

A STUDY OF THE AEROSOL TRANSMISSION OF
FRIEND AND RAUSCHER VIRUS LEUKEMIAS

by

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ABSTRACT

Young adult BALB/c mice were exposed to aerosols of Friend and Rauscher viruses. Ten-fold dilutions, 10^0 to 10^{-3} of the spray suspensions were used and the exposure interval varied between 5-30 minutes. The temperature and relative humidity remained constant throughout the aerosolizations at (25°C) and (80 per cent) respectively. The period of observation extended over 325 days. Spleen weight and microscopic examination of spleen sections were used to determine disease production. Forty animals exposed to Friend and Rauscher virus aerosols, were later challenged with active virus suspension by intraperitoneal injections in an effort to detect the presence of neutralizing antibodies. Hematocrit determinations and differential counts were performed on ten of the animals exposed to Friend virus aerosols.

These studies indicate the following: (1) the transmission of Friend and Rauscher virus leukemia by the aerosol route was not accomplished, (2) a detectable neutralizing antibody response was not observed, (3) both Friend and Rauscher viruses are extremely sensitive to the aerosolization process, and (4) the finding of low hematocrit values as well as the presence of Friend cells in the peripheral blood suggest a sub-clinical form of the disease.

INTRODUCTION

During the early part of this century, numerous references were made suggesting a possible viral etiology of human leukemia. These were, for the most part, conjectural, because many attempts to transmit leukemia to lower animals and man were either unsuccessful or not reproducible. Engelbreth-Holm and Frederiksen (12) concluded this from their research efforts. Within the past twenty years, however, inbred lines of mice have been developed in which leukemic strains with a high incidence of leukemia have evolved through the practice of selective breeding. It is an accepted fact that genetic factors play a significant role in murine leukemia. However, infection is directly responsible for the development of murine leukemia and the infectious agent is a virus. In view of these developments in murine leukemia, it can be asked if human leukemia is also virus-induced. A number of significant studies made during the past ten years would suggest an affirmative answer to this question.

The classic discoveries of the avian leukosis complex by Ellerman and Bang (10) of the chicken sarcoma by Rous (44), and of the rabbit papilloma by Shope (49) have aided in formulating the concept that viral agents may play a role in the induction of human neoplasia. The introduction of new techniques and the use of better established and re-evaluated techniques have contributed considerably to (1) our knowledge of the relationship of viruses to neoplasia, (2) the discovery

of the viral origin of leukemia in mice, and (3) the production of tumors in different species of animals.

The research of Gross (20) in 1951 using AK mice, proved the development of murine leukemia from a cell-free filtrate. The observations of Gross were confirmed by Wolley and Small (57) in 1956. Graffi (18) produced leukemia in mice with extracts from malignant mouse tumors. Latarget (32) accelerated the development of leukemia in AK mice by injections of human leukemic tissue extracts and also induced leukemia in strains of mice with a low spontaneous incidence of leukemia. Friend (15) induced leukemia in adult Swiss mice by injecting cell-free filtrates extracted from malignant tumors. Rauscher (43) induced leukemia in BALB/c mice with cell-free filtrates extracted from neoplastic mouse tissues. Stewart (51) produced multiple tumors in mice from virus-infected tissue culture extracts. Human adenoviruses have been shown to produce tumors when injected into hamsters (26). Magrassi (34) and Mas y Magro (35) were able to produce a leukemia-like disease in guinea pigs with tissue extracts from leukemic patients. Bergoltz (1) reported the induction of leukemia in mice with extracts of leukemic tissues obtained from patients dying of acute leukemia.

The murine leukemia viruses can be grown, stored, and used for the production of experimental leukemia in mice, thus providing a useful research approach to the study of the leukemia problem.

Electron microscopy with the thin sectioning and negative staining techniques have provided evidence that a virus is responsible for the development of murine leukemia. Dmochowski (7) in 1957 reported the demonstration of virus-like particles in human leukemic tissue

similar to those seen in murine leukemic tissue. Grist (19) in 1964 reported the observation of virus particles from tissues of human leukemic patients. Murphy (38) reported the isolation of agents from human leukemic tissue and stated that the electron micrographs of these agents were similar to those reported in murine leukemia. Porter (41) showed the occurrence of electron dense particles in human leukemic tissues similar to those observed in murine leukemic tissues. Dmochowski (9) 1964 confirmed his original observations and presented a greater volume of electron micrographic evidence.

