MASTER

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

PROGRESS REPORT NO. 12 TO THE U. S. ATOMIC ENERGY COMMISSION
ON RESEARCH PERFORMED UNDER CONTRACT AT (11-1)-910
COVERING THE THREE-YEAR PERIOD JULY 1969 - JULY 1972

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April 30, 1972

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PERIOD COVERED BY THIS REPORT:

This report covers the period from July 1969 to July 1972.

LITERATURE REVIEW:

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 Germany³ bovine leukosis is recognized as a major disease problem, and eradication and/or control measures have been initiated. In Germany³ and Sweden⁴ the incidence of tumor cases has been reported as approximately one percent of all slaughtered cattle with even higher incidences common at abattoirs in high incidence areas.

In the United States the incidence of leukemia varies in different parts of the country, but the overall average is approximately 17 to 18 cases yearly per 100,000 cattle.^{5,6} More recent studies on the incidence of bovine leukemia in Minnesota show a minimum incidence estimate of 14.8 cases of the adult form per 100,000 dairy cows at risk per year.⁷ These incidences are quite low in comparison to those reported in Europe, but even so the disease is economically important in the United States, costing the cattle industry an estimated three million dollars annually.⁸ World-wide

* The term bovine leukemia as used in this report is synonymous with the term bovine leukosis used by many European investigators and the terms lymphosarcoma or malignant lymphoma as used by some investigators in the United States. Also, in this report bovine leukemia will refer only to the adult form of the disease.

losses, if known, would of course be of much greater magnitude.

Even if economic importance is not considered, extensive studies of bovine leukemia are justified, for they have and will continue to contribute much to the understanding of naturally occurring leukemias. During the past, suitable animal model systems have contributed much to the understanding of comparable diseases in man. Lymphocytic leukemia is such a disease; however, many questions remain to be answered. Of special interest is the area of etiology and transmission. Here is where comparative studies of bovine leukemia may be most helpful, because in certain ways bovine leukemia more closely resembles leukemia in man than the extensively studied chicken and mouse systems. For instance cattle, like man, are not extensively inbred, have a fairly long life span, and spontaneously develop leukemia with about the same incidence as man.⁹ These qualities make the bovine an ideal animal for use in leukemia transmission experiments that, of course, cannot be performed on man himself.

Epidemiological investigations have provided indirect evidence that bovine leukemia is a transmissible disease. In Germany, during World War II, cattle were transported from regions with a high incidence of leukemia into areas where leukemia was very rare. The result was the continued occurrence of leukosis in offspring of the high incidence animals, plus the appearance of leukemia in the previously leukosis free native cow families.¹⁰ Studies of high incidence herds in Minnesota also show that

leukemia transmission is associated with the movement of animals¹¹ from these herds to apparently leukemia free herds. In southern Sweden the use of a whole blood vaccine for piroplasmosis was⁵ associated with the propagation of leukemia in cattle. In Denmark the geographical distribution of cases is not correlated with the distribution of the different Danish breeds of cattle and the disease has passed from animals of one breed to those of another within the same herd.² These studies and others that show leukemia developing in an unpredictable manner in cow families where each member should be of equal susceptibility on the basis of genetic make-up indicate that genetic factors are not the primary determinant in the etiology of bovine leukemia. Epidemiological investigations do, however, indicate that an infectious agent is directly involved in the etiology of bovine leukemia and that both horizontal and vertical transmission do take place. Cohort analysis studies, performed at Minnesota^{11,12} under AEC contracts, support such a hypothesis.

It is widely hypothesized that the proposed infectious agent of bovine leukemia is viral in nature. Major support for this hypothesis comes from the conclusive demonstration of the role of C-type viruses in the etiology of transmissible lymphoid¹³ neoplasms in the chicken,¹⁴ mouse,¹⁵ cat,¹⁶ and guinea pig.

Reports on the observation of virus like particles in the milk and lymphoid tissues of leukemic cows and cows from high incidence^{17,18} herds provide additional support for a viral etiology. How-

ever, these investigations are not conclusive because the electron micrographs leave much doubt that the observed particles are similar to the C-type viruses known to cause leukemia in other species.

Much more convincing evidence for a viral involvement in lymphocytotic and leukemic cattle has been provided by the examination of cultured lymphocytes. In December of 1969 Miller et al.¹⁹ reported a very significant observation. In many short-term (24 to 72 hour) phytohemagglutinin stimulated cultures of lymphocytes, obtained from the peripheral blood of lymphosarcoma cases, cows¹ from high incidence herds, and² cattle that had been inoculated with lymphosarcoma extracts, they found abundant virus like particles. Unlike the particles observed earlier in milk and tissues, these particle clearly resemble the mature C-type viruses known to cause leukemia in the murine¹⁴ and feline¹⁵ species. This important finding was immediately confirmed by our research group at Minnesota.²⁰ Since that time we have consistently observed particles with C-type size and morphology in 72 hour phytohemagglutinin stimulated cultures of lymphocytes obtained from leukemia cases and animals with a persistant lymphocytosis, but not in stimulated lymphocyte cultures from nonlymphocytotic normal animals.

