agent X_{11}. If no subscripts are shown, its designation can be assumed to be X_{10}.

The C/A type cell metabolite was prepared with the same procedure, however, except BH-RSV inoculation.

II. Intracellular transmissibility of agent X in C/O type cells and the resistance inducing activity.

Methods

Resistance inducing activity of agent X was examined (RIF test with agent X). Five-tenth ml of agent X was used for inoculation of C/O type cells (1.5×10^6) on the first day of experiment. As challenge viruses, BH-RSV, RSV(RAV-1) and RSV(RAV-2) were used. Foci were counted after 7 to 10 day incubation and relative sensitivity of the cells was calculated.

Results

Reactions to BH-RSV, RSV(RAV-1) or RSV(RAV-2) challenge of C/O cells which had been inoculated with agent X are shown in Table 8.

Table 8. Intracellular transmissibility of agent X in C/O cells and the resistance inducing activity.

Tertiary culture of C/O cells transferred to assay plates*	FFU/entire plate due to challenge of:		
	BH-RSV	RSV(RAV-1)	RSV(RAV-2)
Exposed to the agent X-Test	5 (0.0057)[a]	8 (0.0083)	105 (0.1635)
Exposed to the C/A cell metabolite-Control	820 (1.0000)	967 (1.0000)	640 (1.0000)

( )[a] : Fraction of FFU (Test/Control) = Relative sensitivity.

* : The secondary culture of C/O cells was inoculated with agent X or C/A cell metabolite, and 5 days later the cells were transferred to assay plates.

Note: The plate which had only the agent X showed 2 foci on an entire plate without challenge viruses. The number was subtracted from the number of foci produced by virus challenges.

Table 8 shows that tertiary culture of C/O type cells derived from the secondary culture inoculated with agent X revealed 0.0057, 0.0083 or 0.1635 of relative sensitivity to infection of BH-RSV, RSV(RAV-1) or RSV(RAV-2) respectively.

These results indicated that: (1) Agent X was transmissible intracellularly in C/O type cells and gave the cells marked resistance to the BH-RSV or RSV(RAV-1) challenge. Agent X had to contain the virus interfering with BH-RSV or with RSV(RAV-1). Thus, agent X should contain RAV-1. (2) Agent X also should contain RAV-2 in addition to RAV-1 since there was fairly marked interference with the RSV(RAV-2) challenge.

It seems that RAV-1 originated from a phenotypically mixed virus existing in the BH-RSV stock, i.e., RAV-1(RAV-2) and/or RAV-1(RAV-2 plus other virus coat). With the aid of the RAV-2 protein coat, the phenotypically mixed virus could penetrate the C/A type cells, and the RAV-1 genome could produce its progeny, RAV-1 or RAV-1(RAV-1).

III. Identification of agent X by neutralization technique.

Methods

1. Preparation of mixtures of agent X and antiserum:

Antisera against RSV(RAV-1) and RSV(RAV-2) were inactivated at 56°C for 30 minutes. Then tenfold dilutions (10^{-1} and 10^{-2}) were made with maintenance medium. The inactivated antisera diluted to 10^{-2} were mixed with agent X as indicated below and incubated in a 37°C water bath for 40 minutes for neutralization of specific viruses in the agent X.

Tubes or groups	Agent X	Antisera (10^{-2} dilution)
I	0.6 ml of 10^{0}	Anti-RSV(RAV-1), 0.6 ml
II	1 ml of 10^{-1}	1 ml
III	1 ml of 10^{-2}	1 ml
IV	0.6 ml of 10^{0}	Anti-RSV(RAV-2), 0.6 ml
V	1 ml of 10^{-1}	1 ml
VI	1 ml of 10^{-2}	1 ml
VII	0.6 ml of 10^{0}	Normal calf serum, 0.6 ml
VIII	1 ml of 10^{-1}	1 ml
IX	1 ml of 10^{-2}	1 ml

2. Inoculation of mixtures of agent X and antisera, and BH-RSV challenge:

The secondary culture of C/O type cells (1.5 x 10^{6} cells plated per 100 mm plastic dish) were inoculated with 1 ml of a mixture of agent X and antiserum on the first day of the experiment. Two 100 mm dishes were prepared for each of groups I-IX. All plates were incubated at 37°C in CO_2 for 5 days and resistance inducing activity was examined according to the method used for the preceding experiment (II). BH-RSV was used as a challenge virus.

Results

The tertiary culture of C/O type cells derived from the secondary culture cells inoculated with 1 ml of 50-50 mixture of undiluted agent X and anti-RSV(RAV-1) serum at dilution of 10^{-2} revealed 0.3838 of relative sensitivity as shown in Table 9. The virus activity (namely, intracellular

Table 9. Neutralization test for agent X.

Groups	Dilutions of agent X, 0.5 ml of each	Antiserum, 0.5 ml of 10^{-2} dilution	FFU/entire plate due to BH-RSV challenge
I	10^{0}	Anti-RSV(RAV-1)	127 (0.3838)[a]
II	10^{-1}		300 (0.9090)
III	10^{-2}		313 (0.9495)
IV	10^{0}	Anti-RSV(RAV-2)	7 (0.0202)
V	10^{-1}		93 (0.2838)
VI	10^{-2}		297 (0.8990)
VII	10^{0}	Normal calf serum	0 (0.0000)
VIII	10^{-1}		57 (0.1717)
IX	10^{-2}		147 (0.4444)
BH-RSV control (No agent X-antiserum mixture added)		Normal calf serum	330 (1.0000)
Cell control (No agent X-antiserum mixture and challenge virus added)		Normal calf serum	0 (0.0000)

( )[a] : Fraction of FFU (Test, I-IX/BH-RSV control) = Relative sensitivity.

Note : Any plates inoculated with the agent X-antiserum mixtures but not challenged with BH-RSV did not show foci.

resistance inducing activity or RIF activity) of 0.5 ml of the undiluted agent X was neutralized with 0.5 ml of anti-RSV(RAV-1) serum at dilution of 10^{-2} by 38.4% . In other words, the numbers of relative sensitivity could be considered as the fraction of the virus activity lost by neutralization with a specific antiserum or by too low of a concentration of agent X. In this experiment, 0.9 or more of relative sensitivity was taken arbitrarily as an effective loss of virus activity due to specific neutralization (positive reaction) or due to high dilution of virus (pseudo-positive).

When three groups with 0.5 ml of the agent X at dilution of 10^{-1} (Table 9, groups II, V and VIII) were compared, only group II showed a positive reaction (0.9090 of relative sensitivity). This fact indicated that 0.5 ml of agent X at dilution of 10^{-1} was effectively or specifically neutralized by 0.5 ml of anti-RSV(RAV-1) serum at dilution of 10^{-2}. Therefore, a major fraction of agent X was RAV-1 or an antigenically related type.

The comparison of the RIF activity of agent X treated with anti-RSV(RAV-2) with that of agent X treated with normal calf serum (control) demonstrated that a portion of RAV in agent X could be neutralized by anti-RSV(RAV-2) serum. Therefore, it is probable that agent X contains another virus which possesses RAV-2 protein coat. Since the BH-RSV challenge dose is at such a high dilution, virtually all of the focus-forming particles would be RSV(RAV-1). The RAV-1 genome would be required for interference with this type of

virus. The RIF activity eliminated by the anti-RSV(RAV-2) must have been due to a phenotypically mixed RAV-1(RAV-2).

Neutralization tests proved that agent X contained RAV-1 as a major fraction and RAV-1(RAV-2) as a minor fraction.

IV. Presence of RAV-1 in the culture fluids of C/O cells, and absence in the culture fluids of C/A cells inoculated with agent X.

