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

PROGRESS REPORT NO. 11 TO THE U. S. ATOMIC ENERGY COMMISSION

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MASTER

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I. PERIOD COVERED BY THIS REPORT:

This report covers the period from May 1, 1970 to April 30, 1971.

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 Michigan,⁹ 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. In Germany⁴ and Sweden⁵ 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^{1,2} phase of the disease. 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^{1,2,6,10,14} studied by many workers in this country and in Europe.

The Pennsylvania group strongly insists that heredity has a major role in the natural occurrence of lymphocytosis and tumor in high¹⁰ incidence herds. 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.

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 immuno-diffusion and immunofluorescence tests for this virus. In
22
December of 1969 Miller et al. 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 lympho-
23
cytosis. 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 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 10 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 leukemias 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
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 1970-71 contract year:

1. Virologic and Serologic Studies

a. To continue the purification and concentration of C-type virus to provide virus material for transmission and other studies by the following approaches:

1) To continue to culture lymphocytes from leukemic and lymphocytotic animals and to study different methods of the release of C-type virus from lymphocytes as a source for the concentration of C-type virus.

2) To continue studies of adaptation of C-type virus to bovine and other species cell lines and using this virus as a source for concentration of virus particles.

b. Determine the physical and chemical characteristics of the C-type virus.

c. Develop and utilize serologic tests to determine extent of the presence of C-type virus in selected dairy cattle herds.

- d. Attempt to determine the biological and immunological relationship between the syncytial and C-type virus.
- e. To study a helper virus model system in bovine leukemia utilizing a bovine sarcoma-fibroma cell line recently established in our laboratories and C-type virus isolated from bovine leukemia cases.

2. Transmission Studies

- a. 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.
- b. To adapt monitoring techniques such as virus recovery, immunodiffusion, immunofluorescence or other methods or parameters of infectivity in inoculated animals.

3. High Incidence and Control Herd Studies

- a. To obtain additional data on the natural outcome of lymphocytosis and nonlymphocytosis 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 use immunologic procedures described under virologic and serology studies to investigate immunologic differences among cattle in high incidence herds.
- f. To provide a well defined source of animals for transmission studies.

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 social economic influence on the reporting of bovine leukemia advised by an Atomic Energy Commission Review Committee (1966) has 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. The data and progress will be reported under the following headings:

- A. Virus Isolation Studies in Cattle
- B. Virus Purification and Concentration Studies
- C. Cell Culture and Serologic Studies
- D. Ultrastructure Studies
- E. Transmission Studies
- F. High Incidence and Control Herd Studies

A. Virus Isolation Studies in Cattle

Lymphocytes were separated from defibrinated fresh whole blood employing the modified silicone method described in the attached manuscript. Processed lymphocytes were suspended to a concentration of 1 million cells per ml. in media 199 containing 20% bovine fetal serum, antibiotics, and a final concentration of 2% PHA. After 72 hours of incubation at 37° C, pellets were prepared for examination in the electron microscope.

The results of the viral isolation studies for the period May 1, 1969 through April 30, 1971 are shown in table I and 2.

Table 1. Results of Viral Isolation Attempts
During the Period May 1, 1970 through
April 30, 1971

<u>Hematological Classification</u>	<u>Positive</u>	<u>Negative</u>	<u>Total No. of Animals</u>
Leukemia	11	0	11
Persistent Lymphocytotic	1	0	1
Normal (non lymphocytotic from non-leukemic Herd)	0	4	4
	—	—	—
Total	12	4	16

Table 2. Cumulative Results of Viral Isolation
Attempts from April 1969 through
April 30, 1971

<u>Hematological Classification</u>	<u>Positive</u>	<u>Negative</u>	<u>Total No. of Animals</u>
Leukemia	20	2	22
Persistent Lymphocytotic	9	0	9
Normal (non-lymphocytotic from non-leukemic Herd)	0	14	14
	—	—	—
Total	29	16	45

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.

In the course of the last contract year three animals previously reported as lymphocytotic and positive for C-type particles became leukemic. In each case they remained positive for C-type particles. These three well studied animals represent the first experimental evidence in support of our hypothesis that C-type particles together

with a persistent lymphocytosis represents a prodromal stage of leukemia. Our present working hypothesis is that an increase in the percentage of lymphocytes showing nuclear pockets followed by the isolation of C-type particles from mitogen stimulated lymphocyte cultures is the earliest reliable indication of bovine leukemia. We believe that this combination of events preceeds the development of the persistent lymphocytosis. In our transmission studies we expect to experimentally elucidate the sequence and timing of events leading up to bovine leukemia.