The consistent finding of particles with C-type size and morphology in lymphocyte cultures obtained from leukemia cases and cows with a persistant lymphocytosis, plus the conclusive demonstra-

tion of the role of C-type viruses in the etiology of lymphoid neoplasms in other species,^{13,14,15,16} strongly suggests, but does not prove, a viral etiology for bovine leukemia. This proof awaits a conclusive demonstration of transmission by means of a cell free filtrate. A few investigators have reported on^{21,22} successful cell free transmission. However, because of poor experimental designs and the very small number of animals actually developing lymphosarcoma, their studies are not convincing and cannot be considered positive proof of a viral etiology for bovine leukemia. Our goal here at Minnesota is to furnish additional evidence for a viral etiology of bovine leukemia and to investigate the pathogenesis of the disease.

SCIENTIFIC SCOPE AND OBJECTIVES FOR THE THREE-YEAR PERIOD JULY 1969 TO JULY 1972:

The primary objective of our research is to experimentally transmit bovine leukemia to newborn calves and 90-day fetuses and to extensively monitor these animals from the time of inoculation to the time of tumor development. In conjunction with this main goal, electron microscopic examination of cultured lymphocytes, virological investigations on the C-type particles of bovine origin, and studies of high incidence and control herds will be carried out.

I. Objectives of Electron Microscopic Studies of Cultured Lymphocytes.

- A. To demonstrate that C-type particles can be consistently observed in phytohemagglutinin stimulated cultures of

lymphocytes obtained from leukemia cases and animals with a persistent lymphocytosis, but not in cultures from non-lymphocytotic normal animals.

II. Objectives of Virological Investigations on the C-type Particles of Bovine Origin.

- A. To attempt to develop an in vitro continuous source of bovine C-type particles.
- B. To purify the bovine C-type particles so that meaningful studies can be performed to determine their physical, biochemical and serological properties.

III. Objectives of High Incidence and Control Herd Studies.

- A. To obtain data on the natural outcome of lymphocytotic and nonlymphocytotic 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 nonlymphocytotic animals.
- D. To augment our data on the role of genetic factors in the occurrence of lymphocytosis and leukemia.
- E. To provide a well-defined source of animals for transmission studies.

IV. Objectives of the Transmission Studies.

- A. To propagate and concentrate the massive amount of bovine C-type particles needed for transmission studies.

- B. To experimentally transmit bovine leukemia to newborn calves by means of a cell free filtrate containing concentrated C-type particles of bovine origin.
- C. To experimentally transmit bovine leukemia to 90-day-old fetuses by means of a cell free filtrate containing concentrated C-type particles of bovine origin.
- D. To extensively monitor the progress of the transmission studies using parameters that will clarify the pathogenesis of bovine leukemia.

PROGRESS REPORT

I. Electron Microscopic Studies of Cultured Lymphocytes.

We hypothesize: (a) That in cattle, persistent lymphocytosis and the ensuing leukemic condition are manifestations of a viral infection and that the virus can be consistently demonstrated in both conditions but not in normal nonlymphocytotic animals. (b) That a persistent lymphocytosis together with demonstrable C-type particles represents a prodromal stage of leukemia and that this combination of findings is a reliable indication that the animal will become leukemic. To examine these hypotheses three groups of animals have been examined: (1) All confirmed cases of bovine leukemia available in our clinic during the period covered by this report. (2) Several cases of persistent lymphocytosis* showing no clinical signs of leukemia. (3) Hematologically normal animals from non-leukemic herds. As funding has not been specifically granted for this study, the number of animals examined is not extensive but is sufficient to provide evidence in support of our hypotheses.

METHODS:

Blood obtained by venipuncture was defibrinated using glass beads. The lymphocytes were then separated from other blood components by means of the silicone separation method described by Joel.²³ Separated lymphocytes were suspended to a concentration of 1×10^6 cells per ml. in our special medium containing 300 units of penicillin and 150 micrograms of -----

* Lymphocyte count elevated at least three standard deviations above that considered normal for the breed and age. In all cases examined the condition had persisted for at least six months.

dihydrostreptomycin per ml. This medium was supplemented with bovine fetal serum and PHA added to a final concentration of 2%. After incubation for 72 hours at 37°C the cells were pelleted and processed for electron microscopic examination.

RESULTS:

The results of the electron microscopic studies of cultured lymphocytes are summarized in Table 1.

TABLE I RESULTS OF THE ELECTRON MICROSCOPIC
EXAMINATION OF CULTURED LYMPHOCYTES

<u>Hematological Classification</u>	<u>Pos.</u>	<u>Neg.</u>	<u>Total No. of Animals</u>
Leukemia	23	2	25
Persistent Lymphocytotic	10	0	10
Normal (nonlymphocytotic from nonleukemic herd)	0	16	16
	—	—	—
Total	33	18	51

We believe that the two leukemic cows negative for C-type particles represent false negatives due to imperfections in the culturing technique early in the studies.

