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BOVINE LYMPHOCYTIC LEUKEMIA: STUDIES OF
ETIOLOGY, PATHOGENESIS AND MODE OF TRANSMISSION

PROGRESS REPORT NO. 10 TO THE U. S. ATOMIC ENERGY COMMISSION

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Principal Investigator

D. K. Sorensen, Department of Veterinary Medicine

Co-Investigators

S. K. Dutta, Department of Veterinary Medicine
R. F. Hammer, Department of Veterinary Medicine
V. L. Larson, Department of Veterinary Medicine
V. Perman, Department of Veterinary Pathology & Parasitology
R. E. Shope, Department of Veterinary Medicine
A. F. Weber, Department of Veterinary Anatomy

College of Veterinary Medicine
University of Minnesota
St. Paul, Minnesota 55101

April 30, 1970

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I. PERIOD COVERED BY THIS REPORT:

This report covers the period from May 1, 1969 to April 30, 1970.

II. BACKGROUND INFORMATION:

A. Background

On June 1, 1960 a contract between the United States Atomic Energy Commission and the University of Minnesota was negotiated. The initial proposal raised these provocative questions: (1) Is the apparent frequency of occurrence of bovine leukemia in Minnesota real? (2) Is there an increasing prevalence of this disease as is implied in Meat Inspection Reports? (3) Is there a marked geographic variation in the frequency of the occurrence of bovine leukemia, and does it correlate with that noted in human leukemia? (4) Do environmental factors influence the occurrence and distribution of bovine leukemia in this region? (5) Is there a familial relationship in the occurrence of bovine leukemia? These questions were raised at a time when the influence of environmental factors (such as radioactive fallout) were of pressing importance.

At this time, there were no reports of studies conducted in the United States to determine the occurrence and distribution of leukemia in cattle, and no organized attempts had been made to study the relationship of environmental or familial factors to the occurrence of bovine lymphocytic leukemia.

The annual reports of the Meat Inspection Division of the U. S. Department of Agriculture reveal that cattle condemnations attributed to malignant lymphoma showed an apparent increase from 9.7 to 18.1 per 100,000 cattle slaughtered during the years 1952 to 1959. These records further indicate that in 1958 one fifth (18.6%) of all cattle condemnations in the United States were reported from the federally-inspected slaughtering plants in Minnesota and nearby Eau Claire, Wisconsin and further that South St. Paul Meat Inspection Station in 1958 reported 14.0% (464) of the malignant lymphoma condemnations in the United States while slaughtering less than 5.0% of the cattle. Reports for human mortality attributed to leukemia in the United States reveal that Minnesota had one of the highest mortality rates in the nation. Studies of leukemia in man in Minnesota have shown significant geographic variations in the distribution of mortality rates among counties and groups of counties in different areas of the state. Studies to determine the influences of environmental factors on the occurrence and distribution of leukemia in man in Minnesota were in progress.

Because Minnesota had a high mortality attributed to human leukemia and studies on the influence of environmental factors were in progress, it was prudent to initiate studies on the occurrence and distribution of leukemia in cattle and to study the relationship of environmental or familial factors to its occurrence in the state of Minnesota. This was the only comprehensive study of this type being conducted in the United States.

In the United States, extensive studies since 1960 on bovine leukemia have been reported from Minnesota,^{6,7} California,⁸ Michigan⁹ and Pennsylvania¹⁰ with additional reports from other laboratories^{11,12} appearing in more recent years.

The occurrence of bovine lymphocytic leukemia was first described by Leisering in 1871. Since that time many reports of its occurrence including comprehensive studies of the disease have been reported from several countries (see reviews by Bendixen).^{1,2} In Denmark^{1,2} and Germany bovine leukemia is recognized as a major disease problem and eradication and/or control measures have been initiated.⁴ In Germany⁵ and Sweden the incidence of tumor cases has been reported as approximately 1 per cent of slaughtered cattle with higher incidences common at abattoirs in high incidence areas.

The world-wide reports of bovine leukemia provide epidemiologic evidence to indicate that bovine leukemia was a transmissible disease. The pattern of geographic distribution of bovine leukemia in Germany¹³ and Denmark^{1,2} revealed such factors as movement or trade of animals, and vertical transmission from dam to daughter as suggestive evidence for a transmissible agent. The use of a whole blood vaccine for piroplasmosis in southern Sweden was associated with the propagation of leukemia. Results of studies of high incidence herds in Minnesota are consistent with the concept that leukemia transmission is associated with movement of animals from these herds to apparently leukemia free herds.¹⁴

A hypothesis advanced repeatedly by many of these workers is

that horizontal transmission is the major factor in the natural spread of this disease in several countries, while vertical transmission either transplacental or through the mother's milk may be contributory.