B. Virus Purification and Concentration Studies

Virus concentration procedures have been carried out to purify and concentrate the C-type particles for studies on their morphology physical characteristics, serological properties, and principally to provide source material for cell free transmission studies. As described in the 1969-1970 progress report, considerable effort was expended in trying to separate C-type particles from the closely associated cellular material. As a result of this study, the following procedure has been adopted in preparing cultured lymphocytes for zonal centrifugation.

Cultured lymphocytes are frozen directly in the culture media at -90°C . They are stored at this stage until the day of zonal centrifugation. At that time they are thawed down and sonicated at 20 kc/s (70 Watts for two to four seconds. Electron microscopic examination at this time shows few intact lymphocytes, but the virus particles appear undamaged. Many of the viral particles are still associated with small fragments of cellular material but we feel that harsher treatment results in the loss of viral viability.

Zonal Centrifugation

Viral purification and concentration by means of batch type zonal centrifugation is currently carried out as reported by Dutta in the attached paper. Using this technique we have been limited to a sample

size of 200 cc's per run. This has been a major problem in producing sufficient amounts of cell free material for transmission studies. Our progress in this respect will greatly increase in the near future as we have now acquired a continuous flow zonal centrifuge capable of processing 18 liters of material in a single run. This increased capacity to produce purified virus will also allow us to proceed with our serological and physical properties studies.

Preparation of Centrifuged Samples for Inoculation

The purified virus bands in the gradient between 35 and 42% sucrose. Our earlier experience has shown that the injection of material containing more than 5% sucrose can cause a severe reaction in the newborn calf. To eliminate this complication, we now dialyze the collected fractions in 0.1 M phosphate buffer pH 7.2 for 20 hours at 4°C. The dialysis bag is then placed in Polyethylene Glycol flakes (Ave. M.W. 20,000) at 4°C for 3 hours to further concentrate the virus. At this stage the sample is frozen and stored at -90°C. until use.

C. Cell Culture and Serologic Studies

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

Materials and Methods

Cell Culture Methods

Bovine fetal spleen, endocardial, embryo, kidney, lymph node, lungs and thymus cell lines in continuous culture were used. Bovine fetal

endocardial, spleen, kidney, and thymus cell lines were characterized by epithelial-type cells and bovine fetal lungs, lymph node, and embryo cell were characterized by fibroblastic-type cells. The trypsinized cell suspensions were mixed in tissue culture flasks in a ratio of approximately 1 to 5 with the lymphocyte culture suspension from 72 hour lymphocyte cultures containing abundant C-type virus particles. Also these cells were mixed in the same ratio with the frozen and thawed lymphocyte culture suspensions devoid of viable lymphocytes as monitored by trypan blue staining. They were incubated at 37C. for 2 to 3 hours after which Eagles MEM containing 20% bovine fetal serum was added to the flasks to dilute to a concentration of approximately 100,000 trypsinized cells per ml. The flasks were incubated at 37C., on the fourth day the medium was replaced and on the seventh day the first cell passage was done. The cell cultures were examined regularly for any cytological change and were serially passaged on every fifth day. From each passage, floating cells and attached live cells dispersed by trypsinization were pelleted together at 1,000 rpm for 10 minutes, processed for EM and examined for the presence of virus particles.

Serological Methods

Complement fixation, immunodiffusion, immunofluorescence and cytotoxic tests were performed by standard methods. Lymphocyte cultures from leukemic and lymphocytotic cows having abundant C-type virus particles provided the source of the antigen. Sera from leukemic and lymphocytotic cows, positive for C-type virus particles, and hyperimmune guinea pig and rabbit serum were used in the serologic tests. Hyperimmune sera were prepared by 6 weekly subcutaneous inoculations of frozen and thawed preparations of concentrated (50 X) lymphocyte cultures containing abundant

C-type virus particles. The bovine sera used were whole sera and sera fractionated in sephadex G 2000 columns. Lymphocyte cultures and sera from normal cows were used as controls.

Complement fixation tests were performed in tubes by the standard methods. Lymphocyte cultures containing abundant C-type particles were centrifuged and the cells were resuspended with the supernatant fluid to 1/50 of the original volume. These cell suspensions were then frozen and thawed or sonicated in a Bronson sonifier (model 125) at power setting 7 for 15 seconds and used as antigen. The amount of guinea pig complement used was 1.8 units. Complement fixation tests were also performed by Dr. Sarma from the National Institute of Health by microtechnic methods (1) using the above said antigens and sera. In addition CF tests were performed with concentrated bovine C-type virus preparation prepared by zonal centrifugation and broad reacting murine leukemia group specific antisera prepared in rats by the induction of transplanted murine sarcoma virus-induced tumor to determine any possible immunological relationship between bovine leukemia C-type virus and C-type viruses of other known leukemia of different species.