During the course of this study three of the animals with a persistent lymphocytosis and demonstrable C-type particles developed leukemia. In each case they remained positive for C-type particles; however, in every animal, lymphocyte cultures prepared during the lymphocytotic state contained virus like particles in more abundance than cultures prepared after a clinical picture of leukemia developed. These three well-studied animals provided the first experimental evidence in

support of our hypothesis that a persistent lymphocytosis together with demonstrable C-type particles represents a prodromal stage of bovine leukemia.

II. Virological Investigations on the C-Type Particles of Bovine Origin.

A. Development of an InVitro Continuous Source of Bovine C-Type Particles.

It is desirable to have an in vitro continual source of bovine C-type particles. This would eliminate the expenses involved in locating and maintaining source animals, and would eliminate the blood processing currently necessary before even short-term cultures can be set up.

During the past three years we have used three approaches in trying to establish an in vitro source of bovine C-type particles. Attempts have been made to: (1) propagate the viral like particles in previously established bovine cell lines. (2) Establish continuous lymphocyte cell lines using peripheral blood lymphocytes from animals known to have abundant C-type particles. (3) Establish continuous cultures using bone marrow cells aspirated from the sternum of cows whose lymphocytes produce abundant C-type particles in culture.

1. Attempts to propagate the viral like particles in previously established bovine cell lines.

METHODS:

Bovine fetal spleen, fetal endocardial, fetal embryo, fetal kidney, fetal lymph node, fetal lung, and fetal

thymus cell lines were established in continuous culture. Trypsinized suspensions of each cell type were mixed with 72-hour lymphocyte cultures known to be producing many C-type particles. A ratio of one established cell to five viable lymphocytes was used. The mixed cultures were incubated at 37°C for three hours, after which Eagles MEM supplemented with 20% bovine fetal serum was added to give a final concentration of 1×10^5 established cells per ml. After four days nonadherent cells were discarded and the medium replaced. On day seven, and every fifth day thereafter, cells were serially passed. At each passage the cultures were examined for cytological changes and cells were pelleted and processed for electron microscopic examination.

RESULTS:

Our attempts to adapt bovine C-type particles to propagate in established bovine cell lines were largely unsuccessful. Viral like particles were never observed in the inoculated bovine fetal embryo, fetal lymph node, fetal lung, fetal kidney and fetal thymus cell lines. In bovine fetal spleen and fetal endocardial cell lines cytological changes consisting of cell rounding and syncytium formation were occasionally observed. Electron microscopic examination of cells from these cultures revealed a few isolated C-type particles and occasional clusters of particles similar to the syncytial virus

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described by Malmquist. All attempts to increase the number of C-type particles produced by these two cell lines have failed.

2. Attempts to establish continuous lymphocyte suspension cultures using peripheral blood lymphocytes from animals known to have abundant C-type particles.

METHODS:

Lymphocytes from five cows known to produce C-type particles were suspended in R.P.M.I. 1640 medium containing 20% bovine fetal serum. Some cultures were started using lymphocytes from a single animal, but in most cases lymphocytes from several animals were pooled together. It was anticipated that this pooling of genetically unrelated cells would result in the antigenic stimulation of some cells and perhaps induce them to multiply. In both single and pooled cell cultures the initial cell concentration was 2×10^6 cells per ml. Stationary and spinner cultures were tried, as was incubation with and without 5% CO₂ in the atmosphere. Once a week the cells were allowed to settle and one-half of the old medium was removed and replaced with an amount of fresh medium calculated to maintain a cell density of 2×10^6 viable cells per ml.

RESULTS:

Attempts to initiate continuous lymphocyte suspension cultures were not successful. In all cultures, the viable cell count decreased rapidly. By the fourth

medium exchange the volume of all cultures had been reduced to less than 20 cc's. and it was no longer possible to maintain a viable cell density of 2×10^6 cells per ml. In some of the cultures viable cells remained for as long as 60 days but they never became established, and by 120 days cells in all cultures had died out.

3. Attempts to establish continuous cultures using aspirated bone marrow cells.

METHODS:

One cc. of bone marrow was aspirated from the sternum of six lymphocytotic cows known to be good sources of C-type particles. Each bone marrow sample was suspended in two cc's of R.P.M.I. 1640 medium containing 100 units of heparin. Equal portions of each suspension were added to 250 cc. plastic tissue culture flasks along with antibiotics and 20 cc's. of R.P.M.I. 1640 medium supplemented with 20% bovine fetal serum. The 12 flasks were incubated at 37°C in a flat position for one week. At this time the old medium was completely discarded leaving only those cells that adhered to the plastic. These adherent cells were left undisturbed for an additional two weeks.