Early German workers were the first to observe that a lymphocytosis in apparently normal cattle was a feature of high incidence herds. Furthermore, Danish investigators observed in retrospective studies that 90 per cent or more of adult leukemia cases had a persistent lymphocytosis prior to the development of the tumorous phase of the disease.^{1,2} Thus, many investigators in European countries equate persistent lymphocytosis on a herd basis with the tumor phase of the disease. Studies of lymphocytotic animals in high incidence herds indicate that approximately 5 to 10% of these animals develop tumors. In addition, our studies indicate that many lymphocytotic animals are slaughtered for economic reasons¹⁴ prior to entry into high risk years.

The role of hereditary factors in bovine leukemia has been studied by many workers in this country and in Europe.^{1,2,6,10,14}

The Pennsylvania group strongly insists that heredity has a major role in the natural occurrence of lymphocytosis and tumor in high incidence herds.¹⁰ A more plausible explanation of this type of data is vertical transmission, either transplacental or in the milk, of an infectious agent.

Our studies in Minnesota performed under AEC contracts support the epidemiologic evidence for horizontal or vertical transmission

of leukemia by an infectious agent.^{14,16} We have demonstrated that the incidence of leukemia increases markedly with increased age of animals.¹⁵ Further, a marked increase in incidence is associated with increased herd size.¹⁵

The cohort analysis studies lend highly supportive data to the hypothesis of horizontal and vertical transmission for an infectious agent as the major factor in the natural transmission of bovine leukemia.^{14,16}

Successful attempts to transmit bovine leukemia have been reported from Germany and Sweden.^{13,17,18} These transmission studies as well as epidemiologic data indicate a long latency period of at least 2 to 5 years with increasing incidence in older animal populations. Evidence for a viral etiology was strongly suggested by these previous studies and knowledge from comparative studies of leukemia in other animals.

Several laboratories have attempted to isolate and/or define virus particles from lymphocytosis or leukemic animals (see Progress Report No. 9). In addition, successful transplantation of bovine leukemia to young calves following total body gamma irradiation or antilymphocyte serum has been accomplished by the Pennsylvania group.

During the past year, in 1969, a major breakthrough occurred in regards to the viral etiology of bovine leukemia. It appeared that the hypothesis of a viral etiology of bovine leukemia was closer to being confirmed.¹¹ Malmquist et al. from Iowa, reported in January of 1969 on the isolation of a syncytial virus from lymphosarcomatous

and apparently normal cattle. They also reported on the development of immunodiffusion and immunofluorescence tests for this virus. In December of 1969 Miller et al.²² of Wisconsin reported on the demonstration of C-type virus particles in phytohemagglutinin stimulated lymphocyte cultures obtained from cows with lymphosarcoma (leukemia) or from cattle that had been inoculated with lymphosarcoma material. During 1969 our own research group independently also demonstrated C-type virus particles in phytohemagglutinin stimulated lymphocyte cultures from leukemic cattle and cattle with a persistent lymphocytosis.²³ The relationship of bovine syncytial virus to bovine leukemia remains to be determined. The relationship of C-type virus to bovine leukemia appears to be better associated with disease based upon the preliminary studies conducted by the Minnesota and Wisconsin research groups than does the syncytial virus.

These preliminary studies show that C-type virus particles can consistently be demonstrated from leukemic and lymphocytotic animals and not from non-lymphocytotic or apparently normal animals.

The relationship of bovine syncytial virus and C-type particles in PHA-stimulated lymphocyte cultures remains to be determined. It seems unlikely that they are identical based upon morphological characteristics.

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B. General Information

The approach to the study and the procedures used are described in some detail in Progress Reports 1 and 9 to the U. S. Atomic Energy Commission [see Contract AT (11-1)-910], and will not be repeated in this report. Additional procedures initiated are described in the various sections.

A number of terms need definition in the general introduction as they will be used in various sections of this report

1. Juvenile leukemia - a consistent clinical form of leukemia occurring in young animals usually under 6 months of age but up to one year.
2. Thymic-lymphosarcoma - a consistent clinical form of leukemia occurring in young animals from 6 months to 2½ years of age, approximately.
3. Adult leukemia - leukemia with variable clinical and pathological manifestations occurring in animals usually over 2 years of age. The adult type typified the type of leukemia found in multiple case herds.
4. Multiple case herd data refers to data obtained from herds where more than one case of leukemia (adult type) has occurred in one year.
5. Lymphocytosis - referred to as a preclinical phase of the tumor form of bovine leukemia. The lymphocytosis is characterized by an elevation of lymphocyte count above 3 standard deviations greater than the normal count for animals for that age. The lymphocytes consist of a morphologically normal

population of cells and are found in normal animals in leukemia herds. Counts to 30,000 or more in a number of animals (mean 12.5%) are usual. The lymphocytosis is not associated with any demonstrable pathologic process or tumor growth and appears somewhat analagous to the early stages of chronic lymphocytic leukemia of man.