Methods

Primary cells used were prepared from a pool of 3 to 5 ten-day-old embryos of line 15 I (C/O type) or line 7 (C/A type) chickens. Secondary cultures of each line, 2 x 10^6, plated in 100 mm plastic dishes were inoculated with 0.5 ml of the agent X on the first day. After 4 days of incubation at 37°C in CO_2, the culture fluids were poured off, the cell sheets were rinsed once with BSS and replaced with 10 ml of fresh maintenance medium. The following day, the culture fluids of C/O and C/A type cells (test materials) were harvested. The culture fluids having free viruses harvested from the C/O or C/A type cells were designated as agent X_{11} or agent X_{20} respectively. The C/O and C/A type cell metabolites were prepared with the same procedure except inoculation of agent X.

The secondary culture of C/O type cells in concentration of 1 x 10^6 was plated in assay plates. The following day, preceding complete monolayer formation, one ml of agent X_{11} or X_{20} plus 3 ml of maintenance medium was added to each assay plate. Following two days incubation at 37°C in CO_2, the cell sheet was rinsed and challenged with a measured amount of BH-RSV, and the foci counted.

Results

Table 10 demonstrates that C/O type cells in assay plates exposed to agent X_{11} revealed 0.0389 of relative sensitivity. However, C/O type cells exposed to agent X_{20} showed 0.9437 of relative sensitivity.

These facts indicated the agent X could be multiplied in C/O type cells and RAV-1 was released into the fluid (agent X_{11}) and the RAV-1 interfered with BH-RSV. But C/A type cells did not support the agent X multiplication intracellularly. In the preceding experiment, RAV-1(RAV-2) was shown to be present in agent X in sufficient concentration to contribute to interference in C/O type cells. But, the concentration seems to be insufficient for producing a productive infection in C/A type cells.

Table 10. Transmissibility of agent X by means of culture fluid in C/O cells and interference with BH-RSV challenge.

Secondary culture of C/O cells plated in assay plates	FFU/entire plate due to BH-RSV challenge
Exposed to one ml of the culture fluid of C/O cells inoculated with agent X (agent X_{11}) - Test group	27 (0.0389)[a]
Exposed to one ml of the C/O cell metabolite - Control	685 (1.0000)
Exposed to one ml of the culture fluid of C/A cells inoculated with agent X (agent X_{20}) - Test group	503 (0.9437)
Exposed to one ml of the C/A cell metabolite - Control	533 (1.0000)

( )[a] : Fraction of FFU (Test/Control) = Relative sensitivity.

Note : All plates only exposed to the test materials but not challenged with BH-RSV did not show any foci.

V. Successive intracellular transmissibility of agent X in C/O cells and the resistance inducing activity, and failure in C/A cells.

Methods

Primary cells used were prepared from a pool of 3 to 5 ten-day-old embryos of line 15 I (C/O type) or line 7 (C/A type) chickens. The secondary culture of each line was prepared in 150 mm plastic dishes (5×10^6 cells in 25 ml of growth medium) and was inoculated with 1 ml of agent X. Four days after inoculation, the growth medium was replaced with fresh maintenance medium which was harvested 24 hours later. The culture fluids harvested from the C/O or C/A type cells were referred to as agent X_{11} or agent X_{20} respectively. Culture fluids of C/O and C/A type cells were prepared by using the same procedure omitting the agent X inoculation, these were termed cell metabolites.

Secondary culture of C/O type cells prepared in 100 mm plastic dishes (1.5×10^6) were inoculated with 1 ml of either test material or metabolite. The intracellular transmissibility of the test material (agent X_{11} or agent X_{20}) and RIF activity were examined.

Results

Table 11 shows that: (1) The test cells, tertiary culture of the C/O type cells derived from the secondary culture which had been inoculated with agent X_{11}, showed no foci when these were challenged with BH-RSV or RSV(RAV-1). The test cells showed 0.3592 of relative sensitivity to RSV(RAV-2) infection.

(2) However, the test cells treated with agent X_{20} showed practically the same numbers of foci found in the controls. The relative sensitivity was 1.0787, 0.9016 or 1.1000 to infection of BH-RSV, RSV(RAV-1) or RSV(RAV-2) respectively. Therefore, the interfering agent could propagate in C/O type cells successively but not in C/A type cells.

Table 11. Successive passage of resistance inducing activity of agent X in C/O cells and failure in C/A cells.

Tertiary culture of C/O cells transferred to assay plates*	FFU/entire plate due to challenge of:		
	BH-RSV	RSV(RAV-1)	RSV(RAV-2)
Exposed to the culture fluid of C/O cells inoculated with the agent X (agent X_{11}) - Test	0 (0.0000)[a]	0 (0.0000)	170 (0.3592)
Exposed to the culture fluid of C/O cells - Metabolite control	1003.33 (1.0000)	1100.00 (1.0000)	473 (1.0000)
Exposed to the culture fluid of C/A cells inoculated with the agent X (agent X_{20}) - Test	913.33 (1.0787)	1100.00 (0.9016)	587 (1.1000)
Exposed to the culture fluid of C/A cells - Metabolite control	846.67 (1.0000)	1220.00 (1.0000)	533 (1.0000)

( )[a] : Fraction of FFU (Test/Control) = Relative sensitivity.

* : The secondary culture of C/O cells was inoculated with the test material, and 5 days later the cells were transferred to assay plates.

Note : All assay plates only exposed to the test materials but not challenged with viruses did not show any foci.

B. Experiments with the free viruses from C/A type cells (a pool of line 7 chicken embryo cells) inoculated with RSV(RAV-1) stock.

I. Preparation of test material - agent Y.

Primary cells used were prepared from a pool of 3 to 5 ten-day-old embryos of line 7 (C/A type) chickens. The secondary culture of the cells was inoculated with RSV(RAV-1) as follows:

Experiment number	Number of cells	Input virus (Titer in C/O cells in FFU)	Ratio of the virus to the cells: input virus/no. of cells.
287	2×10^6/100 mm dish	2×10^5	0.1
322	10×10^6/150 mm dish	1.7×10^6	0.17
401	1.5×10^6/100 mm dish	8×10^5	0.53

Cell culture fluids of Experiments 287, 322 and 401 were harvested according to the procedure for the preparation of agent X (Part A, Section I). The culture fluids having free viruses were designated as Agent Y Nos. 287, 322 and 401.

II. Intracellular transmissibility of agent Y in C/O type cells and the resistance inducing activity.

Methods

Secondary culture of C/O type cells (1.5×10^6 cells plated per 100 mm plastic dish) were inoculated with 0.5 ml of agent Y. The intracellular transmissibility of the agent Y and RIF activity were examined.

Results

Table 12 shows that: (1) The tertiary culture of C/O type cells derived from the secondary culture cells inoculated with the agent Y No. 287 revealed 0.0000, 0.0100 or 0.7557 of relative sensitivity when the tertiary cells were challenged with a measured amount of BH-RSV, RSV(RAV-1) or RSV(RAV-2) respectively. (2) Tertiary cells treated with the agent Y No. 322 showed 0.4122, 0.4766 or 1.4080 of relative sensitivity to infection of BH-RSV, RSV(RAV-1) or RSV(RAV-2) respectively. (3) Tertiary cells treated with the agent Y No. 401 showed 0.0069, 0.0000 or 0.3478 of relative sensitivity to challenge of BH-RSV, RSV(RAV-1) or RSV(RAV-2) respectively.