Immunological methods have been employed to demonstrate the infectious origin of leukemia. Schwartz (48) reported on the basis of positive agglutination and precipitation reactions using leukemic tissues from AKR mice and an anti-leukemic factor, that the leukemic response of the host represented the result of an infectious agent. Calaresu (5) observed a cross-agglutination reaction of red blood cells among various human leukemias and murine leukemias. He suggested that the agent or agents may be etiologically similar. Dmochowski (7), Negroni (40), Murphy (38), and Prince (42) have reported (1) the observation of virus-like particles, and (2) the isolation of virus-like particles from biopsies of human leukemic tissue. Inman (29) 1964, reported the observation of virus-like particles in tissue culture from human leukemic bone marrow extracts. Stewart (51) reported the induction of leukemia in mice by inoculating mouse tumor tissue culture extracts into these animals.

The occurrence of two phenomena in nature concerning leukemia, namely "clusters" and Burkitt's Lymphoma, add to the evidence in support of the role of viruses in human leukemia. The studies in the provinces of Kenya and Uganda, Africa, by Burkitt (4) showed that in these areas in contrast to the findings elsewhere, malignant lymphoma of the jaw in children is a very common disease. It comprises 51 per cent of the total incidence of cancer in African children. The extremely high frequency, limited geographical distribution, specific age and sex characteristics, anatomical distribution, and abundance of arthropod vectors indicate an environmental influence and suggest a local etiological factor responsible for the transmission of this lymphoma. Higginson (27) reported a similar cancer incidence in the Bantu and "Cape Colored" races of South Africa. Dalldorf (6) observed that this common lymphoma of Africa children has a certain relationship with the acute lymphoblastic leukemia found in American children. The frequencies susceptibility to chemotherapy radiation therapy, age of peak incidence, and sex characteristic are essentially identical. Dalldorf suggests that the difference between the two malignancies lie basically in the incident form of the disease.

The viral concept of leukemia has received support more recently from the increasing frequency occurrence of leukemia in clusters. Although cursory reports appeared as early as 1932, most of the documented clusters have been reported since 1957 (23). The significance of clustering is obvious. There are a limited number of plausible hypotheses for this phenomenon, and a viral etiology seems most likely. Clusters have been reported in ten different locations in the United

States and the most important one was in Niles, Illinois. Eight cases of childhood leukemia were diagnosed in this small Illinois community during the three-year span of 1957 to 1960, as reported by Schwartz (48). All of the children lived in the same neighborhood and all children attended the same school or had siblings that attended that school. Antibody studies revealed that the sera of relatives of these children contained specific anti-leukemic antibodies. The sera of the mother or the sibling closest in age to the leukemic child reacted positively with the highest frequency. The occurrence of antibody in relatives and in laboratory workers exposed to leukemic materials suggest the infectious nature of a leukemogenic agent. (48) Mustacchi (39) reported the clustering effect observed in San Francisco, where a higher incidence of leukemia was found in families with a previous leukemic background and where the economic situation was conducive to infectious disease. He also observed that ethnic differences did not play a role in the incidence of leukemia. However, Knudson (31) was in disagreement, since he observed a definite association of granulocytic leukemia with persons of Spanish-American background. The methods used in the assessment of clustering were subjected to the criticism that the statistical evaluation was not valid. Knox (30) 1964, however, observed that re-analysis of the data by another method suggested clustering. Confirmation was also presented by Meighan (36) 1964, when he reported a cluster of leukemia cases in Oregon. Meighan reported that 258 cases of leukemia were diagnosed within an eleven year period and that there was an excessive number of those cases in which the patients resided

within two miles of each other and had developed the disease within 250 days of each other.

Assuming that the concept of a viral etiology of neoplastic diseases is an acceptable theory, the question to be answered is how the virus is transmitted in nature. It has been reported that healthy animals residing in the same cage with overt leukemic animals over long periods of time fail to develop leukemia. (16) The fact that the murine leukemias can be easily transmitted to healthy animals by various routes of inoculation has also been reported. (15) (43) The experimental transmission of murine leukemias by the aerosol route had not been adequately studied.

During the past century, the validity of the concept of airborne infection has been constantly challenged with the results leading to avowed proponents of its importance in the disease process or its lack of importance. Within the past 25 years, this field has taken on new directions and a broader scope. With the present knowledge of disease in which the airborne route of infection has been substantiated, there exists adequate basis for a theory of airborne infection.