RESULTS:

In many of the cultures the adherent cells multiplied, producing a monolayer. By three weeks some monolayers became confluent and began to shed cells into the medium. These cells were spun down and resuspended in fresh medium

where they grew in suspension. Every week the cells were counted, spun down and resuspended in fresh medium to a concentration of 2×10^6 viable cells per ml. The monolayer cultures were refed every two weeks and passed when confluent. The long-term fate of the monolayer and suspension cultures was as follows: (a) By ten weeks the monolayer cultures had died out. (b) In five of the six suspension cultures, the viable cell density gradually decreased and eventually they had to be pooled together to maintain a cell density of 2×10^6 viable cells per ml. This pooled suspension culture remained at this cell density for four weeks and then rapidly died out. (c) One suspension culture multiplied, reaching a level of 4×10^6 viable cells per ml. during the first week. This culture was used to start four new cultures. Two of these cultures again reached a density of 4×10^6 viable cells per ml. One culture was used to start additional cultures; the other was pelleted and processed for electron microscopic examination. Electron microscopic examination demonstrated that the propagating cells were not the expected lymphocytes, but atypical cells of the granulocytic series. No C-type particles were observed and propagation of these nonlymphocytic cells was not pursued.

II. Studies on the Purification of Bovine C-Type Particles.

To determine the physical properties, do meaningful biochemical studies, or to prepare viral antigens for immuni-

zation, it is necessary to have relatively pure viral preparations. Early in this reporting period considerable effort was expended in trying to purify the bovine C-type particles.

A. Attempts to Release Bovine C-Type Particles from Cellular Material.

Early in our studies we discovered that stimulated lymphocytes producing abundant C-type particles do not release these particles into the medium. Instead the particles become attached to the cells and to cellular debris. Many attempts were made to release them.

METHODS:

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

1. By mechanical means, such as shaking and sonication at various intensities for various lengths of time.
2. With enzymes, such as trypsin, pronase, hyaluronidase and neuraminidase at various concentrations for various lengths of time.
3. With detergents, such as sodium deoxycholate and EDTA at different concentrations for various lengths of time.
4. By alternate freezing and thawing.
5. It was thought that the attachment of the C-type particles to the cellular material could be the result of an antigen-antibody complex. In view of this possibility lymphocyte cultures were treated with urea,

subjected to low pH, or treated with high salt concentrations for different lengths of time in an effort to break this potential complex.

After each of the above-mentioned treatments, the cells were pelleted and processed for electron microscopic examination.

RESULTS:

With the exception of treatment with urea and decreasing pH, the techniques described above were not successful in releasing the C-type particles. The limited success obtained with urea and decreased pH indicates that the binding is indeed similar to that of antigen-antibody reactions.

B. Density Gradient Studies of Bovine C-Type Particles.

METHODS:

Mitogen stimulated lymphocyte cultures containing abundant C-type particles were subjected to one freeze-thaw cycle and then brief sonication in an attempt to free particles from the cellular material.* This material was then pumped into the core of a Beckman TI-15 zonal rotor containing a linear sucrose gradient varying from 10% at the core to 60% at the periphery. The rotor was then accelerated from the 3000 rpm speed used for loading to 30,000 rpm's. After two hours the rotor was slowed to 3,000 rpm's and its contents displaced through the core by pumping 60% sucrose in through the wall. Fractions

*These experiments were performed before the more effective urea and low pH methods were discovered.

were collected and their density determined with a refractometer. Each 20 ml. fraction was dialyzed against buffer to eliminate the sucrose and then pelleted in an ultracentrifuge. The resultant pellets were processed and then examined with the electron microscope.

RESULTS:

The C-type particles were observed in all fractions with densities of 1.15 to 1.18 g/cm³. and were found still attached to fragments of cellular material. Free, individual, unattached particles banded at a density of 1.15 to 1.16 g/cm³. This range is similar to that reported for other oncogenic viruses.

III. High Incidence and Control Herd Studies.

A. 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 10 years. Unfortunately, several herds studied in previous years are no longer available for study due to herd dispersals. The present herds have had at least two confirmed cases of bovine leukemia.

RESULTS OF MULTIPLE CASE HERD STUDIES:

As in previous years additional "new cases" of leukemia

have occurred in herds which had been under study.

Thirty-one (91%) of these cases had exhibited persistent lymphocytosis for periods up to five years prior to the development of tumorous clinical leukemia.

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 herd type. Familial and temporal-spatial relationships reported in past years are still noted in studies of leukemia cases seen in these herds.

B. Control Herd Studies.

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 five years. During the past several years at least 10 herds of this type have been located and studied. Presently five of the larger of these herds are under more intensive study and have served as source herds for experimental animals used in our transmission studies. Additionally, during the past year cattle from these herds have been used as controls for virus isolation studies.

IV. Transmission Studies.

A. Propagation of the Massive Numbers of Bovine C-Type Particles Needed for the Experimental Transmission Studies.

1. Calculation of the approximate number of C-type particles needed for the inoculation of ten newborn calves and ten fetuses.