These results indicated that the agent Y Nos. 287 and 401 contained RAV-1 as a major fraction and RAV-2 as a minor fraction. These viruses could be transmissible intracellularly, and could induce resistance in C/O type cells against the challenge viruses. The agent Y No. 322 contained less RAV-1 compared with the other agents. Increase of FFU due to the RSV(RAV-2) challenge (1.4080 in fraction) in Experiment 322 was further evidence for the cells possessing RAV-1 (Hanafusa, 1965).

RAV-1 must have originated from a phenotypically mixed virus existing in RSV(RAV-1) stock, i.e., RAV-1(RAV-2) and/or RAV-1(RAV-2 plus other).

III. Propagation of RAV-1 of agent Y No. 322.

Methods

Since agent Y No. 322 showed less RAV activity when

Table 12. Intracellular transmissibility of agent Y in C/O cells and the resistance inducing activity.

Experiment number	Tertiary culture of C/O cells transferred to assay plates*	FFU/entire plate due to challenge of: BH-RSV	RSV(RAV-1)	RSV(RAV-2)
287	Exposed to the agent Y-Test	0 (0.0000)[a]	3 (0.0100)	217 (0.7558)
	Exposed to the C/A cell metabolite-Control	313 (1.0000)	333 (1.0000)	287 (1.0000)
322**	Test	157 (0.4122)	170 (0.4766)	357 (1.4080)
	Control	380 (1.0000)	357 (1.0000)	253 (1.0000)
401	Test	10 (0.0069)	0 (0.0000)	833 (0.3478)
	Control	1450 (1.0000)	1517 (1.0000)	2540 (1.0000)

( )[a] : Fraction of FFU (Test/Control) = Relative sensitivity.

* : The secondary culture of C/O type cells was inoculated with agent Y or C/A cell metabolite, and 5 days later the cells were transferred to assay plates.

** : With a separate experiment, the agent Y No. 322 was passed in C/O cells and tested the increasing of RAV activity.

Note : All plates which had only test materials but not RSV challenges did not show foci.

inoculated to C/O type cells (Table 12), attempts were made to propagate virus in the C/O type cells.

The secondary culture of C/O type cells (5×10^6 per 150 mm plastic dish) was inoculated with 2 ml of the agent Y No. 322. On the 3rd day postinoculation, the culture fluid

was discarded, cell sheet was rinsed once with BSS and replaced with 25 ml of maintenance medium. The following day the culture fluid was harvested and the virus extracted by three successive freezing and thawing of the cells. The cell debris was removed by centrifugation at 400 x g for 15 minutes and the extract (supernatant fluid) was pooled with the culture fluid harvested from these cells. The pool was designated as agent Y No. 322-1. This procedure was repeated three times more starting with the agent Y No. 322-1, and agents Y Nos. 322-2, -3, and -4 were prepared.

One ml of each agent Y No. 322-1, -2, -3 or -4, was added to the sparsely formed C/O type cell monolayers prepared in assay plates. The four plates were inoculated with each agent. After 2 days of incubation, 3 out of 4 plates from each group was challenged with BH-RSV, and the foci were counted. The other one was used as a test control without a BH-RSV challenge.

Results

Table 13 shows that the C/O type cells in assay plates exposed to the agent Y No. 322-1, -2, -3 or -4 revealed 0.2105, 0.0789, 0.0921 or 0.0526 of relative sensitivity respectively when the cells were challenged with BH-RSV.

These results indicated that the viruses in the agent Y No. 322 could be propagated by successive passage in C/O type cells.

IV. Identification of agent Y by neutralization technique.

Methods

The materials, i.e., cells, antisera and challenge virus, and the method were the same as the neutralization

Table 13. Propagation of RAV-1 of agent Y No. 322 in C/O cells.

Viral agents propagated in C/O cells and added to the assay plates of C/O cells.	FFU/entire plate due to BH-RSV challenge.
Agent Y, 322-1	53 (0.2105)[a]
Agent Y, 322-2	20 (0.0789)
Agent Y, 322-3	23 (0.0921)
Agent Y, 322-4	13 (0.0526)
No. (cell control)	253 (1.0000)

( )[a] : Fraction of FFU (Test/Control) = Relative sensitivity.

Note : All plates which had only test materials but not BH-RSV challenge did not show any foci.

test with agent X, except for the substitution of agent Y for agent X. For this experiment, agent Y No. 287 was employed.

Results

Table 14 shows that tertiary culture of C/O type cells derived from the secondary culture inoculated with 1 ml of 50-50 mixture of undiluted agent Y and the anti-RSV(RAV-1) serum at a dilution of 10^{-2} (group I) revealed 0.7394 of relative sensitivity. This result indicates that viral activity (namely RIF activity) of 0.5 ml of the undiluted agent Y was neutralized with 0.5 ml of anti-RSV(RAV-1) serum at dilution of 10^{-2} by 73.9 %.

Five-tenth ml of the agent Y at dilution of 10^{-1} was neutralized with the same amount of anti-RSV(RAV-1) serum by 77.3 % (group II).

Table 14. Neutralization test for agent Y.

Groups	Dilutions of the agent Y, 0.5 ml of each	Antiserum, 0.5 ml of 10^{-2} dilution	FFU/entire plate due to BH-RSV challenge
I	10^{0}	Anti-RSV(RAV-1)	293 (0.7394)[a]
II	10^{-1}		307 (0.7731)
III	10^{-2}		337 (0.8487)
IV	10^{0}	Anti-RSV(RAV-2)	0 (0.0000)
V	10^{-1}		0 (0.0000)
VI	10^{-2}		83 (0.2101)
VII	10^{0}	Normal calf serum	0 (0.0000)
VIII	10^{-1}		3 (0.0084)
IX	10^{-2}		80 (0.2017)
BH-RSV control (No agent Y-antiserum mixture added)		Normal calf serum	397 (1.0000)
Cell control (No agent X-antiserum mixture and challenge virus added)		Normal calf serum	0 (0.0000)

( )[a] : Fraction of FFU (Test, I-IX/BH-RSV control) = Relative sensitivity.

Note : All plates inoculated with only the agent X-antiserum mixtures but not challenged with BH-RSV did not show any foci.

The comparison of the RIF activity of agent Y treated with anti-RSV(RAV-2) serum with that of agent Y treated with normal calf serum demonstrated no differences. The free viruses in agent Y No. 287 were not neutralized with anti-RSV(RAV-2) serum.

Neutralization tests proved the agent Y contained RAV-1 as a major fraction.

V. Successive intracellular transmissibility of agent Y in C/O cells and the resistance inducing activity, and failure in C/A cells.

Methods

The primary cultures used were prepared from a pool of 3 to 5 ten-day-old embryos of line 15 I (C/O type) or line 7 (C/A type) chickens. The secondary cultures of each line of cells, prepared in 100 mm plastic dishes (1.5×10^6 cells per dish) were inoculated with 0.5 ml of the agent Y No. 287 on the first day of experiment. Four days after inoculation the cell sheet was rinsed and replaced with maintenance medium. On the following day the culture fluids were harvested. The culture fluid harvested from C/O type cells was designated as agent Y_{11}. Cell associated viruses were extracted from C/A type cells by three successive freezing and thawing of the cells. The cell debris was removed by centrifugation and the supernatant fluid was pooled with the culture fluid harvested from C/A type cells. The pool was referred to as agent Y_{20}. Cell metabolites of each line were prepared and used as control materials.

RIF tests were performed using agent Y_{11} and agent Y_{20} (1 ml of each).