C. Scientific Scope and Objectives of the Study Including Modifications

The initial contract to study bovine lymphocytic leukemia was negotiated June 1, 1960. This project proposed to study the occurrence and distribution of bovine lymphocytic leukemia in Minnesota and adjacent areas, with particular reference to the investigation of environmental and familial factors. This project included clinical, hematologic and pathologic studies to provide definitive diagnoses and to characterize and relate the morphological types with the occurrence and distribution of the disease.

The specific aims of these studies (1960) were:

1. To study the frequency of occurrence and the geographic distribution of bovine lymphocytic leukemia in Minnesota and adjacent areas of surrounding states:
 - a. to obtain information regarding incidence and prevalence in relation to age, sex, breed, familial relationships and the movement of animals from one population group (herd) to another;
 - b. to evaluate within the limitations of reporting any changes in incidence during the next several years, in

relation to the "apparent" increase reported by the U. S. Department of Agriculture Meat Inspection Division records and a sample of practicing veterinarians in Minnesota;

- c. to determine if there is any statistical relationship between selected environmental factors and the frequency of occurrence and distribution of bovine leukemia, particularly in herds where multiple cases occur, and emphasis will be given to herd management practices and history of previous disease, soil types and available geologic information, and the use of agricultural and other chemicals which may be known or suspected carcinogens.
2. Clinical, hematologic and pathologic studies will be made:
- a. to establish a definitive diagnosis for the cases of the disease as they occur and are reported in Minnesota and adjacent study areas;
 - b. to ascertain whether lymphocytic leukemia can be classified into acute and chronic courses, and if any relationship exists between the course of the disease and the age of the animal;
 - c. to obtain more definitive and complete information regarding the manifestations of spontaneously occurring bovine lymphocytic leukemia by detailed study of a limited number of selected herds and cases where multiple cases of the disease occur.

The above were the initial objectives of the research project.

Essentially there was no major deviation from these objectives during the first five years of study. We did, however, change our procedures somewhat and broaden our efforts in certain areas. These changes of procedure include the use of the electron microscope in the pathologic studies and the initiation of hematologic studies in selected cattle populations to better define the role of a lymphocytosis in bovine leukemia.

The change of procedure and the additional studies initiated were (sixth renewal request, 1965):

1. It was proposed to complete the studies on the occurrence and distribution of bovine lymphocytic leukemia in Minnesota and the adjacent areas:
 - a. to do limited retrospective studies on socio-economic factors that may influence the reporting of leukemia and effect the data obtained on incidence and rates as determined by age, herd size, breed distribution and geographic distribution;
 - b. to obtain data on environmental factors on the selected normal herd for comparison to the single case herds;
 - c. to complete the statistical analysis and evaluation of accumulated data on occurrence and distribution and the role of environmental factors in bovine lymphocytic leukemia.
2. It was proposed to continue limited studies on multiple case herds:

- a. to investigate the relationship between lymphocytosis and the occurrence and course of new cases of leukemia in the herds;
 - b. to analyze and evaluate the data on the role of genetic factors as they may influence lymphocytosis and/or tumor development.
3. It was proposed to obtain additional hematologic data on selected normal herds and single case herds:
- a. to complete base line parameters for the evaluation of single case herds characterized by leukemia occurring in young animals and adult animals;
 - b. to complete the comparison studies of the single case herds to multiple case herds.
4. It was proposed to shift the emphasis of the intensive clinical, hematologic, biochemical and pathologic studies on leukemic animals to animals with a marked lymphocytosis (pretumor phase):
- a. to determine the relationship of lymphocytosis to the tumor phase of bovine leukemia;
 - b. to determine the eventual natural outcome of animals with a marked lymphocytosis (pretumor phase) by intensive clinical and pathologic studies;
 - c. to attempt to increase the incidence of tumor formation in lymphocytotic animals by various known promoter factors.