In our experimental cell free transmission studies it was considered highly desirable to meet the following dosage related criteria.

- a. The dose should be large enough to produce leukemia in 100% of the animals inoculated.
- b. In nature the peak incidence of bovine leukemia is not reached until 7 years of age, probably because the naturally occurring disease has a very long latent period. Experimental cell free transmission studies performed with other species have indicated that the latent period is dose dependant and can be considerably shortened by increasing the dose. We feel that our dose should be large enough to produce the tumorous stage of bovine leukemia within 30 months.
- c. The dose should be defined to the extent that it can be reproduced in this laboratory and in others.

OBTAINING 100% INCIDENCE:

In meeting criteria a and b we have relied heavily on work performed by others studying feline and murine leukemia. Experiment 3, Table 1, in Moloney's article²⁵ has been especially helpful. Using data from this experiment it was calculated that 4×10^{-5} gram equivalents of tumorous lymph node tissue is needed per gram of newborn

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mouse to give a 100% incidence of leukemia. To maintain this per gram dosage level in newborn Holstein calves an inoculum containing 1.82 gram equivalents of tumor tissue would be required.* In our transmission experiments we have decided to increase this to a minimum of 2.82 gram equivalents for a newborn calf and to use 0.70 gram equivalents for the fetal inoculations.

LATENT PERIOD:

If the natural life span of the laboratory mouse is taken as 2.5 years and that of Holstein cattle as 20 years, then each month of life for the mouse is equivalent to 8 months of life in cattle. From Table 1 in Moloney's²⁵ article, the average time from viral inoculation (done during the first day of life) to death from leukemia would be 4.0 months in a mouse receiving 4×10^{-5} gram equivalents of tumorous lymph node tissue per gram of body weight. Thus by maintaining this per gram dosage level in newborn Holstein calves we can expect a latent period (time from inoculation of virus to tumor development) of 32.0 months. Actually, we expect our latent period to be shorter than this because: (1) By using 2.82 instead of 1.82 gram equivalents per calf, we are using a dose considerably larger than 4×10^{-5} gram equivalents per gram. (2) We feel that 1 gram of tissue from our phytohemagglutinin stimulated lymphocyte cultures

 *For this calculation the weight of a newborn mouse was taken as 1.25 grams, that of a newborn Holstein calf as 45.5 kgs.

contains 10 to 100 times as many C-type particles as a gram of the tumorous lymph node tissue used by Moloney. From Table 1 in Moloney's article a 10-fold increase in dose size shortens the latent period by 0.6 of a month, this is equivalent to 4.8 months in cattle and thus our expected latent period becomes 27.2 months.

With the fetal inoculations the number of virus like particles injected per gram of body weight will be even greater; consequently, an even shorter latent period is expected.

DEFINING THE DOSE:

The number of gram equivalents used per calf or per fetus is a very rough measurement of the viral dose because the number of C-type particles per gram of cultured lymphocytes is not constant. For instance, the number of viral like particles per gram of lymphocytes varies greatly when different animals are used as the lymphocyte source. Thus, to more clearly define our dose, we are using the following procedure. When the PHA stimulated cultures are harvested, the number of viable cells per cc. is determined and cells are pelleted for electron microscopic examination. During this examination the percentage of viable cells with cell associated C-type particles is determined. Multiplying the total number of viable cells times the percentage with cell associated C-type particles gives the total number

of cells with attached viral like particles. Using this type of calculation the cultures processed for one newborn calf dose contain, on the average, 8.4×10^8 cells with attached viral like particles.

The thin section examined in the electron microscope allow one to see much less than 1/100 th. of the cell's total thickness; therefore each cell actually has at least 100 times more C-type particles attached to it than observed in a single thin section. Thus, our calf doses contain at the very minimum 8.4×10^{10} C-type particles. Using the same reasoning, our fetal doses contain 2.1×10^{10} C-type particles. Of course, we presently have no way of knowing how many, if any, of these particles have biological activity.

2. Viral like particle production at the start of this reporting period.

Three years ago the number of viable cells in the 72 hour PHA stimulated cultures averaged 25% at the time of harvest. The proportion of viable cells with C-type particles adhering to their surface averaged 20%. At this level of production we would need to culture 210 liters to produce the 1.05×10^{12} C-type particles needed for our proposed transmission studies. When we bleed a cow for the purpose of initiating a large 72 hour lymphocyte culture, we draw a maximum of 250 cc's. of blood. After processing this volume yields, on the

average, 0.8 liter of culture. Thus, to produce 210 liters of culture we would have to draw and process blood 263 times. Maintaining a reasonable hematocrit value in the limited number of source animals (seldom more than two), it would take two years just to complete the culturing for this study. For this reason efforts were expended to try and increase the C-type particle output of the cultures.