Results

Table 15 shows that: (1) The test cells, tertiary culture of C/O type cells derived from the secondary

culture inoculated with agent Y_{11}, did not reveal any foci when these were challenged with BH-RSV or RSV(RAV-1). The test cells showed 0.4980 of relative sensitivity to RSV(RAV-2) infection. (2) However, tertiary culture of C/O type cells derived from the secondary culture inoculated with agent Y_{20} showed 0.9290, 0.9192 or 0.8398 of relative sensitivity when the cells were challenged with BH-RSV, RSV(RAV-1) and RSV(RAV-2) respectively.

The results indicated that the viruses in the agent Y could be transmitted intracellularly, successively in C/O type cells, but not in C/A type cells.

VI. Failure of RAV-1 production in C/A cells inoculated with RSV(RAV-1) stock neutralized by anti-RSV(RAV-2) serum.

Methods

Since the preceding experiments indicated that RSV(RAV-1) stock contained phenotypically mixed virus, RAV-1(RAV-2) and/or RAV-1(RAV-2 plus other), an attempt was made to eliminate from the stock the viruses having the RAV-2 protein coat. Equal amounts of RSV(RAV-1) stock and anti-RSV(RAV-2) serum at dilution of 10^{-1} were mixed. The mixture was incubated in 37°C water bath for 40 minutes.

The secondary culture of C/A type cells (1.5×10^6 per 10 ml of growth medium) was inoculated with 0.4 ml of the virus-antiserum mixture (0.2 ml of the virus stock contained 2×10^5 FFU). On the 5th day of postinoculation the culture fluid was harvested according to the method used for the preparation of agent X (Part A, Section I) or agent Y (Part B, Section I). Using this material, intracellular

Table 15. Successive intracellular transmissibility of agent Y in C/O cells and the resistance inducing activity and failure in C/A cells.

Tertiary culture of C/O cells transferred to assay plates*	FFU/entire plate due to challenge of:		
	BH-RSV	RSV(RAV-1)	RSV(RAV-2)
Exposed to the culture fluid of C/O cells inoculated with the agent Y(agent Y_{11})-Test	0 (0.0000)[a]	0 (0.0000)	417 (0.4980)
Exposed to the culture fluid of C/O cells-Metabolite control	803 (1.0000)	1250 (1.0000)	837 (1.0000)
Exposed to the culture fluid plus extract of C/A cells inoculated with agent Y(agent Y_{20})-Test	1133 (0.9290)	1100 (0.9192)	1013 (0.8398)
Exposed to the culture fluid plus extract of C/A cells-Metabolite control	1220 (1.0000)	1197 (1.0000)	1207 (1.0000)

( )[a] : Fraction of FFU (Test/Control) = Relative sensitivity.

* : Secondary culture of C/O cells was inoculated with the test material, 5 days later the cells were transferred to assay plates.

Note : All assay plates exposed to the test materials but not challenged with viruses did not show foci.

transmissibility of the agent in C/O type cells and RIF activity were examined.

Results

Table 16 shows that relative sensitivity of C/O type test cells to BH-RSV, RSV(RAV-1) or RSV(RAV-2) challenge was 0.8708, 0.9605 or 1.0251 respectively.

Table 16. Absence of virus activity in the culture fluid of C/A cells inoculated with RSV(RAV-1) stock neutralized by anti-RSV(RAV-2) serum.

Tertiary culture of C/O cells transferred to assay plates*	FFU/entire plate due to challenge of:		
	BH-RSV	RSV(RAV-1)	RSV(RAV-2)
Exposed to anti-RSV(RAV-2) serum treated RSV(RAV-1) stock-Test	607 (0.8708)[a]	973 (0.9605)	817 (1.0251)
Exposed to C/A cell metabolite-Control	697 (1.0000)	1013 (1.0000)	797 (1.0000)

( )[a] : Fraction of FFU (Test/Control) = Relative sensitivity.

* : The secondary culture of C/O cells was inoculated with test material or C/A cell metabolite and 5 days later the cells were transferred to assay plates.

Note : All assay plates exposed to the test material but not challenged with viruses did not show foci.

Results indicate that anti-RSV(RAV-2) serum neutralized the RAV-2 protein coated viruses found in the RSV(RAV-1) stock. Therefore, RAV-1 or RAV-2 could not be recovered from the culture fluid of C/A type cells inoculated with RSV(RAV-1) stock neutralized by the antiserum.

C. Experiments with the free viruses from C/A type cells (individual line 7 chicken embryo cells) inoculated with RSV(RAV-1) stock.

I. Preparation of test material - agent Z.

Since line 7 chickens were not homogeneous, an attempt was made to examine the relationship between the individual

embryo and its ability to support the RAV-1 growth.

The primary cultures used were derived from 15 individual ten-day-old embryos of line 7 chickens. Secondary cultures from each individual embryo cells ($1.5 - 2 \times 10^6$ per 100 mm plastic dish) were inoculated with RSV(RAV-1) stock. The ratio of the virus to the cell, FFU of input virus/number of cells, was kept between 0.1 - 0.5. On the 5th day of postinoculation the culture fluid was harvested according to the procedure used for preparation of agent X (Part A, Section I) or agent Y (Part B, Section I), and the culture fluid having free viruses was designated as agent Z.

Susceptibility to RSV(RAV-2) infection of the individual embryos was determined. The tertiary culture of the individual embryo cells prepared in assay plates was challenged with $1 - 4 \times 10^3$ FFU of RSV(RAV-2). If the assay plates showed foci, the cells were considered as C/A type. Twelve out of 15 embryo cells were C/A type and the remainder, C/AB type (Table 17).

II. Intracellular transmissibility of agent Z in C/O type cells and the resistance inducing activity.

Methods

Secondary culture of C/O type cells, 1.5×10^6 cells plated in 100 mm plastic dish, was inoculated with 0.5 ml of agent Z. The intracellular transmissibility of the agent Z and RIF activity were examined.

Results

Fifteen embryos were classified into two groups:

group I composed of 12 C/A type and group II composed of 3 C/AB type (Table 17).

Table 17 shows that: (1) The agent Z preparations derived from C/A type cells (group I) were quite effective in inhibiting focus formation by BH-RSV and by RSV(RAV-1). In addition considerable inhibition of infection with RSV (RAV-2) was also produced. Relative sensitivity of the test cells to BH-RSV, RSV(RAV-1) or RSV(RAV-2) infection was 0 to 0.0737 (average 0.0313), 0 to 0.0851 (average 0.0172) or 0.1415 to 0.6951 (average 0.3818) respectively. These results indicated that the agent Z prepared from C/A type cells contained RAV-1 as a major fraction and RAV-2 as a minor fraction. (2) The agent Z derived from C/AB type cells (group II) shows only a little inhibitory activity for all 3 challenge viruses based on the average values. Relative sensitivity to BH-RSV, RSV(RAV-1) or RSV(RAV-2) was 0.5645 to 1.0208 (average 0.7532), 0.6848 to 1.0062 (average 0.8303) or 0.6679 to 1.0411 (average 0.8721) respectively. These indicated that agent Z prepared from C/AB type embryo cells contained a negligible amount or no RAV-1 and RAV-2.

These results indicated that the RSV(RAV-1) stock used for preparation of agent Z contained phenotypically mixed viruses RAV-1(RAV-2) and/or RAV-1(RAV-2 plus other) in addition to normal viruses such as RAV-1(RAV-1).

III. Identification of agent Z by neutralization technique.

Methods

Materials and methods were the same as the neutralization tests with the agent X or Y.

Table 17. Determination of embryo type and testing of agent Z derived from the cell preparations for its resistance inducing activity.