In 1966 our leukemia research team requested a review and

critical evaluation by a review team. This evaluation was requested because it was our thought that some of the objectives of our research had been met. This was particularly true of certain aspects of the studies on occurrence and distribution and on the relationship of environmental factors to the occurrence and distribution of leukemia. It seemed apparent that sufficient data had been collected to fulfill the objectives of the epidemiologic studies and that statistical analysis of the data should be completed. Further, it was suggested that we increase our efforts on determining the significance of lymphocytosis and initiate studies on the transmission of bovine leukemia.

The review team was composed of Dr. Donald Anderson, Richard Barnes, Douglas Grahn, Leo Whitehair and Bibbs.

The initial objectives of this study were modified in consultation with the review team. A brief summary of these changes in objectives follows:

1. To complete the studies on the occurrence and distribution of bovine leukemia and the possible influence of environmental factors. Since the Atomic Energy Commission site visit committee posed the question of the role of socio-economic factors on the reporting of bovine leukemia, it was deemed necessary to initiate studies on the role of socio-economic influence on the reporting of bovine leukemia to validate data on the occurrence and distribution.
2. To continue limited studies on multiple case herds to determine the relationship between lymphocytosis and tumor cases and to evaluate the role of genetic factors.

3. To obtain additional data on selected normal herds and single case herds to complete base line parameters for the evaluation of the single case and multiple case herds.
4. To shift the emphasis of the intensive clinical, hematologic, biochemical and pathologic studies on leukemic animals to animals with a marked lymphocytosis to determine the natural outcome of lymphocytotic animals, to attempt to increase the incidence of tumor formation, to determine the nature and significance of ultrastructural differences in bovine lymphocyte cells of normal, preleukemic and leukemic animals.
5. Following suggestions of the Atomic Energy Commission site visit committee to initiate preliminary studies on attempts of transmission of bovine leukemia.

In 1968 we received a critical evaluation by a U. S. Atomic Energy Commission outside review team. The review team consisted of Drs. N. P. Page, F. T. Brooks, R. W. Touchberry, R. R. Marshak, and G. H. Theilen. On the basis of this review and our own opinions a number of changes were made. The scope of the project was narrowed down to include only two areas of research. These were virologic and transmission studies and studies on high incidence and control herds. The title of the project was changed to "Bovine Lymphocytic Leukemia: Studies of Etiology, Pathogenesis and Mode of Transmission" to more clearly identify the scope of the project.

Following are the specific research objectives of the 1969-70 contract year:

1. Virologic and Serologic Studies

- a. attempt to infect bovine and other species' cell lines with C-type virus;
- b. determine the physical and chemical characteristics of the C-type virus;
- c. develop and utilize serologic tests to determine extent of infection of C-type virus in selected dairy cattle herds;
- d. attempt to determine the biological and immunological relationship between the syncytial and the C-type virus;
- e. attempt to determine the relationship of a helper virus utilizing a recently established bovine sarcoma-fibroma cell line and the C-type virus.

2. Transmission Studies:

- a. concentrate C-type virus particles for transmission studies by differential centrifugation;
- b. to continue transmission studies by administration of a defined cell free, virus rich, inoculum to 2-3 month fetuses and colostrum deprived newborn female calves;
- c. to adapt monitoring techniques such as virus recovery, immunodiffusion, immunofluorescence or other methods or parameters of infectivity in inoculated animals.

3. High Incidence Herd Studies:

- a. to obtain additional data on the natural outcome of lymphocytotic and non-lymphocytotic animals;
- b. to provide a genetically defined source of animals for virus isolation studies;

- c. to continue to study the neonatal temporal-spatial relationships between leukemic, lymphocytotic and non-lymphocytotic animals;
- d. to augment our data on the role of genetic factors in the occurrence of lymphocytosis and leukemia;
- e. to use immunologic procedures described under virologic and serology studies to study immunologic differences among cattle in high incidence herds.

III. PROGRESS REPORT

General Information

The initial objectives to study the occurrence and distribution of bovine leukemia and the possible influence of environmental factors have been completed. The studies on the role of bovine leukemia advised by an AEC Review Committee (1966) have been completed. The emphasis of the research studies have been primarily on virologic and transmission studies the past year, but preparation and publication of results of the epidemiologic studies was also carried out and will be reported. The data and progress will be reported under the following headings:

- A. Virus Isolation Studies in Cattle
- B. Virus Concentration Studies
- C. Cell Culture Studies
- D. Serologic Studies
- E. Transmission Studies
- F. Ultrastructure Studies

G. High Incidence Herd Studies

H. Epidemiologic Studies

I. Hematologic Studies

A. Virus Isolation Studies in Cattle

The most significant progress this past year has been the demonstration of C-type particles from leukemic and lymphocytotic animals. Preliminary results were reported in last year's progress report. This breakthrough was accomplished by stimulating lymphocyte cultures obtained from these animals with phytohemagglutinin and then subjecting these cultures to electron microscopy for demonstration of the virus. The results of this phase of the research contract were reported at the fourth International Symposium on Comparative Leukemia which was held at Cherry Hill, New Jersey in September 1969. This paper will be published in the proceedings of this symposium. Copies of this paper will be attached to this progress report.