The great epidemiologist William Farr (13) contributed to the confirmation of this hypothesis during the cholera epidemic of 1849 in London, in which he correlated the elevation of areas within the city to the incidence and mortality of cholera. William F. Wells (56), eighty-five years later, developed the concept of the droplet nucleus when he showed that a number of pathogenic organisms could be atomized into an aerosol in which they remained viable for days. Although

many of the epidemiological studies failed, a great deal of knowledge of the basic mechanisms of airborne infection was accumulated. These accumulative data revealed the governing principles and laws involved in airborne infections: (1) the principles of generation of natural and artificial aerosols; (2) the techniques of exposing man and animals to aerosols under experimental conditions; (3) the capacity of routine laboratory procedures to create aerosols, thus infecting laboratory workers; (4) the capacity of infected persons to either contaminate their environment or serve as dangerous carriers; (5) the discernment of portals of entry of infection into the respiratory tract; and (6) the crucial importance of particle size in determining penetration and retention of inhaled particles. The studies of airborne infection utilizing artificial aerosols were limited until Henderson (25) in 1952, designed an apparatus which could be used in the laboratory for studying the effects of airborne agents. Viruses, rickettsiae, bacteria, and fungi have been shown to be transmissible by the aerosol route. Loosli (33) reported that influenza virus could be transmitted by the aerosol route. This was confirmed by Schulman (47), Hood (28), and Hamre (21). Beutler (2) reported a high incidence of infection when irradiated mice were subjected to an aerosol of influenza virus. Gogolak (17) showed that mice could be infected with murine pneumonitis virus by the aerosol route, using a cloud chamber regulated for specific relative humidity and intra-chamber pressures. In 1963, Miller (37) reported that both yellow fever and Rift Valley fever viruses were stable in experimental aerosols. The yellow fever virus appeared to better survive the aerosolization.

process. Both viruses produced characteristic disease in Rhesus monkeys which were exposed to these aerosols. Suptel (53) reported infectivity in mice exposed to aerosols of Cocksackie viruses. Slepishkin (50) and Sulkin (52) reported that accidentally created aerosols have infected many laboratory workers, some fatally.

The leukemogenic Friend and Rauscher viruses and the leukemias they produce have been previously characterized. Friend isolated an infections agent from mice that had been inoculated, when newborn, with cell-free extracts from Ehrlich mouse ascites carcinoma. This virus produces a characteristic leukemia when injected into a susceptible murine host. The disease is characterized by a marked proliferation of immature mononuclear cells which invade the hematopoietic system. These cells can be seen in various stages of mitosis in the tissues they infiltrate. The infected mice develop spleens so enlarged that they are easily detected by abdominal palpation. Terminally, the mice are inactive, anemic, and emaciated. They have elevated white blood cell counts, enlarged hemorrhagic spleens, and massive intraperitoneal hemorrhage. Mice can be infected by the intraperitoneal, intranasal, intracerebral, intramuscular, and subcutaneous routes; however, in the latter four, the onset of the disease is delayed several days. The virus has an RNA component and a diameter of 120mp. It is resistant to massive doses of radiation, active after storage at -70°C for six months, or after lyophilization and storage at room temperature for three months. The infectivity of the virus is destroyed by heating at 56°C for thirty minutes, overnight exposure to ether at 4°C , and by treatment with a 1:2000 dilution of formalin.

While studying the effects of a virus-induced leukemia reported by Schoolman et. al. (46), Rauscher observed that one BALB/c mouse developed a tumor at the site of the intraperitoneal injection of an extract prepared from pools of infected tissue from mice previously inoculated with this viral agent. The tumor was readily transplantable, producing an invasive lymphoblastic disease. This disease is characterized by (1) extreme splenomegaly with marked invasion of the red pulp by erthroblastic cells, (2) enlargement of the liver and lymph nodes with accumulations of nucleated red cells and granulocytes in the respective tissues, and (3) a marked infiltration of spleen, thymus, and lymph nodes by large mononuclear basophilic cells in those mice developing lymphocytic leukemia. Mice are susceptible to the virus by the intraperitoneal, intravenous, intranasal, intracerebral, and subcutaneous route; virus initiates a response through the first two routes, however, much more quickly than through the other routes. Electron micrographs reveal the virus to be bounded by a double-limiting membrane and to have a diameter of 100 mμ. The "nucleoid" of the mature particle measures 55 mμ. The virus is stable at -70°C and 4°C for thirty days; it is active following lyophilization. The infectivity of the virus is destroyed by heating at 56°C for thirty minutes and by overnight exposure to ether at 4°C.

STATEMENT OF PROBLEM

Previous investigations of the aerosol transmissibility of leukemogenic viruses were concerned primarily with the animal to animal contact exposure. The possibility that these neoplastic diseases may be transmitted by the aerosol route has been frequently suggested. Friend and Rauscher viruses are known to be leukemogenic when injected into highly susceptible BALB/c mice. The objective of this investigation was to determine if these viruses could be transmitted to healthy animals by the aerosol route under controlled conditions of temperature and relative humidity and subsequently produce leukemia.