3. Viral like particle production in our present culturing system.

Presently the number of viable cells in our 72 hour PHA stimulated cultures averages 75% at the time of harvest. The proportion of viable cells with C-type particles adhering to their surface averages 30%. At this level of production we now need to culture only 50 liters to produce the required 1.05×10^{12} C-type particles. This reduces the number of times that we must draw and process blood to 63 and allows us to complete the culturing within one year.

4. Progress in culturing.

The paragraphs above detail the tremendous progress made in improving our culturing technique. The practical result is that we have completed all culturing necessary for the inoculation of 10 calves and 10 fetuses. In fact, we have cultured enough material to inoculate two additional calves. The cultured material is stored at -90°F until it is concentrated for inoculation.

5. Processing the cultured material for inoculation.

It is not the purpose of this report to completely describe our very effective processing method. Instead we will briefly describe what each step accomplishes.

- a. The frozen cell cultures are thawed and the cells completely homogenized.
- b. The larger pieces of cellular material are removed by low speed centrifugation.
- c. The remaining cellular components and the virus like particles are pelleted against the wall of a Beckman CF-35 rotor.
- d. The pelleted material is resuspended and filtered through a 1.2 micron pore size filter if it is to be used for a calf dose, or through a .45 micron filter if it is for a fetal dose.
- e. The processed material is stored at -90°F until the time of inoculation.

SPECIAL FEATURES OF OUR PROCESSING SYSTEM:

- a. It is our assumption that the observed C-type particles are viral in nature and that they are temperature sensitive to the extent that they lose infectivity when not frozen. For this reason we have made every effort to attain rapid processing, at a low temperature, to assure a minimal loss of infective titer. The total processing time can now be kept to less than 12 hours. During 10 of these hours the

sample is kept at less than 5°C. For the other two hours the temperature remains less than 15°C.

b. With the Beckman L2-65B ultracentrifuge and accessory CF-35 continuous flow zonal rotor, we can pellet out all of the C-type particles contained in 8,000 cc's. of culture and resuspend them in 8 cc's. Thus we can effectively concentrate the particles 1,000 fold.

c. The filtration step assures that our inoculum is absolutely cell free.

6. Progress in processing the cultured material for inoculation.

Prior to October of 1971, only slight progress was made in the concentration of cultured material for inoculation. Two calf doses and one fetal dose were prepared at the Oak Ridge National Laboratories using their K-III continuous flow zonal ultracentrifuge. These three doses are inferior to the ones currently prepared with our continuous flow ultracentrifuge because: (a) They contain an estimated 10 to 100 fold fewer C-type particles. This is because the dosage criteria were formulated after these doses were prepared. (b) They were kept in a nonfrozen state for a much longer period of time.

A year of effort was expended in trying to process our cultured cells using a batch type zonal centrifuge.

With this set up a maximum of 200 cc's. of culture can be concentrated in a single run, thus it would take 20 all day runs to process the amount of material that we now use for inoculating a single calf. To reduce this to within reason, each liter of culture was first concentrated to 200 cc's. before being processed in the batch type rotor. With this set up we were able to process enough material to inoculate three additional calves. However, for the same reasons mentioned in the preceeding paragraph, these three doses are inferior to those prepared using our continuous flow ultracentrifuge.

In October of 1971 our Beckman L2-65B ultracentrifuge and accessory CF-35 continuous flow rotor became functional. Since that time there has been no problem in concentrating material for inoculation. Between November, 1971 and February, 1972 we concentrated enough material to inoculate 7 calves and 8 fetuses with each dose receiving approximately the number of C-type particles detailed previously. Thus, combined with the doses prepared in the other centrifuges, we now have processed more than the amount of material needed to complete the proposed transmission studies.

B. Transmission Studies Using Newborn Calves.

METHODS:

All calves used in this study were obtained from the

defined control herds described earlier in this report. They were all deprived of colostrum and were all inoculated within 30 hours of birth. Prior to inoculation, and at monthly intervals thereafter, blood was drawn and evaluated as described in the section on the monitoring of inoculated animals. The dose (previously defined) was administered in two parts. Three-fifths of it was given intravenously by way of the jugular vein; the other two-fifths was administered via the intraperitoneal route. All calves were housed in isolation units for the first two to four months of life. They were then transferred to a university owned farm where they were in contact with each other but not in direct contact with other animals.

PROGRESS IN CALF TRANSMISSION STUDIES:

The ten proposed calf inoculations were completed in March of 1972. Two of the original ten animals died and were subsequently replaced by the inoculation of two additional calves in April. Thus we have actually inoculated 12 newborn calves. The two deaths were attributable to severe coliform infections with subsequent diarrhea and septicemia. The control of this problem in the colostrum deprived calves has not been each and we still have not solved it. For this reason the newborn colostrum deprived calves cannot be considered as being successfully inoculated until they are at least six weeks old. Of the ten calves still living two were inoculated with the material processed at Oak Ridge, two with material prepared in the batch type rotor and six with a defined inoculum

prepared in our continuous flow rotor.