Groups	Individual embryos			Relative sensitivity of C/O cells against challenge of:		
	Egg No.	Hen No.	Susceptibility to RSV(RAV-2)	BH-RSV	RSV(RAV-1)	RSV(RAV-2)
I: C/A type cells	1	Pen 19	+	0.0185	0.0000	0.5181
	2	Pen 19	+	0.0694	0.0851	0.6951
	3	Pen 19	+	0.0263	0.0000	0.5152
	15	577	+	0.0000	0.0268	0.2938
	26	580	+	0.0127	0.0022	0.3079
	27	582	+	0.0063	0.0003	0.4917
	28	584	+	0.0209	0.0045	0.4479
	29	589	+	0.0084	0.0020	0.4054
	30	576	+	0.0067	0.0000	0.2150
	31	578	+	0.0386	0.0141	0.1415
	32	580	+	0.0464	0.0040	0.2304
	35	599	+	0.0737	0.0679	0.3147
	Average			0.0313	0.0172	0.3818
II: C/AB type cells	16	580	-	0.7744	0.8000	0.9073
	17	582	-	0.5645	0.6848	0.6679
	34	593	-	1.0208	1.0062	1.0411
	Average			0.7532	0.8303	0.8721

Note : Some assay plates with test cells showed a few foci in the entire plate without virus challenges. These numbers of foci were substracted from the numbers of foci of virus challenged plates.

Agent Z prepared from the line 7 cells of the embryo No. 1 was used.

Results

Table 18 shows that: (1) The tertiary culture of C/O type cells derived from the secondary culture inoculated with 1 ml of 50-50 mixture of undiluted agent Z and the anti-RSV(RAV-1) serum dilution of 10^{-2} revealed relative sensitivity of 0.7532 (group I). (2) The same amount of the agent Z, 0.5 ml of undiluted, was not neutralized with the 0.5 ml of anti-RSV(RAV-2) serum at dilution 10^{-2} (group IV). This indicates that the agent Z was neutralized by anti-RSV(RAV-1) serum but not by anti-RSV(RAV-2) serum.

When RSV(RAV-2) serum treated groups (groups IV, V and VI) and normal calf serum treated groups (groups VII, VIII and IX) were compared, groups V or VI showed more foci than groups VIII or IX respectively. Therefore, it is probable that agent Z contains another virus which neutralized with anti-RSV(RAV-2) serum. The RIF activity eliminated by the serum must have been due to a phenotypically mixed RSV-1(RAV-2).

It is concluded that agent Z contained the virus RAV-1(RAV-1) as a major fraction and a low concentration of RAV-1(RAV-2) virus. Similar results were shown repeatedly by the neutralization tests for the agent X (Table 9) and for agent Y (Table 14).

Table 18. Neutralization test for agent Z.

Groups	Dilutions of the agent Z, 0.5 ml of each	Antiserum, 0.5 ml of 10^{-2} dilution	FFU/entire plate due to BH-RSV challenge
I	10^{0}	Anti-RSV(RAV-1)	387 (0.7532)[a]
II	10^{-1}		510 (0.9935)
III	10^{-2}		530 (1.0325)
IV	10^{0}	Anti-RSV(RAV-2)	0 (0.0000)
V	10^{-1}		210 (0.4091)
VI	10^{-2}		567 (1.1039)
VII	10^{0}	Normal calf serum	0 (0.0000)
VIII	10^{-1}		123 (0.2403)
IX	10^{-2}		407 (0.7922)
BH-RSV control (No agent X-antiserum mixture added)		Normal calf serum	513 (1.0000)
Cell control (No agent Y-antiserum mixture and challenge virus added)		Normal calf serum	0 (0.0000)

( )[a] : Fraction of FFU (Test, I-IX/BH-RSV control) = Relative sensitivity.

Note : Some plates showed a few foci without BH-RSV challenge. Those numbers were substracted from the numbers of foci of virus challenged plates.

IV. Attempt to pass the inhibitory factor of agent Z in a second passage in C/A type cells.

Methods

The primary culture of C/A type cells was prepared from a pool of 3 to 5 ten-day-old line 7 chicken embryos. The secondary culture of the cells in suspension (2×10^6 cells per 100 mm plastic dish) was inoculated with 0.5 ml of agent Z, prepared from the embryo No. 1, on the first day of experiment. Four days after inoculation the cell sheet was rinsed, and 10 ml of maintenance medium was added. On the following day the culture fluid and cell associated viruses were harvested and designated as agent Z_{20}. Cell metabolite was prepared by the same procedure except agent Z inoculation.

Resistance inducing activity of agent Z_{20} (used 1 ml) was examined. Cell metabolite was used for control test.

Results

Table 19 shows that the test cells, tertiary culture of C/O type cells derived from the secondary culture inoculated with agent Z_{20} revealed 0.7132, 0.7428 or 0.7856 of relative sensitivity when the cells were challenged with BH-RSV, RSV(RAV-1) or RSV(RAV-2) respectively.

Results indicated that viruses of agent Z could not be transmitted intracellularly in C/A type cells.

Table 19. Resistance inducing activity of agent Z after a second attempted passage of the factor in C/A type cells (Agent Z_{20}).

Tertiary culture of C/O cells transferred to assay plates*	FFU/entire plate due to challenge of:		
	BH-RSV	RSV(RAV-1)	RSV(RAV-2)
Exposed to the culture fluid plus extract of C/A cells inoculated with the agent (agent Z_{20}) Z-Test	613 (0.7132)[a]	770 (0.7428)	895 (0.7956)
Exposed to the culture fluid plus extract of C/A cells-Metabolite control	860 (1.0000)	1037 (1.0000)	1125 (1.0000)

( )[a] : Fraction of FFU (Test/Control) = Relative sensitivity.

* : The secondary culture of C/O cells was inoculated with agent Z_{20}, and 5 days later, the cells were transferred to assay plates.

Note : All plates only exposed to the test materials but not challenged with viruses did not show any foci.

DISCUSSION

Reduction of FFU of BH-RSV in Mixed Cultures of Chicken Embryo Cells

An obvious reduction of FFU occurred when BH-RSV was grown in a mixture of genetically susceptible chicken embryonic cells (C/O type) and a small number of genetically resistant chicken cells (C/A type). A significant lowering of BH-RSV titer was also found when the virus was titrated in a mixture of two cell types (the virus was exposed to a small number of C/A type cells in assay plates one hour before adding C/O type cells).

To interpret these results several possibilities may be considered under the following headings:

1. Interferon or other inhibitory substances produced by C/A type cells: Gresser and Enders (1962) demonstrated the production of interferon by an established human amnionic cell line (WS cells), which was resistant to Sindbis virus. When this line was mixed with Sindbis virus and susceptible human amnionic cells (HA cells), the HA cells were protected by the interferon. Bader (1962) demonstrated production of interferon by chicken embryo cells exposed to RSV. No attempt was made in Bader's study to determine whether the cell population of an interferon-treated culture consisted of resistant and susceptible cells or only partially resistant cells. More recently, Steck and Rubin (1966) showed that the protection of the chicken embryo cells from the RSV(RAV-1)

challenge is attributed to the intact RAV-1 particles or native subunits of the viral coat but not interferon.

The author's unpublished data showed that the supernatant fluid (centrifugation at 100,000 x g for 2 hours) of culture fluid from C/A type cells exposed to BH-RSV protected C/O type cells from the BH-RSV infection. The nature of the substance is being examined.

Thus, the possibility of production of interferon or other virus growth inhibitory substance by genetically resistant chicken embryo cells exposed to BH-RSV is remained.

2. Latent infection of C/A type cells: No evidence was obtained indicating that C/A type cells used were latently infected with either bacteria, fungi or viruses, which might interfere with BH-RSV, since no growth of such organisms occurred when culturing these cells for long periods at 37°C in appropriate media.