Immunodiffusion tests were performed as described (2) using the same antigens and sera used in the complement fixation test. In addition to the above antigens and sera, syncytial virus positive antigen and positive sera obtained from Dr. Van der Maaten (National Animal Disease Laboratory, Ames, Iowa) were used.

In immunofluorescence studies, gamma globulin from sera precipitated by 50% saturated ammonium sulfate was conjugated with fluorescein isothiocyanate. The unconjugated fluorescein was removed by passing the conjugate through sephadex G 25 column (3,4). The conjugates were absorbed with bovine liver powder before use. Smears of cell pellet

suspensions from the lymphocyte cultures prepared on coverslips were stained with the conjugates.

Cytotoxic tests were performed according to the procedure described (5)

Results

Cell Culture Studies

Attempts to adapt C-type virus to bovine cell lines were largely unsuccessful, cytological changes were not observed in inoculated bovine fetal embryo, lymph node, lung, kidney, and thymus cell lines. On EM examination virus particles were not observed in these cell cultures. Limited success was observed in bovine fetal spleen and endocardial cell lines inoculated with viable lymphocytes containing C-type virus particles. Many attempts to infect these two cell lines were made. In a few cases isolated cytological changes with rounding of cells and occasionally syncytial type changes were observed after 3 or 4 serial cell passages. Some of the cells became detached and floated in the medium. On EM no virus was observed in intact live cells. But, in the debris of degenerated cells occasionally there were isolated clusters of virus similar to bovine syncytial virus. In some instances a few isolated C-type virus particles were observed in the debris of degenerated cells. Serial passages of these two cell lines were done in an effort to increase the number of C-type virus particles but was unsuccessful, in fact the number of C-type particles decreased and was lost with subsequent passages.

Similar studies with these two cell lines using frozen and thawed lymphocyte culture suspension were also unsuccessful.

Serological Studies

The objective of the serological studies was to attempt to demonstrate immunologic relationships between virus rich lymphocyte culture antigen

and various sera. More specifically to develop a serologic procedure to determine infection with C-type virus particles.

Complement fixation tests with bovine lymphocyte culture viral antigen and sera from leukemia, lymphocytotic, and normal cattle resulted in no reaction. Also, there was no reaction with hyperimmune guinea pig and rabbit sera. The broad reacting murine leukemia group specific antisera reacted (titer 1:4) with the bovine leukemia C-type virus indicating a possible immunological relationship between C-type virus of bovine leukemia and C-type viruses of other known leukemia of different species.

The lymphocyte culture viral antigen and sera from leukemic, lymphocytotic, and normal cattle did not react in immunodiffusion tests, nor did the hyperimmune guinea pig or rabbit sera. Also, there was no reaction between this antigen and serum positive for syncytial virus antibody. Moreover, 7 lymphocytotic and 10 normal cows studied failed to react with syncytial virus antigen.

There was no immunological reactions evident in the immunofluorescence and cytotoxic tests using the lymphocyte culture viral antigen and sera from leukemia, lymphocytotic, and normal cattle.

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D. Ultrastructural Studies

The ultrastructural investigations of bovine leukemia carried on was directed into 2 areas: 1) Further study of the distribution of lymphocytic nuclear pockets among lymphocytotic and non-lymphocytotic cattle and their relation to C-type virus production, 2) A statistical analysis of the observable ultrastructural differences found between the peripheral blood agranulocytes of lymphocytotic cattle from multiple case herds and cattle with normal hemograms from leukemia-free herds.

1. Ultrastructural Studies of the Association of Increased Occurrence of Agranulocytic Nuclear Pockets in Peripheral Blood of Cattle with the Appearance of C-type Particles in Cultured Blood Lymphocytes

Introduction

In previous work in our laboratory (Weber, et al., J. Nat. Cancer Inst.: 43:1307-1315, 1969) it was found that the incidence of lymphocytic nuclear pockets was highly significantly increased ($P < 0.001$) in animals from multiple case herds with persistent lymphocytosis, and in those showing signs and symptoms of clinical bovine leukemia as versus animals from leukemia-free herds with normal hemograms. But, a few animals with normal hemograms from herds with a history of leukemia also showed a mild to marked increase in the incidence of nuclear pockets. It thus appeared that an increased incidence of nuclear pockets may occur before the event of persistent lymphocytosis.