During the past year a major effort was made on these virus isolation studies. It was necessary to further improve the lymphocyte culture techniques for virus isolation.

The technique which proved to be most efficient was to obtain fresh whole blood from leukemic and lymphocytotic animals, defibrinate and separate the lymphocyte by the silicone method. The lymphocytes were suspended in Eagles MEM with 20% bovine fetal serum to a concentration of 1 million cells per ml., a mitogen was then added and the lymphocyte culture was incubated.

In 72 hour mitogen stimulated cultures, virus particles were observed. Phytohemagglutinin and concanavalin were used as mitogens

and both were satisfactory for inducing virus production. Lymphocyte cultures from cases having high lymphocyte counts with immature cell types yielded more virus particles than did cultures established from cases having normal or lower total lymphocyte counts.

C-type mature virus particles are 95-125 m μ in diameter with a central dense nucleoid surrounded by a single or rarely a double membrane. These particles were most frequently observed extracellularly in clusters closely adjacent to viable lymphocytes. They were always attached to some sort of cellular material. Matured C-type virus particles and A-type virus were seen intracellularly on rare occasions, and when observed were always in the cytoplasmic vacuoles. Budding of virus particles from the plasma membrane into the cytoplasmic vacuoles and on the cell surface were observed.

C-type particles were isolated from nine of eleven leukemic cows including one thymic form in a calf. Failure to isolate virus particles from two leukemic cases may have been due to imperfection in our isolation technique during the early phase of our isolation studies and therefore, probably are more appropriately false negatives. Eight out of eight lymphocytotic cows yielded C-type particles. All ten normal control cows studied were negative for any C-type virus particle.

A more detailed description of the methods used, the results and discussion of these virus isolation studies is presented in a second manuscript which is being prepared for publication. A copy of this manuscript will be attached to this progress report.

Electron micrographs depicting the various types of virus particles are appended to this report (figures 1-5). C-type, A-type and budding particles are shown in these micrographs. These micrographs also demonstrate the presence of the extra cellular material to which the virus particles remain attached.

B. Virus Concentration Studies

The primary objective of the virus concentration studies was to improve the methods for the isolation, separation and concentration of C-type virus. It was observed that very few virus particles were present in the supernatant tissue culture fluid and most of them were attached to the cellular material. In an effort to obtain a greater yield of virus, studies were conducted to obtain a release of virus from the cellular material.

Mitogen stimulated lymphocyte cultures containing C-type virus particles were treated as follows:

1. Mechanical means such as shaking and sonication at various intensities for various lengths of time were used.
2. Treatment with enzymes such as trypsin, pronase, hyaluronidase and neuraminidase at various concentrations for different lengths of time were employed.
3. Treatment with detergents such as sodium deoxycholate, dipotassium ethylenediamine tetracetate (EDTA) were used. Maximum concentration which did not damage the cells or the virus was used on this experiment for various lengths of time.
4. It was thought possible that the attachment of the virus to the cellular material may be an antigen - antibody complex. In view

of this possibility the lymphocyte cultures were treated with urea, subjected to low pH with acetic acid and treated with high salt concentration for different lengths of time in an effort to break this potential complex.

In each case after the various treatments, the cells were pelleted and examined with electron microscopy to determine whether virus release had occurred.

It was not possible by employing the above described techniques to cause release of the virus from the cellular material; however, treatment with urea and decreasing the pH did cause release of some virus particles.

During the past year considerable time and effort was devoted to this study because the concentration of virus particles was necessary to initiate the transmission and other studies.

C. Cell Culture Studies

The primary objective of the cell culture studies was to adapt and propagate C-type virus in cell culture systems. This would permit and facilitate many additional studies with this virus.

Various bovine cell lines such as spleen, endocardial, and kidney were inoculated with the cells from 72 hour lymphocyte cultures which were positive for C-type virus particles. They were passaged serially at regular intervals. After 3 or 4 serial passages cytopathogenic changes appeared in bovine spleen and bovine spleen and bovine endocardial cell lines. Some of the infected cells came off the glass of the tissue culture bottle.