To date (April 13, 1972) seven of the inoculated calves are over two months of age and are in good health. Three calves are under one month of age and diarrhea is currently a problem in all three. Thus, they cannot be considered as successfully inoculated until they survive the first six weeks. None of the calves show any clinical signs of leukemia. However, it must be emphasized that of the calves inoculated with inoculum prepared in our continuous flow ultracentrifuge, the oldest is now only three months of age. Thus it is much too early to expect tumor development in these animals.* We feel that it may take even longer in the animals inoculated with the Oak Ridge or batch processed inoculum. Of course we expect many changes to occur before tumor development. Our methods for detecting some of these changes and the results to date are described in the section on the monitoring of inoculated animals.

C. Transmission Studies Involving Fetal Inoculations.

The pregnant cows used in this study were from the defined leukemia free control herds described earlier in this report. Evaluation of their past hematological records and complete testing using the methods described in the section on the monitoring of inoculated calves showed these animals to be leukemia free by the following criteria: (1) No record of lymphocytosis. (2) No C-type

*See our previous discussion on the expected latent period where we explain our reasons for thinking that it may take up to 27 months for tumors to appear at this dosage level.

particles demonstrable in PHA stimulated lymphocyte cultures. (3) Less than 0.5% of their lymphocytes show nuclear pockets.

At approximately 90 days of gestation the fetuses were inoculated. The 2.1×10^{10} C-type particles required for a fetal dose were concentrated by ultracentrifugation to a volume of 3 cc's. or less. The injections were made intraperitoneally, through the uterine wall which was exteriorized through a lateral abdominal incision.

PROGRESS IN FETAL TRANSMISSION STUDIES:

Of the ten proposed fetal inoculations, two have been completed as of April 1, 1972. Two additional fetuses will be inoculated before the current contract period expires in July. The doses for the six other proposed animals have been prepared but cannot be administered because we lack the funds necessary for the purchase of six pregnant cows.

Shortly after the first fetal inoculation several animals in the clinic developed bovine viral diarrhea infections. Our experimental animal was included in this group and we attribute the abortion directly to this infection. The cause of the second abortion is unknown. Grossly the fetus was normal with the exception of a generalized edema. There was no evidence of injection trauma or of a bacterial infection. Microscopic examination of tissues was not possible because of the extensive post mortem autolysis.

Because the failure of the first two fetal inoculations cannot be traced directly to the inoculation procedure, an additional fetus will be inoculated before procedural changes are made. If abortion continues to be a problem the dose will be decreased or the fetuses will be inoculated at a later stage of gestation.

D. Monitoring of the Inoculated Animals.

One of the major goals of our project is to produce a better understanding of the pathogenesis of bovine leukemia. By routinely testing every inoculated calf and fetus once each month from the time of its birth until it dies from leukemia, we hope to get a clearer picture of the events that occur prior to the development of leukemia. By carefully searching for certain changes in the lymphocytes we expect to show that reliable indications of ensuing leukemia can be detected considerably before the appearance of persistent lymphocytosis.

We are currently monitoring the inoculated calves and fetuses to detect changes in the following: (1) The complete blood count which includes the packed cell volume, grams of hemoglobin per 100 cc's., red cell count, white cell count, differential count, and the total proteins in grams per 100 cc's. (2) The percentage of lymphocytes showing nuclear pockets. (3) The percentage of cultured lymphocytes with C-type particles adherent to their surface. (4) The percentage of cells with a non-diploid chromosomal make up.

COMPLETE BLOOD COUNTS OF INOCULATED CALVES:

It is widely accepted that persistent lymphocytosis represents an early precancerous stage of bovine leukemia. By monitoring the complete blood count in all of our calves we will be able to see when this sign develops in relation to changes in the other parameters which we are simultaneously studying. In doing this, we hope to show that reliable indications of leukemia appear prior to the development of a persistent lymphocytosis.

RESULTS OF COMPLETE BLOOD COUNTS:

A detailed description of the results of all complete blood counts is beyond the scope of this report. Thus we will consider only the absolute lymphocyte counts from the most recent samplings. These results are summarized in Table II.

TABLE II

RESULTS OF THE COMPLETE BLOOD COUNTS

<u>Calf No.</u>	<u>Age</u>	<u>Type of Inoculum</u>	<u>Lymphocytes per mm³</u>
202	15.5 mo.	Oak Ridge	7,600
203	15.5 mo.	Oak Ridge	8,300
222	10.5 mo.	Batch type	8,700
245	8.5 mo.	Batch type	7,200
301	3.0 mo.	CF-35	4,500
302	2.0 mo.	CF-35	3,700
303	2.0 mo.	CF-35	4,500

As can be seen from Table II, none of the inoculated animals are currently lymphocytotic. We do not know when lymphocytosis will appear but we would predict that the time of appearance will be directly related to the number of biologically active C-type particles administered. Thus we would predict that those animals inoculated with the CF-35 processed material will become lymphocytotic at an earlier age than those receiving inoculum prepared at Oak Ridge or in the batch type rotor.