MATERIALS AND METHODS

A. Animals

The animals employed throughout this investigation were inbred BALB/c mice obtained from a colony maintained at the University of Arizona. The original families were derived from the offspring of BALB/c pregnant mice purchased from Simonsen's Laboratories, Gilroy, California. The animals were weaned not earlier than 21 days and not later than 25 days. Males and females were separated and those used in the study were between five and eight weeks of age. The families were fed Purina breeder chow pellets and the Purina Laboratory chow pellets for experimental mice. The animal room was maintained at a room temperature of 70°F and a relative humidity of 10 to 15 per cent.

B. Virus Suspensions

The Friend and Rauscher viruses suspended in BALB/c mouse spleen homogenates were obtained from Dr. Howard A. Fieldsteel, Stanford Research Institute, Menlo Park, California. The vials were shipped in dry ice and arrived in the frozen state. They were quickly thawed by gentle agitation in a 37°C water bath, and 0.2 ml. of the suspension was injected intraperitoneally into each of ten young adult BALB/c mice. The animals were sacrificed 21 days later and the infected tissues harvested. A virus pool was prepared using the following procedure:

- (1) the spleens were removed with sterile instruments.

They were pooled, weighed, and a 20 per cent suspension

prepared with a sucrose stabilizer solution suggested by Bovarnick, Miller, and Snyder (3), and later modified by Fieldsteel (14).

- (2) the whole spleens suspended in sucrose stabilizer solution were homogenized in a Virtis Homogenizer for two minutes, allowed to cool for two minutes and again homogenized for two minutes. The homogenization was performed in an ice bath.
- (3) the homogenate was dispensed into 5 ml. vials and quickly frozen in a dry ice-alcohol bath and stored at -65°C until needed.
- (4) for use, the homogenate was quickly thawed by gentle agitation at 37°C and centrifuged in a refrigerated angle-head centrifuge at 2000 rpm for ten minutes.
- (5) the supernatant was removed and employed as the virus suspension throughout the experimentation.

C. Sucrose Stabilizer Solution

The sucrose stabilizer solution used in the preparation of splenic homogenates was prepared in our laboratory. The constituents were added in succession and the solution filtered through a Hormann asbestos pad filter under positive nitrogen gas pressure. The constituents of a 2X preparation were as follows:

KOH	0.548 g
L-Glutaric acid	1.440 g
K_2HPO_4	2.508 g

KH_2PO_4	1.034 g
Sucrose	150.0 g
Water	QS to 1000 ml

D. Virus Titrations

1. Friend Virus Spray Suspension

The virus suspension was diluted serially, 10^0 to 10^{-6} . The dilutions were carried out in a 4°C ice bath, and 0.2 ml. quantities of each dilution were injected intraperitoneally into each of six normal animals. The mice were observed for 35 days. They were palpated daily, and at the end of the observation period they were sacrificed. The spleens were removed with sterile instruments and placed in 10 per cent formalin. After fixation, the spleens were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. The histological examination was used to verify the gross diagnosis of the disease. The titer of the virus suspension was calculated by the Reed-Muench method (44). To ensure an accurate endpoint, six two-fold dilutions were made from 10^{-4} to $10^{-5.5}$. The dilutions were carried out in a 4°C ice bath, and 0.2 ml. quantities of each dilution were injected intraperitoneally into each of ten animals. The same criteria and method were used to determine the titer. Control animals were divided into two groups, uninoculated and inoculated intraperitoneally with 0.2 ml. sucrose stabilizer.

2. Rauscher Virus Spray Suspension

The same procedure was followed as in (1) above except for the virus employed.

3. Friend Virus Impinger Sample

The impinger collection was diluted to a final dilution of 1:243, utilizing a three-fold dilution scheme. The dilutions were carried out in a 4° ice bath, and 0.2 ml. quantities of each dilution were injected intraperitoneally into each of ten mice. The observation period was 35 days, and the same method was used to determine the titer.

4. Rauscher Virus Impinger Sample

The same procedure was utilized as in (3) above except for the virus employed.

E. Intranasal Instillations

1. Friend Virus

Five animals were anesthetized with ether, and 0.05 ml. of the undiluted virus suspension was instilled by drop-let with a tuberculin syringe into the nasal passages of each animal. The animals were placed in a cage for future observation.

2. Rauscher Virus

The same procedure was utilized as in (1) above with the exception of the virus employed.

F. Aerosol Apparatus

The apparatus consists of a Collison atomizer, dispensing a particle 1μ - 5μ in size, enclosed in an air tight 250 cubic foot safety cabinet with an animal exposure box attached approximately five feet from the atomizer. The aerosol was pulled past the animals by reduced pressure created by a vacuum pump and exhausted through a series of absolute filters into the outside atmosphere. An AGI-30 model impinger, used to sample the aerosol, was placed downstream of the animal. The temperature, relative humidity, and total flow rate were varied to suit the conditions of experiment design. The equipment is pictured in figure 1.