3. Exhaustion of medium by cells: Frequent replacement of medium between BH-RSV inoculation and harvesting of the culture fluids would eliminate this possibility.

4. Effect of C/A type cells on adsorption of BH-RSV: This possibility was examined thoroughly, and it was found that C/A type cells adsorb BH-RSV at a similar efficiency as do C/O type cells. Furthermore, C/A type cells supported the multiplication of RAV-1 which interferes with RSV(RAV-1).

In summary, when referring to the reduction of FFU of BH-RSV in mixed chicken cell cultures, it was concluded

that: (1) The genetically resistant cells (C/A type) adsorb the BH-RSV, thereby reducing the viral population which can infect the susceptible cells (C/O type). (2) C/A type cells inoculated with BH-RSV stock produce RAV-1 which protects C/O type cells from the infection of RSV(RAV-1). (3) The production of interferon or other virus growth inhibitory substance by C/A type cells exposed to BH-RSV is, indeed, another possibility.

Adsorption of BH-RSV or RSV(RAV-1) by C/A Type Chicken Embryo Cells

Rous associated virus type 1 or RAV-1 and RSV(RAV-1) are the major viruses of BH-RSV stock. Minor components, namely RAV-2 and RSV(RAV-2), or RAV-3 and RSV(RAV-3) are also present. Rous associated virus type 1 or RAV-1, RSV(RAV-1), RAV-3 and RSV(RAV-3) belong to subgroup A of avian sarcoma and leukosis complex and RAV-2 and RSV(RAV-2) to subgroup B (Crittenden et al.,1963; 1964; Vogt and Ishizaki, 1965; 1966; Ishizaki and Vogt, 1966).

Highly inbred line 15 I chickens are susceptible to the viruses belonging to subgroups A and B (C/O). Theoretically, three-fourths of line 7 chickens are resistant to subgroup A and susceptible to subgroup B viruses (C/A). One-fourth is resistant against both subgroups A and B (C/AB). The genetic determination of susceptibility is clearly manifest at the embryonic and cellular level, and parallels the strain characteristics observed in young and adult chickens (Crittenden et al., 1963; Vogt and Ishizaki, 1965).

According to the information mentioned above, a low concentration of BH-RSV supposedly contains only RAV-1 and RSV(RAV-1). Therefore, this low concentration of BH-RSV would not be adsorbed by C/A type chicken cells. However, the experimental evidence showed unexpected results which indicate that C/A type cells adsorb the viruses belonging to subgroup A.

A measured amount of BH-RSV was allowed to remain in contact with monolayers of C/A or C/O type cells at 37°C. After a certain period of adsorption, the fluids containing the unadsorbed fraction of BH-RSV were removed from both C/A and C/O type cells. When the unadsorbed BH-RSV fractions were titrated in C/O type cells, no significant differences were found. This indicated that the genetically resistant C/A type cells adsorb the BH-RSV as well as the genetically susceptible C/O type cells. Furthermore, using the same experimental procedure with a pseudotype of BH-RSV, RSV(RAV-1), at 37°C and 5°C the investigator found that C/A and C/O type cells adsorb the virus with similar efficiencies.

Vogt and Ishizaki (1965) indicated that the genetic resistance of chicken embryo cells to avian tumor viruses appeared to introduce a barrier in one of the early steps of infection, namely, adsorption, penetration or uncoating of the virus. The author's data showed that the genetically resistant chicken embryo cells adsorb BH-RSV or RSV(RAV-1). Steck and Rubin (1966) also indicated that primary adsorption of RAV-1 or RSV(RAV-1) occurs at a

normal rate even on genetically resistant cells. However, this unexpected adsorption phenomenon is not known whether it represents a highly specific interaction between RSV (RAV-1) and the host cells or a non-specific one.

Existence of Phenotypically Mixed Rous Associated Virus in BH-RSV or RSV(RAV-1) Stock

When two different viruses infect the same cell some of the progeny exhibit phenotypic mixing: that is, the genome of one parent may be enclosed in a coat determined, at least in part, by the genome of the other parent. Thus the phenotype may not correspond to the genotype. This anomaly disappears after one cycle of growth, if further mixed infections are avoided. The phenotypic mixing was first detected in a bacteriophage (Novick and Szilard, 1951). This bacteriophage was phenotypically an E. coli phage T4 but genotypically a phage T2. Phenotypic mixing has subsequently been observed in polioviruses (Ledinko and Hirst, 1961), myxoviruses: Newcastle disease virus (Granoff, 1959; 1962), enteroviruses: poliovirus and Coxsackie B_1 virus (Cords and Holland, 1964a, 1964b; Holland and Cords, 1964), and group A arboviruses: Sindbis virus, western equine encephalitis virus and eastern equine encephalitis virus (Burge and Pfefferkorn, 1966). It was demonstrated that phenotypically mixed Rous associated viruses existed in the BH-RSV or its pseudotype, RSV(RAV-1) stock.

Culture fluid having free viruses of the C/A type cells (prepared by pooling 3 to 5 ten-day-old chicken embryos) inoculated with BH-RSV was designated as agent X.

Agent X illustrated that: (1) This contained RAV-1 as well as RAV-2. Tertiary culture of C/O type cells derived from the secondary culture inoculated with agent X showed strong resistance against BH-RSV, RSV(RAV-1) or RSV(RAV-2) challenge. The seed BH-RSV stock contained a phenotypically mixed virus, i.e., RAV-1 genome enveloped in RAV-2 protein coat, at least in part. This virus penetrated C/A type cells with the aid of RAV-2 protein coat, and RAV-1 genome produced its own progeny, i.e., RAV-1 or RAV-1(RAV-1). (2) A neutralization test proved that agent X contains RAV-1 as the major fraction, and that the agent X contains another phenotypically mixed virus, i.e., RAV-1(RAV-2). When C/A type cells were inoculated with BH-RSV, RAV-1(RAV-2) and RAV-2(RAV-2), which existed in the seed stock, infected the same cell and RAV-1(RAV-2) was produced. Concurrently, another virus, RAV-2(RAV-1), could be made to appear in agent X. (3) Experiments showed that the most viruses present in agent X were enveloped in RAV-1 protein coat, i.e., RAV-1(RAV-1) and RAV-2(RAV-1). When C/O type cells were inoculated with the culture fluid of C/O type cells infected with the agent X (agent X_{11}), the subsequent passage of the cells became resistant against BH-RSV, RSV(RAV-1) and RSV(RAV-2) challenges. However, C/O type cells inoculated with the second passage fluid of

BH-RSV in C/A type cells (agent X_{20}) did not show interference with the challenge viruses.

Since there was unexpected evidence indicating RAV-1, a subgroup A virus, multiplied in the genetically resistant C/A type cells, an attempt was made to infect the C/A type cells with RSV(RAV-1). The culture fluid of C/A type cells (a pool of line 7 chicken embryo cells) inoculated with RSV(RAV-1) stock was harvested on the 5th day following infection. Culture fluid having free viruses (agent Y) showed results similar to those of agent X, for example: (1) Agent Y contains strong RAV-1 activity and some RAV-2 activity. (2) Neutralization test also proves that agent Y is composed mostly of RAV-1. (3) Agent Y can be transmissible in C/O type cells without loosing the RAV-1 and RAV-2 activities. However, agent Y does not infect the C/A cells repeatedly. (4) The results indicated above were not quantitatively similar among 3 experiments (Table 12, Experiments 287, 322 and 401), e.g., experiment 322 shows less effective results. The differences are attributed to differences in the cell population of heterogeneous line 7 chicken embryos. In addition, another experiment shows that (Table 16): (5) When RSV(RAV-1) stock is neutralized with anti-RSV (RAV-2) serum before inoculation of C/A type cells for the preparation of agent Y, the culture fluid shows no RAV-1 or RAV-2 activity. Result indicates that the viruses having RAV-2 coat protein are needed to penetrate C/A type cells.