In infected bovine spleen cell lines, on electron microscopy, the presence of syncytial virus particles were observed. These particles were both extracellular and intracytoplasmic and 95-110 mu diameter. Intracytoplasmic budding particles were also observed.

In infected endocardial cell line cultures, on electron microscopy, both syncytial virus and C-type virus particles were observed with syncytial virus predominating. Both of these virus particles were seen more frequently in cells which came off the glass of the tissue culture bottles.

In bovine kidney cell line cultures no cytopathogenic changes were observed, nor were any virus particles demonstrated on electron microscopy.

Uninfected and also mitogen stimulated bovine spleen, endocardial and kidney cell lines served as controls and were negative for any cytopathogenic change or any virus particles on electron microscopy.

The results of the attempts to infect and propagate C-type virus in bovine endocardial cells was successful. These results were complicated by the fact that, in addition, syncytial virus was also present and propagated in the same endocardial cell lines. In an attempt to further study this phenomena, endocardial cells were cultivated with lymphocyte cell cultures containing C-type particles. The lymphocyte cells in these cultures were ruptured by alternate freezing and thawing prior to co-cultivation so that no intact lymphocyte cells remained when examined by electron microscopy.

There were, though, many intact C-type particles observed. Upon serial passages these inoculated endocardial cell lines revealed no virus particles until the fourth passage. On the fourth passage budding of virus particles was observed. These studies are still in progress and definitive evidence that these budding virus particles are C-type has not been demonstrated.

D. Serologic Studies

The primary objectives of the serological studies were to adapt existing serological techniques for use with the C-type virus and to attempt to determine if antibodies to C-type virus existed in known infected animals.

Complement fixation, immunodiffusion, immunofluorescence and cytotoxic test were preformed. Cells from mitogen stimulated lymphocyte cultures from leukemic and lymphocytotic cows containing abundant C-type virus particles were used as antigens. Sera and sera fractionated by Sephadex G 200 columns from leukemic and lymphocytotic cows were used. Lymphocyte cultures and sera from normal cows were used as controls. In addition, for complement fixation tests, complement fixing antibodies of murine, feline and avian species were used to determine any possible immunological relationship.

There was no immunological reaction between the antigen and sera from the leukemic, lymphocytotic and normal cattle. Also, no reaction was observed between these antigens and complement fixing antibodies of murine, feline and avian species.

The conclusion to these preliminary studies is that since no reaction occurred with the antigen antibody tests used in their

homologous systems, antibodies against C-type virus were not present in leukemic, lymphocytotic or normal cattle with the methods employed as outlined above.

E. Transmission Studies

It was proposed last year that transmission studies would be initiated when C-type particles from lymphocyte cultures were concentrated in a defined cell free and virus rich inoculum. As stated in the previous sections the concentration studies have not, as yet, been as successful as anticipated. Therefore, the proposed transmission studies have not been initiated.

Progress has been made toward the anticipated transmission studies in that source herds (herds with no history of leukemia and with hematologically normal cattle) for experimental animals have been identified and defined. The experimental animals in these studies will be as previously proposed i.e. immunologically incompetent (60-90 days old) fetuses and immunologically immature and defined (colostrum deprived) newborn calves.

Two transmission studies (fetal inoculation and foster nursing studies) initiated in previous years will be reported in this section. Our presently successful virus isolation procedures have made it possible to virologically evaluate animals from those studies.

1. Foster Nursing Studies:

Reciprocal foster nursing studies on bovine leukemia were initiated in 1967. These studies were undertaken to prove the hypothesis of leukemia transmission via milk which was formulated on the basis of epidemiological studies of high incidence herds. These studies have been terminated during the

past year at the recommendation of the Review Committee, due to insufficient available funds. The study is included in this progress report only to report the results of virological studies on the animals prior to their disposal.

Materials and Methods:

For these studies, two groups of calves were used: 1) progeny of lymphocytotic cows from high incidence leukemia herds 2) progeny of "normal" cows, i.e., cows with normal lymphocyte counts from herds with no history of leukemia and which had no lymphocytotic cattle. Colostrum and milk used in this experiment were obtained from the dams of the above groups and also milk from clinical cases of bovine leukemia. This colostrum and milk was either fed shortly after obtained or was stored at -70° C. until newborn calves were available.

Progeny of lymphocytotic cows were removed from their dams immediately after birth and housed separately. These calves were fed only colostrum from "normal" cows for 2 days and thereafter milk replacer until a roughage diet was fed.

Progeny of "normal" cows were removed from their dams after birth and housed separately. These calves were fed either colostrum from lymphocytotic cows or milk from clinic cases of bovine leukemia.