G. Aerosolization Process

1. Series 1-Friend Virus

Eight animals were placed in the exposure box and given a ten-minute air wash, after which an aerosol was generated from a 10^0 dilution of the virus suspension. Exposure time was five minutes. The animals were air washed for ten minutes, removed, and placed in cages. The same procedure was followed for virus dilutions 10^{-1} through 10^{-3} . The fifth aerosolization involved twelve animals, 10^0 dilution of virus suspension, and exposure for thirty minutes. The physical conditions of temperature (25°C), relative humidity (80 per cent), and total flow rate (95 liters per minute) were the same for this series.



Figure 1. Photograph of Aerosol Apparatus

2. Series 1-Rauscher Virus

Nine animals were placed in the exposure box and given a ten minute air wash, after which an aerosol was generated from the virus suspension. Exposure time was fifteen minutes. The animals were air washed for ten minutes, removed, and placed in cages. This procedure was followed for the 10^{-1} and 10^{-3} dilutions of the virus suspension. The third aerosolization involved nine animals, 10^0 dilution of the virus suspension, and five minutes exposure. The fourth aerosolization involved fourteen animals, 10^0 dilution of the virus suspension, and thirty minutes exposure. The physical conditions of temperature (25°C), relative humidity (80 per cent), and total flow rate (24 liters per minute) were the same for this series.

3. Series 2-Friend Virus

Fifty animals were placed in the exposure box and given a ten-minute air wash, after which an aerosol was generated from a 10^0 dilution of virus suspension. Exposure time was thirty minutes. The animals were air washed for ten minutes, removed, and placed in cages. The impinger collection was divided into three portions. Portion one was injected in 0.2 ml. quantities into 24 healthy animals by the intraperitoneal route. Portion two was serially diluted and titrated by animal inoculation. Portion three was utilized in the assay of the physical tracer. The physical conditions of temperature (25°C), relative

humidity (50 per cent), and total flow rate (24 liters per minute) were the same for this series.

4. Series 2-Rauscher Virus

The identical procedure was utilized as in (3) above with the exception of the virus employed.

H. Assay of Physical Tracer

Phenol red indicator and acid hematin were used as physical tracers to, (1) determine the physical amount of virus spray suspension that came in contact with the animals, and (2) calculate the estimated respiratory dose.

1. Phenol red indicator was added to the virus spray suspension in a concentration of 100µg/ml.. Various concentrations of Phenol red in a 0.1 M borate buffer, pH 9.2, were placed in a Beckman DU Spectrophotometer and were read at 540mµ. A standard curve was constructed from this data. Aliquots of the impinger samples were placed in the spectrophotometer, and the values obtained were compared with the standard curve.
2. The spray suspension was serially diluted in N/10 hydrochloric acid, and from the acid hematin content of the spray suspension a standard curve was constructed, utilizing a Beckman DU Spectrophotometer set at 540mµ. Aliquots of the impinger sample were placed in the spectrophotometer, and the values obtained were compared with the standard curve.

I. Animal Challenge

1. Friend Virus

Twenty of the previously exposed animals were divided into two groups of ten animals each. On the 175th day following aerosolization, these animals were challenged with different concentrations of virus to detect the presence of neutralizing antibody. All animals were injected by the intraperitoneal route. Group one received 10 ID50's per 0.2 ml. quantity, and group two received 100 ID50's per 0.2 ml. quantity. Control animals included four groups of six animals each. Groups one through three received one hundred, ten, and one ID50's, respectively; the fourth group were the normal controls. The animals were placed in cages and observed for 35 days. They were palpated daily, and sacrificed at the end of the observation period. The spleens were removed with sterile instruments and weighed. Spleens greater in weight than 0.5 g. were considered positive. The results of each group were compared by means of a contingency table to determine whether a significant difference existed among the test groups.

2. Rauscher Virus

The same procedure was utilized as in (1) above with the exception of the virus employed.

J. Hematocrit Studies

1. On the 300th day following aerosolization, ten animals, aerosolized with Friend virus, were sacrificed by cervical

fracture and the pleural cavity opened. Blood was collected directly from the heart. Hematocrit estimations were performed with Drummond microhematocrit capillary tubes.

The tubes were centrifuged at 7100 rpm for 10 minutes in an IEC table model centrifuge and were read on a microhematocrit tube reader. Blood films were made from heart blood, stained with Wright's stain, and differential counts carried out.