Since the line 7 chicken embryos are not homogeneous, and the experiments discussed above revealed the quantitative differences among 3 experiments, 15 individual line 7 embryos were examined. Free viruses in the culture fluids of line 7 cells (prepared from individual embryos) inoculated with RSV(RAV-1) stock (agent Z) was examined. Twelve out of 15 individual embryos (Table 17, group I) was C/A type. The agent Z prepared from these embryonic cells demonstrated that: (1) Agent Z has strong RAV-1 activity and some RAV-2 activity, namely intracellular resistance inducing activity. (2) A neutralization test demonstrates that agent Z is composed primarily of RAV-1. In addition agent Z contains a low concentration of RAV-1(RAV-2) virus. (3) Viruses in agent Z can not multiply successively in C/A type cells. These results coincide with the agent X or Y discussed above. The results are quite similar to those reported by Cords and Holland (1964). They observed that poliovirus RNA enclosed in capsid protein of Coxsackie B_1 virus was able to undergo a single cycle of replication in mouse cells which were not susceptible to poliovirus.

Three of the 15 embryos was C/AB type (Table 17, group II). The genotype of line 7 embryos is homozygous resistant to subgroup A virus infection and heterozygous susceptible to subgroup B virus infection, i.e., aa Bb. Susceptibility is dominant over resistance. Therefore, the mating of line 7 chickens, aa Bb x aa Bb, will give progeny with aa BB: 2 aa Bb: aa bb genotypes. Thus, the

phenotypic expression of the first 3 out of 4 is C/A, i.e., resistant against subgroup A but susceptible to B viruses. The last one is C/AB, i.e., resistant against both subgroups A and B viruses (Stone, H., 1967). These theoretical explanations coincided with the author's findings.

Agent Z prepared from 3 of 15 embryos (Table 17, group II) revealed negligible or negative RAV-1 and RAV-2 activities. Using the results of group I and II of Table 17, conclusion can be made indicating that only embryo cells having the receptor for subgroup B (C/A type) show RAV-1 and RAV-2 activities. C/AB type embryos (Table 17, group II) do not demonstrate these activities. This was strong evidence that RAV-1 contained in agent X, Y or Z must have originated from a phenotypically mixed virus. The virus must have been enclosed by RAV-2 protein coat (at least in part), i.e., RAV-1(RAV-2) and/or RAV-1(RAV-2 plus other). This phenotypically mixed virus was present in BH-RSV or RSV(RAV-1) stocks used for preparation of X, Y or Z. Since agent X, Y or Z also revealed RAV-2 activity, the seed inoculum, BH-RSV stock for agent X or RSV(RAV-1) stock for agent Y or Z was assumed to contain RAV-2(RAV-2) and/or RAV-2(RAV-2 plus other) as a minor group.

This series of investigations demonstrated the presence of phenotypically mixed virus: RAV-1(RAV-2) and/or RAV-1(RAV-2 plus other) in BH-RSV or in RSV(RAV-1) stock. The author's unpublished data also showed the

presence of phenotypically mixed viruses in a RSV(RAV-2) stock. When C/A type cells were inoculated with the RSV(RAV-2) stock, the culture fluid demonstrated RAV-1 activity in addition to RAV-2 activity.

Origin of the phenotypically mixed virus existing in BH-RSV stock, i.e., subgroup A RAV genome enveloped in subgroup B RAV coat or vice versa, must have been as follow: in the process of BH-RSV preparation in young C/O type chicken, two viruses, RAV-1 and RAV-2, infected the same cell and their progeny could have exhibited phenotypic mixing. The possibility remained that the genome of the phenotypically mixed virus originated from RAV-3, but unlikely since RAV-3 is an extremely minor fraction of BH-RSV when compared with RAV-1 or RAV-2.

There are theoretical possibilities that BH-RSV stock contains more phenotypically mixed viruses other than RAV-1(RAV-2), i.e., RAV-2(RAV-1), RAV-2(RAV-3) and RAV-3(RAV-2).

The origin of the phenotypically mixed viruses present in the RSV(RAV-1) stock must be attributed to the RAV-1 stock which was used for activation of NP cells to produce infectious RSV(RAV-1). This RAV-1 stock probably contained RAV-2(RAV-1) in addition to RAV-1(RAV-1). When NP cells, derived from the C/O type cells infected with BH-RSV, was activated by the RAV-1 stock containing RAV-1(RAV-1) and RAV-2(RAV-1), the following viral particles were synthesized: RAV-1(RAV-1), RSV(RAV-1), RAV-2(RAV-2), RSV(RAV-2), RAV-1(RAV-2), RAV-2(RAV-1), RSV(RAV-1 plus

RAV-2), RAV-1(RAV-1 plus RAV-2) and RAV-2(RAV-1 plus RAV-2). The underlined phenotypically mixed viruses infected the C/A type cells and the culture fluid revealed RAV-1 and RAV-2 activities.

The culture fluids of C/O type cells exposed to agent X or Y also demonstrated RAV-2 activity in addition to RAV-1 activity (Tables 11 and 15) while the similarly treated C/A type cell culture fluid did not. To account for these facts, two assumptions were made: (1) RAV-2 (RAV-2) existing in the agent X or Y was an extremely minor fraction when compared with RAV-1(RAV-1); (2) most of RAV-2 was enclosed in RAV-1 coat protein, namely RAV-2(RAV-1), which is produced by phenotypic mixing when C/A type cells are inoculated with BH-RSV or RSV (RAV-1) stock.

Avian tumor viruses belonging to the same subgroup strongly interfere with each other (Hanafusa et al., 1964b; Vogt and Ishizaki, 1966), and this interference would minimize the chance for multiple infections of the same cell required to produce phenotypically mixed progeny. Therefore, phenotypic mixing among avian tumor viruses occurs more frequently between viruses of different subgroups than between viruses belonging to the same subgroup.

The available data do not rule out the existence of heterozygotes and/or recombinants of both subgroups A and B viruses in BH-RSV or RSV(RAV-1) stocks. However,

the experimental results showed that the C/A type cells supported only a single cycle of growth of RAV-1, that is, the phenotypic expression (possessing of RAV-2 coat protein) was unstable. Protein coat of RAV-1(RAV-2) was lost by a single passage in C/A type cell. Therefore, the data favor phenotypic mixing rather than possibilities of presence of heterozygotes and/or recombinants.

The phenotypic mixing has been observed as a very common phenomenon. The progeny from most of the mixedly infected cells with poliovirus types 1 and 2 showed a high degree of phenotypic mixing (frequently 100%), and in some of these cells there was no tangible evidence for the presence of one of the infecting genotypes (Ledinko and Hirst, 1961). Mixed infections of chicken embryo cells with mutants of two strains of Newcastle disease virus at low multiplicities yielded five to ten times more phenotypically mixed viruses as could be accounted for by the number of cells expected to cause mixed infection by two plaque-forming particles (Granoff, 1959).

Phenotypic mixing resulted in the expansion of host range of viruses. As discussed above, RAV-1(RAV-1) does not infect C/A type cell, but when in a phenotypically mixed state, i.e., RAV-1(RAV-2) or RAV-1(RAV-2 plus other), penetrate the C/A type cell and produce additional RAV-1(RAV-1). Although phenotypic mixing occurred between serologically related viruses, it is envisioned that

an avian tumor virus genome can be enclosed in a mammalian virus capsid protein and that this phenotypically mixed virus could cross a species barrier.