The 2 groups of calves were housed separately until they became adults.

Results

The following number of calves were in each experimental group:

1. 5 - progeny of lymphocytotic cows which received colostrum from "normal" cows.
2. 5 - progeny of "normal" cows which received colostrum from lymphocytotic cows.
3. 5 - progeny of "normal" cows which received milk from clinical cases of bovine leukemia.

The calves in these 3 groups ranged in age from 24 to 36 months and were in apparent good health clinically at the time of virological study.

Virological studies on blood from these 15 animals revealed only one of them to have C-type virus particles present. The positive animal was from group 2 above, i.e. a progeny of a normal cow which had received colostrum from a lymphocytotic cow.

2. Fetal Inoculation Studies:

Bovine leukemia transplantation experiments using intra-uterine inoculation of fetuses were initiated in 1967. These transplantation or cellular transmissions were attempted, at that time, due to a lack of a defined concentrated viral inoculum. These studies were terminated over a year ago but will be reported again because of the virological studies of animals from these experiments.

Materials and Methods:

The experimental animals in these studies were bovine fetuses which had not reached the age of immune competence. Previous progress reports have included reports of studies done by this group to determine the age at which immune competence occurs in

the bovine. These studies demonstrated that the bovine fetus is always immunologically tolerant during the first trimester, i.e. the first 90 days of gestation.

The fetuses used in this experiment are the progeny of cows and bulls which originated from herds which had no history of leukemia. All adult animals in these source herds were examined hematologically for persistent lymphocytosis. Only animals from lymphocytosis free herds were selected.

The inoculated material was obtained surgically from tumor masses of cases of bovine leukemia (adult type). The inoculum consisted of 0.5 gm. of tumor tissue homogenate suspended in 3 cc. of Eagles media and 20% bovine fetal serum. The fetuses were injected intraperitoneally through the wall of the uterus which was exteriorized through an abdominal incision.

Results:

Four calves were born which had been inoculated in-utero by the above method. They are now 2 years of age and are in apparent good health.

Blood from these animals was examined, during the past year, for presence of virus and all 4 animals were found to have large numbers of C-type virus particles present. The procedures used for the demonstration of virus in these animals will be explained in detail in this progress report under the heading of virologic studies.

F. Ultrastructure Studies

An ultrastructural study of peripheral blood agranulocytes of normal and preleukemic cattle was initiated. The objective of the study was to determine if agranulocytes of normal and preleukemic animals differ in a significant manner on the basis of various ultrastructural attributes and if any one or more than one of these attributes could serve as a diagnostic criterion for the preleukemic state. The cattle selected for study were all 5 years of age or older. Five normal cows were selected from leukemia free herds and seven preleukemic animals from multiple case herds. The blocks which were sectioned were carefully selected so that representative numbers of all agranulocytes present in the buffy coat of a centrifuged blood sample could be evaluated. All the cells examined were categorized into one of six classes of cells. Five of the six classes were established on the basis of commonly accepted ultrastructural criteria for such cells. The six classes used were as follows:

1. Inactive lymphocytes
2. Active lymphocytes
3. Monocytes
4. Immature plasma cells
5. Mature plasma cells
6. Light cells

The last category, light cells, is the only novel one. It consists of cells which have very light cytoplasm with a remarkable paucity of organelles and which often appear moribund. About 200 cells were classified from each of the 12 animals and the population

of organelles present in each cell was recorded in the following schema:

1. Number of nucleoli
2. Number of nuclear bodies
3. Number of nuclear pockets
4. Fine or dense chromatin
5. Number of intracellular tears
6. Number of cell to cell contacts
7. Amount and size of ergastoplasm
8. Presence of free or polyribosomes
9. Presence or absence of glycogen
10. Number of cytoplasmic granules
11. Number of mitochondria

The attributes of each cell examined were punched into a data card and a numerical analysis was begun with a computer. Since the cells were scored on the basis of ultrastructural attributes, the data was analyzed by using a contingency table and the chi-square test for independence. The analysis of the data has not been completed but some observations which were made are useful. Seventy-five percent of the agranulocytes were found to be inactive lymphocytes in the preleukemic animals as opposed to 65% in normal animals. Intracellular tears were prevalent in preleukemic lymphocytes while virtually none were found in normal lymphocytes. The tears are consistently found to follow ergastoplasmic sacs or the nuclear envelope. The tears most probably result from the application of centrifugal force during sample preparation, but the dichotomy between normal and

preleukemic animals is striking. The presence of tears is consistent with the observation that leukocytes from chronic lymphocytic leukemia of humans are more fragile than those of normal humans.