2. Fifteen control animals, ten months of age, were drawn at random from the colony at the University of Arizona.

Hematocrit estimations and differential counts were performed by the same procedure as in (1) above.

RESULTS

Gross Observations

In those animals receiving the Friend and Rauscher viruses by the intraperitoneal route, the disease process was the same as that described by Friend (1957) and Rauscher (1962). The diseases were characterized by a short latent period. The spleen enlarged rapidly and weighed as much as 5.0 g. by the 35th day, in contrast to normal spleen weight of 0.2 g.. Hemorrhagic infraction of the spleen was a common finding.

Microscopic Observations

The spleens and livers in Friend and Rauscher leukemia were microscopically indistinguishable. The diseases were characterized by the formation of nests of reticulum cells originating in the red pulp and spreading to the white pulp of the spleen. In the later stages, massive infiltration of the spleen and liver with neoplastic reticulum cells occurred.

Virus Titrations

1. Friend Virus-Spray Suspension

The number of positive animals was determined on the basis of both gross and microscopic observations. The titer of the virus suspension was calculated by the Reed-Muench method. All spleens weighing more than 0.5 g. or showing

neoplastic activity were considered positive. The titer was calculated to be $10^{5.3}$ per ml. of virus suspension.

2. Rauscher-Virus-Spray Suspension

The same criteria for determining positive leukemic animals as in (1) above were utilized for the Rauscher virus suspension. The titer was calculated to be $10^{4.3}$ per ml. of virus suspension.

3. Friend Virus-Impinger Sample

The same criteria for determining positive animals as in (1) above were utilized. The titer of the impinger sample was calculated to be $10^{1.7}$ per ml. of impinger sample collected.

4. Rauscher Virus-Impinger Sample

The same criteria for determining positive animals as in (1) above were utilized. The titer of the impinger sample calculated to be $10^{1.5}$ per ml. of impinger sample collected.

Intranasal Instillations

1. Friend Virus

The animals inoculated by intranasal instillation were observed for 307 days. During the observation period, spleens considered positive by palpation were aseptically removed and weighed. In cases where splenic infectivity as determined by weight was questionable, the spleens were fixed in 10 per cent formalin, sectioned, stained with hematoxylin and eosin, and examined.

On the 44th day, one mouse was sacrificed. Microscopic examination proved negative. Two animals were found to be positive on the 87th day. One animal died on the 162th day. Necropsy proved negative. The last animal was sacrificed on the 307th day. Necropsy proved negative.

2. Rauscher Virus

The animals instilled intranasally were observed for 307 days. On the 87th day, one mouse was sacrificed and necropsy proved negative. Two animals died on the 163rd day. Necropsy showed one animal to be positive and one animal to be negative. The last two animals were sacrificed on the 307th day. One animal was found to be positive and the other negative.

Aerosols

1. Friend Virus-Series 1

The animals, exposed to aerosols, were observed for 180 days. During the observation period, gross signs of leukemia did not appear in any of the animals. See Table 1. At the end of the observation period, the animals were sacrificed by cervical fracture. Spleens were aseptically removed, placed in 10 per cent formalin, sectioned, stained with hematoxylin and eosin, and examined. Examination revealed no signs of leukemia. Control animals injected intraperitoneally with a 10^0 dilution of the virus

Table 1. Response of Animals Exposed to Friend Virus Aerosols*

aerosol	virus dil. in spray suspension	exposure time in min.	total flow rate in l./min.	infect/ exposed	control** infect/ inject.
1	10^0	5	95	0/8	6/6
2	10^{-1}	5	95	0/8	6/6
3	10^{-2}	5	95	0/8	6/6
4	10^{-3}	5	95	0/8	6/6
5	10^0	30	95	0/12	6/6
6	10^0	30	24	0/50	10/10

* temperature and relative humidity remained constant at 25°C and 80 per cent respectively.

** control animals for each dilution were injected intraperitoneally with 0.2 ml. of virus spray suspension.

suspensions proved 100 per cent infective. The normal control animals remained healthy throughout the observation period.

2. Rauscher Virus-Series 1

The animals, exposed to aerosols, were observed for 100 days. During the observation period, gross signs of leukemia did not appear in any of the animals. See Table 2. Microscopic examination revealed no signs of leukemia. However, the animals injected intraperitoneally with 0.2 ml. of the impinger collection became infected. The latent period was advanced from 12 to 35 days. The control animals injected intraperitoneally with a 10^0 dilution of virus spray suspension proved to be 100 per cent infected. The normal control animals remained healthy throughout the observation period.