NUCLEAR POCKET STUDIES IN INOCULATED CALVES:

Nuclear pockets have been shown to occur with increased frequency in peripheral blood lymphocytes of lymphocytotic²⁶ and leukemic cattle as opposed to normal animals. A study of 53 Holstein bulls has shown that animals with normal lymphocyte counts frequently display appreciable²⁶ numbers of lymphocytes with nuclear pockets. PHA stimulated cultures of peripheral blood lymphocytes from lymphocytotic cattle invariably produce C-type virus particles.²⁷ This study demonstrated that peripheral blood lymphocyte cultures from nonlymphocytotic animals will produce virus if at least 0.5% of the lymphocytes contain nuclear pockets. An extrapolation of the plot of the number of virus particles per cell against the frequency of lymphocytes containing nuclear pockets in a blood sample indicate that some threshold number of nuclear pockets must be present for C-type virus particle production. When nuclear pocket frequency in lymphocytes falls

below 0.5% of the cells, the cell cultures may contain so few virus producing cells that the number of particles are so few that they are usually not observed with the electron microscope. On the basis of the above information one can hypothesize that lymphocytic nuclear pockets and C-type virus production should antedate the onset of lymphocytosis and tumorous leukemia in the course of our transmission studies.

RESULTS OF NUCLEAR POCKET STUDIES:

To date, lymphocyte samples from five of the inoculated calves have shown nuclear pockets. In three of these animals (202, 302 & 303) the percentage of lymphocytes showing such pockets has been greater than 0.5%. We consider this level significant and expect these animals to show significant numbers of C-type particles in the near future. As expected, nuclear pockets are appearing earlier in calves inoculated with the more concentrated material prepared in our continuous flow ultracentrifuge.

DEMONSTRATION OF C-TYPE PARTICLES IN INOCULATED ANIMALS:

Earlier in this report we present our evidence indicating that persistent lymphocytosis and the ensuing leukemic condition are the manifestations of a viral infection and we show that this C-type virus can be consistently demonstrated in both conditions but not in normal nonlymphocytotic cattle. We have also described the relationships we find between the percentage of lymphocytes showing

nuclear pockets, the presence of C-type particles and persistent lymphocytosis. From all of our evidence we believe that an increased percentage of nuclear pockets combined with the demonstration of C-type particles in mitogen stimulated cultures is a reliable early sign of ensuing bovine leukemia. To substantiate this claim and to determine the sequence of events leading up to the tumorous stage of leukemia, we are monitoring all inoculated calves for the appearance of C-type particles.

In monitoring the inoculated animals for the production of C-type particles we expect to show a latent period during which no C-type particles can be demonstrated followed by a period in which the percentage of cells with adherent particles gradually increases. We would predict that the latent period will be the shortest in those calves receiving inoculum prepared in the CF-35 rotor.

RESULTS OF OUR ATTEMPTS TO DEMONSTRATE C-TYPE PARTICLES IN INOCULATED ANIMALS:

Initial preinoculation samples from all calves showed no C-type particles and as expected the early samples were also negative. Also, as expected, the length of this latent period was the shortest in those animals receiving the more potent CF-35 prepared inoculum. Currently viral like particles have been observed in lymphocyte cultures of every inoculated animal with the exception of the three animals inoculated within the past month. Somewhat unexpectedly, the percentage of cells showing adherent C-type particles was initially low and has

remained low in all inoculated animals. However, it must be remembered that these studies are still in the early stages with the oldest calves inoculated with the CF-35 prepared material still less than four months old.

CHROMOSOMAL STUDIES ON INOCULATED CALVES:

Although chromosomal abnormalities are commonly found in histopathologically confirmed cases of bovine leukemia,²⁸ no single change in the chromosomal pattern is consistent enough to be diagnostic for this disease. However, since most of the recognized bovine chromosomal abnormalities do involve a change in the number of chromosomes, we have chosen to monitor this parameter in our inoculated animals. We predict that chromosomal abnormalities will be observed before tumors develop. It is our goal to determine when these chromosomal abnormalities first appear and to relate their appearance to any changes found in the other parameters which we are monitoring.

RESULTS OF CHROMOSOMAL STUDIES ON INOCULATED CALVES:

In monitoring the inoculated calves for chromosomal changes, both hypodiploid and hyperdiploid chromosomal spreads have been observed. Hypodiploid spreads have been very rare and may be due to the occasional loss of a chromosome in the slide making process. Hyperdiploid spreads, on the other hand, are not artifacts and are found quite frequently. Most of these hyperdiploid spreads have been tetraploid or near tetraploid with only a few showing chromosomal numbers in the near diploid range. These hyperdiploid spreads are found in both preinoc-

ulation and postinoculation samples, thus they cannot be attributable to an infection of the calves by our C-type particles. However, we are finding a higher percentage of tetraploid and near tetraploid spreads as our study progresses and we do expect to find significant changes in the chromosomal patterns before tumors develop.

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