The number of nuclear bodies present in lymphocytes of preleukemic animals is significantly higher ($P < 0.005$) than in normal cells, but this fact may not be of diagnostic value.

G. Herd Studies

1. Multiple Case Herd Studies:

The general description, criteria for selection and monitoring procedures for high incidence leukemia herds have been reported in previous progress reports and will not be discussed here.

The multiple case herd study presently consists of the study of six herds. These herds have been studied for periods up to 9 years. Unfortunately several herds studied in previous years are no longer available for study due to herd dispersals. Additional herds will be added to our study in the next year. The present herds range in size from 30 to 110 adult dairy cows. All of these herds have had at least 2 confirmed cases of bovine leukemia.

As in previous years additional "new cases" of leukemia have continued to occur in study herds this past year. Four "new cases" occurred in three of the herds. During the past ten years of herd studies a total of thirty "new cases" of leukemia have occurred in herds which had been under study. Twenty-seven (90%) of these cases had exhibited persistent

lymphocytosis for periods up to five years prior to the development of tumorous clinical leukemia. Two of the three (10%) new cases which did not exhibit preclinical lymphocytosis were seen this past year. These cows had never exhibited lymphocytosis on semiannual hematological examinations for three and five years respectively.

Hematological data accumulated during the past year on adult cattle in the study herds is comparable to that reported in previous years. High incidences of persistent lymphocytosis continues to be a characteristic of this type of herd.

2. Control Herds

Control herds by our definition are herds of cattle which have had no cases of leukemia, do not contain cows with lymphocytosis and have had no new animal introduction for at least 5 years. During the past several years at least 10 herds of this type have been located and studied. Presently 2 of the larger of these herds are under more intensive study and will serve as source herds for experimental animals on future transmission studies. Additionally, during the past year cattle from these herds have been used as controls for virus isolation and immunological studies.

H. Epidemiologic Studies

The epidemiologic studies on the occurrence and distribution of bovine leukemia in Minnesota were concluded previously. Four manuscripts have been accepted for publication and are entitled as follows:

1) Selected Epidemiological Aspects of Bovine Leukemia in Minnesota 1961 to 1965. 2) Association of Veterinary Medical Service with Reporting of Bovine Leukemia in Selected Minnesota Dairy Herds. 3) Epidemiological Studies on the Natural Transmission of Bovine Leukemia. Copies of these 3 manuscripts will accompany this progress report. The fourth paper is entitled "Farm Environmental Factors Associated with Reporting of Bovine Leukemia in Selected Minnesota Dairy Herds." This manuscript is currently being revised and will be submitted for publication very shortly.

I. Hematologic Studies

During the past contract year no additional hematologic studies were conducted except that associated with the continued study of the high incidence and control herds. This is briefly reported in the section which deals with the herd studies. Part of the hematologic data collected previously on this project was prepared in a manuscript for publication. A paper entitled "Statistical Evaluation of Lymphocyte Parameters on Minnesota Dairy Cattle" was accepted for publication in the American Journal of Veterinary Research. This paper dealt with the study to establish normal lymphocyte values for dairy cattle in Minnesota and the development of a Minnesota Key. This key can be used as a basis to evaluate lymphocytosis in dairy cattle and its relationship to leukemia. A copy of this paper is attached to this report.

Additional hematologic data on lymphocytotic and leukemic animals is being analyzed and in preparation for publication.

Fig. 1 - Leukemic cow; mitogen stimulated lymphocytes in culture. Note the presence of extracellular C-type virus particles (arrow). These virus particles are in clusters and are attached to cellular material. 25,000 X

Fig. 2 - Lymphocytotic cow; mitogen stimulated lymphocytes in culture. A section of a lymphocyte; note the presence of extracellular C-type virus particles in clusters attached to cellular material. 25,000 X

Fig. 3 - Lymphocytotic cow; mitogen stimulated lymphocytes in culture. C-type virus particles are present intracellularly in the cytoplasmic vacuole. Extracellular C-type virus particles (arrows) are present. 25,000 X

Fig. 4 - Lymphocytotic cow; mitogen stimulated lymphocytes in culture. Note a surface budding of a virus particle (B). Extracellular clusters of C-type virus particles (C) attached to cellular material. 25,000 X

Fig. 5 - Lymphocytotic cow; mitogen stimulated lymphocytes in culture. Note the intracellular presence of different types of virus particles in the cytoplasm; budding of virus particles (B) from the endoplasmic reticulum, A-type virus particles (A) and C-type virus particles (C). 50,000 X