3. Friend Virus-Series 2

The animals, exposed to the aerosol, were observed for 320 days. Five animals are still living, and observation of these animals will continue indefinitely. During the observation period, six animals died and were necropsied. There were no gross signs of leukemia.

The animals challenged with virus to detect the presence of possible neutralizing antibodies were sacrificed at the end of the 35 day observation period. Spleens were removed aseptically and weighed. All spleens weighing more than 0.5 g. were considered positive. Eight of ten

Table 2. Response of Animals Exposed to
Rauscher Virus Aerosols*

aerosol	virus dil. in spray suspension	exposure time in min.	total flow rate in l./min.	infect/ exposed	control** infect/ inject.
1	10^{-1}	15	24	0/9	6/6
2	10^{-3}	15	24	0/9	6/6
3	10^0	5	24	0/9	4/6
4	10^0	30	24	0/14	6/6
5	10^0	30	24	0/50	10/10

* temperature and relative humidity remained constant at 25°C and 80 per cent respectively.

** control animals for each dilution were injected intraperitoneally with 0.2 ml. of virus spray suspension.

animals challenged with 100 ID₅₀'s and 10 ID₅₀'s were infected, whereas ten of ten control animals injected intraperitoneally with the same dose of virus were infected. See Table 3. Statistical analysis of the data indicated that there was no significant difference between challenged and control animals.

On the 285th day, nine animals were sacrificed and hematocrit studies performed. The studies of Elliott (11) showed that there is a definite linear association of hematocrit levels and Friend virus infection. As the disease process increased in intensity, the hematocrit values decreased from a normal value of 44 to a twelve-week infection level of 24. The hematocrit values of the animals exposed to the aerosol ranged between 32 and 40, with an average value of 38. The hematocrit values of the control animals, all ten months of age, ranged between 42 and 51, with an average of 46.

At the time the animals were sacrificed, one animal was found to have an enlarged spleen. The spleen weighed 1.01 g. and was homogenized in a Virtis homogenizer. The homogenate was centrifuged at 2000 rpm for ten minutes. Each of nine BALB/c mice was injected intraperitoneally with 0.4 ml. of the supernatant. These animals, at the time of this writing, have been observed for 50 days, and the characteristic signs of leukemia have not appeared. It was also noted that this animal had the lowest hematocrit

value recorded, and a differential count revealed 26 per cent Friend cells. Differential counts on three other animals with lower than normal hematocrit values showed the presence of Friend cells. Splenomegaly was not observed in these animals.

On the 300th day, ten animals were sacrificed and necropsied. There were no gross signs of leukemia. The impinger samples were 100 per cent infective but the latent period was advanced from 12 to 47 days.

4. Rauscher Virus-Series 2

The aerosolized animals were observed for 325 days. Five animals are still living, and observations of these animals will continue indefinitely. During the observation period, four animals died. The animals were necropsied and revealed no gross signs of leukemia. The animals challenged were sacrificed at the end of the observation period. Spleens were removed aseptically and weighed. All spleens weighing more than 0.5 g. were considered positive. Eight of ten animals challenged with 100 ID₅₀'s and seven of ten animals challenged with 10 ID₅₀'s were infected. All of the ten control animals injected intraperitoneally with 100 ID₅₀'s and 10 ID₅₀'s became infected. See Table 4. Statistical analysis of the data revealed that there was no significant difference between challenged and control animals.

On the 300th day, ten animals were sacrificed and necropsied and revealed no gross signs of leukemia.

Table 3. Response of Animals Exposed to Friend Virus Aerosol and Subsequently Challenged.*

ID50's injected	positive aerosol animals/ total animals injected	positive control animals/ total animals injected
100	8/10	6/6
10	8/10	6/6
1	----	3/6

* control and challenged animals were injected intraperitoneally with 0.2 ml. of virus suspension.

Table 4. Response of Animals Exposed to Rauscher Virus Aerosol and Subsequently Challenged.*

ID50's injected	positive aerosol animals/ total animals injected	positive control animals/ total animals injected
100	8/10	6/6
10	7/10	6/6
1	----	4/6

* control and challenged animals were injected intraperitoneally with 0.2 ml. of virus suspension.

Eight animals were sacrificed on the 300th day and three animals, thought to have splenomegaly, were sacrificed on the 325th day. Necropsy of these animals revealed no gross signs of leukemia. Impinger samples were 100 per cent infective; however, the latent period was advanced from 12 to 53 days.