SUMMARY

1. When BH-RSV was grown in a mixture of 1 x 10^6 of C/O and 2 x 10^5 of C/A type chicken embryo cells, the culture fluid contained between 27.3 and 45.9% of the virus forming the foci in the controls (BH-RSV grown in 1 x 10^6 of C/O type cells).

2. When BH-RSV was titrated in pure or mixed cells of C/O and C/A type chicken embryos, the titer was lowered to 29% of the control assay plates, if 2 x 10^5 of C/A type cells were inoculated with the virus one hour prior to the addition of 1 x 10^6 of C/O type cells.

3. There was evidence that C/A type cells adsorbed BH-RSV or RSV(RAV-1) stock at 37°C. The unadsorbed fractions of input BH-RSV by C/A type cells during the 90 minute adsorption period at 37°C were between 71.4 and 48.4% of the input virus, while those fractions by C/O type cells were between 55.3 and 45.3%.

The unadsorbed fraction of input RSV(RAV-1) by C/A or C/O cells for 90 minutes at 37°C was 59.8 or 52.4% of control respectively.

4. C/A type cells also adsorbed RSV(RAV-1) at 5°C as did C/O type cells. The unadsorbed fraction of input RSV(RAV-1) by C/A or C/O type cells for a 60 minute period at 5°C was 29.6 or 26.4% of the control respectively.

5. Evidence for the existence of phenotypically mixed Rous associated viruses in BH-RSV stock was demonstrated. The culture fluid of line 7 chicken

embryo cells, prepared from a pool of 3 to 5 ten-day-old embryos, inoculated with BH-RSV was designated as agent X and revealed the following properties:

a) Tertiary culture of C/O type cells derived from the secondary culture inoculated with agent X interfered strongly with BH-RSV or RSV(RAV-1) challenge, i.e., more than 99% foci reduction (less than 0.01 of relative sensitivity). This indicated that the BH-RSV stock contained phenotypically mixed virus, e.g., RAV-1(RAV-2) and/or RAV-1(RAV-2 plus other). This virus penetrated C/A type cells with the aid of RAV-2 protein coat, and RAV-1 genome produced homologous progeny, i.e., RAV-1 (RAV-1).

The tertiary culture of C/O cells also interfered with RSV(RAV-2) challenge, i.e., 83.7% foci reduction (0.1635 of relative sensitivity), possibly caused by the presence of RAV-2(RAV-2) or RAV-2(RAV-1) in agent X.

b) Neutralization test indicated that agent X contained RAV-1(RAV-1) as a major fraction and RAV-1 (RAV-2) as a minor fraction.

c) Viruses in agent X multiplied in C/O type cells. RAV-1 was released in the culture fluid only by C/O type cells. Two groups of C/O type cells were prepared in assay plates: Each plate of the first group was inoculated with one ml of the culture fluid of C/O type cells which had been previously infected with agent X (agent X_{11}), and the second group was inoculated with the second passage of BH-RSV in C/A

type cells (agent X_{20}). After 2 days incubation, the assay plates of both groups were challenged with BH-RSV. The first and second groups had 0.0389 and 0.9437 of relative sensitivities respectively (Table 10) demonstrating that most viruses in agent X were enveloped in RAV-1 protein coats that could penetrate C/O type cells and not C/A type cells.

d) The culture fluids of C/O type cells inoculated with agent X (agent X_{11}) again could have been transmissible intracellularly in C/O type cells. The subsequent passage of cells become strongly resistant against BH-RSV or RSV(RAV-1) challenge. The cells revealed fairly marked resistance against RSV(RAV-2) challenge (0.3592 of relative sensitivity) as shown in Table 11. However, the culture fluid of C/A type cells inoculated with agent X (agent X_{20}) showed no evidence for virus multiplication intracellularly.

These results (5, a - d) supported the idea that BH-RSV contained abnormal viruses, so-called phenotypically mixed viruses, i.e., RAV-1(RAV-2) and/or RAV-1(RAV-2 and other) in addition to normal viruses (genome and protein coat from a homologous virus).

6. Evidence for the existence of phenotypically mixed Rous associated viruses in RSV(RAV-1) stock was demonstrated. The culture fluid of line 7 chicken embryo cells (prepared from a pool of 3 to 5 ten-day-old embryos) inoculated with RSV(RAV-1) stock was designated as agent Y and revealed the following properties:

a) Viruses in agent Y could be transmissible intracellularly in C/O type cells and the subsequent passage of cells showed strong interference with BH-RSV or RSV(RAV-1) challenge (less than 0.01 of relative sensitivity in 2 experiments out of 3, Table 12), and some interference with RSV(RAV-2) challenge.

b) Differences of agent Y's resistance inducing activity (RIF activity) among 3 different experiments must be attributed to the number of C/A cells in line 7 chicken cell population used for the preparation of agent Y. One of 3 experiments showed less effectiveness (No. 322 in Table 12), however, the RIF activity could be increased by passing C/O type cells repeatedly as shown in Table 13.

c) Neutralization test indicated that the virus in agent Y was RAV-1(RAV-1).

d) The culture fluids of C/O type cells inoculated with agent Y (agent Y_{11}) could be again transmissible intracellularly in C/O type cells and the subsequent passage cells became resistant strongly against BH-RSV or RSV(RAV-1). The cells revealed fairly marked resistance against RSV(RAV-2) challenge (0.4980 of relative sensitivity). However, the culture fluids of C/A type cells inoculated with agent Y (agent Y_{20}) did not show any indication of virus multiplication.

e) When C/A type cells were inoculated with RSV(RAV-1) stock neutralized with anti-RSV(RAV-2) serum, the culture fluid demonstrated no viral activity when

inoculated in C/O type cells, and the subsequent passage of the C/O cells showed no resistance against BH-RSV, RSV(RAV-1) or RSV(RAV-2) challenge, i.e., 0.8708, 0.9605 or 1.0251 of relative sensitivity respectively, as shown in Table 16.

The preceding data (6, a - e) demonstrated that RSV(RAV-1) stock contained some phenotypically mixed viruses, i.e., RAV-1(RAV-2) and/or RAV-1(RAV-2 plus other).

7. Evidence was presented that showed only C/A type cells could be infected with RAV-1(RAV-2) existing in the RSV(RAV-1) stock. Culture fluids of 15 individual line 7 embryo cells infected with RSV(RAV-1) stock, designated as agent Z, showed the following properties:

a) Viruses in the agent Z, prepared from C/A type embryonic cells (12 of 15 individual embryos), were transmissible intracellularly in C/O type cells. Subsequent passage of these cells showed strong interference against BH-RSV or RSV(RAV-1) challenge (less than 0.04 of relative sensitivity in average) and against RSV(RAV-2) challenge (0.3818 of relative sensitivity in average) as shown in Table 17, group I.

The virus was identified as RAV-1(RAV-1) the major constituent (by neutralization test).

Agent Z was successively transmissible intracellularly only in C/O type cells.

The culture fluid of C/A type cells inoculated with agent Z (the second passage of RSV(RAV-1) in C/A

type cells: agent Z_{20}) did not show any evidence for virus multiplication. Tertiary culture of C/O type cells, derived from the secondary culture inoculated with agent Z_{20}, showed 0.7132, 0.7428 or 0.7956 of relative sensitivity when the cells were challenged with BH-RSV, RSV(RAV-1) or RSV(RAV-2) respectively.

b) Three of the 15 individual embryo cells having C/AB type phenotypic expression could not be infected with RSV(RAV-1) stock. The culture fluids showed negligible or negative RAV-1 or RAV-2 actively as shown in Table 17, group II.

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