DISCUSSION

With a proven test system for studying leukemogenic viruses in susceptible animals, one would think it possible to transmit the murine virus leukemia by the aerosol route. This would apply particularly, when these viruses are infective for numerous murine hosts and capable of being transmitted by various routes of inoculation. The fact that animal to animal transmission has never been reported for the murine leukemias would appear to negate the possibility of an aerosol route of transmission for these viruses. However, experimentation to date concerned with this aspect is not very well documented. Having evaluated the occurrence of spontaneous leukemia, one would expect an extremely low incidence with an accompanying extended latent period. It is therefore possible that an incidence of leukemia transmitted by animal to animal contact has passed undetected. The acceptance of this possibility suggested further experimentation in accordance with the original hypothesis.

In comparing the titers of spray suspension and impinger samples, it was found that there was a great loss of infectivity. Some loss was to be expected, but the amount of loss experienced surpassed all expectation. The extreme sensitivity of these viruses to the aerosolization process at high relative humidity is not in accordance with the general stability of these viruses and the reports concerning other viruses in the airborne state. Both Friend and Rauscher viruses are

considered relatively stable and in some respects, Friend virus is considered resistant. Webb (55) reported that Rous sarcoma virus is stable at high relative humidity but extremely sensitive at low relative humidity in the airborne state. Hemmes (24) observed that polio viruses are stable at high relative humidity and sensitive at low relative humidity when subjected to aerosols. Watkins (54) observed that vesicular stomatitis virus was stable at high relative humidity. Miller (37) reported that both yellow fever and Rift valley fever viruses are stable at high relative humidity. This sensitivity, or "rate of decay", in aerosol characterization is a highly critical parameter. The decay parameter consists of two components: (1) the rate of physical decay, and (2) the death rate of the organism.

Particle size is another parameter that is closely associated with physical decay. The particle size must be small enough to be maintained in an airborne state. However, virus contained within extremely small particles is subject to desiccation within the aerosol cloud, thereby affecting a high death rate of the organism. A large particle of 12 μ is subject to gravitational forces and random coalescence and may not reach the animals. It must also be noted that the optimum particle size for retention in the lower respiratory tract lies between 1-5 μ . (22) Particles larger than 5 μ have a greater probability of deposition in the upper respiratory tract, nasal passages, thus seldom reach the lungs. The Collison atomizer utilized in our experiments produces particles of 1-5 μ in diameter, a size range optimum for deposition in the lungs. Although the particle size

employed in our experimental situation was of such dimensions, these particles were subjected to the physical factors previously mentioned. Then though the decay rate was greater than anticipated and obviously affected the concentration of virus reaching the animals, the animals were subjected to 1 ID₅₀ during the exposure interval. Analysis of the physical tracer data revealed that 99.9 per cent of Friend virus infectivity and 98.7 per cent of Rauscher virus infectivity was lost during the aerosolization process. However, it can be seen from the impinger sample titration and impinger sample injections that active virus was present and did survive the aerosolization process.

It was also noted that the hematocrit values obtained for three of the animals studied suggested that an infective process was in progress. This is borne out by the presence of Friend cells found in the peripheral blood of three of these animals. It has been generally accepted that the presence of Friend cells in the peripheral blood is indicative of Friend virus infection. However, the ratio of number of Friend cells to degree of infectivity has not been quantitatively established. Nevertheless, it is believed that 10-26 per cent Friend cells do constitute a valid supposition that a Friend virus leukemic process exists. The criteria for determining the presence of Friend virus leukemia, i.e., spleen weight, hematocrit value, and presence of Friend cells, were fulfilled by one of the exposed animals. Admittedly, one of fifty animals is an extremely low incidence. However, it is an incidence that would be expected when taking into account the sensitivity of the virus and the function of latency.

The challenged animals were apparently infected, but no significant difference in protection was found. Friend (16) reported that a classical antibody is formed as a result of injection of a formalin-inactivated virus preparation. This would suggest that an active viral process would also produce an antibody that could be detected by the neutralization test. These results do not necessarily indicate that antibody was not present in the animals challenged, but that the antibody may have been in an insufficient concentration to be detectable by the test employed. Further studies are needed in order to answer this question unequivocally.

SUMMARY

The transmission of Friend and Rauscher virus leukemia by the aerosol route was not accomplished, under the conditions of temperature, relative humidity, exposure interval, and concentration of virus utilized in this study. This is also supported by the absence of a detectable neutralizing antibody in the sera of the animals exposed to aerosols containing the viruses. In addition to the parameters mentioned, the virus titer of the spray suspension and the rate of decay of the virus aerosol must be considered in the evaluation of the experimental results.

Only three of the animals exposed to Friend virus aerosols did not follow the observed pattern. The low hematocrit values and the presence of Friend cells in these three animals suggests that virus survived the aerosol, gained entrance, and initiated an infective process. However, the infectivity of the virus was reduced sufficiently to produce only a sub-clinical form of the disease.

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