Previous work in a parallel study at Minnesota had shown that lymphocytes of leukemic and persistently lymphocytic cows

... would produce C-type leukemia virus particles in 72 hours PHA stimulated cell cultures. This finding and results of our other studies suggested the hypothesis that nuclear pockets may signify the presence of leukemia virus infected cells.

We were interested in knowing the minimal level of lymphocytic nuclear pockets at which it would be possible to propagate C-type particles from cultured lymphocytes in any given animal. Information on this point is important to obtain because of a possible use of an increased occurrence of lymphocytic nuclear pockets as a diagnostic test for bovine lymphocytic leukemia.

To obtain information on this point, a herd of 59 Holstein bulls was selected, which had a history of one recent case of bovine lymphocytic leukemia, but where all animals were known, in general, to have normal hemograms, and to be generally clinically healthy.

Design of the Experiment

a. A hematological survey was made of all of the animals in the herd to determine their status with regard to the lymphocytosis key.

b. Simultaneously with the hematological survey, lymphocytic nuclear pocket incidence values were determined for each animal by means of the Anderson buffy coat technique.

Determinations were made by EM evaluation of thin-sectioned profiles of 500 lymphocytes at random per animal.

All animals with 1% and above of lymphocyte profiles having one or more nuclear pockets, were placed in the

positive category. The level of 1% was chosen arbitrarily on the basis of the results of our previous work. A matching number of animals from the same herd with the lowest values for these anomalous forms were similarly studied as controls. Both groups were re-evaluated 3 to 6 months later.

c. Lymphocyte culture preparations were made, using silicone isolation technique of Joel and associates in 1969, and pelleted material from 72 hour cultures was embedded in Epon-Araldite. Thin-sectioned material was evaluated for the presence of cell associated C-type virus particles. For this purpose 50-75 cells were observed in blind studies at random and determinations were made of a) the % of cells which had associated virus C-type particles and b) the average number of C-type particles per involved cell.

Results

The hematological survey of the bulls used in this study showed none to be lymphocytotic, i.e., in Type III category of the lymphocytosis key on the basis of only 2 hematological observations 3 to 6 months apart. One animal (#H238) had a slightly elevated lymphocyte count that fell just beyond the confidence limit for the Type I or normal hemogram category (Fig. 1)

Fig. 1 Minnesota Lymphocytosis Key scattergram with 5% and 1% confidence limits showing lymphocyte absolute values in relation to age.

Eight bulls showed lymphocytic nuclear pocket counts in the 1% and above category (1.0-4.3%; see Table 1). In a few instances monocytes were observed to contain nuclear pockets; these values were excluded from the counts on the basis that definitive monocytes were also excluded from the counts.

In the lymphocyte culture studies it was found that all animals with incidences of lymphocytic nuclear pockets above 1% (1.0-4.3%) consistently had lymphocyte associated C-type particles (Table 1) while those with less than 1% (0.0-0.4%) were essentially negative in this respect. Only one C-type virus particle was observed in association with one cultured lymphocyte in the blood of one of the 8 animals studied.

The results of this study when combined with previous results indicate that the occurrence of increased incidence of lymphocyte nuclear pockets coincides with that of lymphocytosis, and most likely precedes it. It also indicates that the ability of cultured lymphocytes to produce C-type leukemia virus particles is related to the presence of nuclear pockets in these cells. These results lend support to the proposition that the occurrence of an increased incidence of nuclear pockets may be efficacious as a diagnostic test for incipient bovine lymphocytic leukemia.

Table 1. Association of the Occurrence of Agranulocytic Nuclear Pockets and the Production of C-type Virus Particles in PHA Stimulated Lymphocyte Cultures from Cattle with Normal Hemograms.

Group	An.No.	Age (Yrs)	Nuclear Pocket Counts		Virus Particles in Lymph. Cultures	
			Initial	Second	Cells* with Assoc. Virus Particles Virus (%)	Per Cell (Av.)
I Controls (0.0-0.9% Nuclear Pockets/Lymphocyte Thin Section Profile)	H219	6	0.0	0.4	2	1
	H227	5-1/2	0.2	0.8	0	0
	H233	2-1/4	0.4	0.2	0	0
	H235	2-1/4	0.2	0.6	0	0
	H243	2	0.0	0.0	0	0
	H320	4-3/4	0.4	0.4	0	0
	H337	2-1/2	0.0	0.2	0	0
	H339	2-1/2	0.3	0.2	0	0
	H340	2-1/2	0.2	0.6	0	0
Averages			0.2	0.4	0	0
II 1.0% and Above Nuclear Pockets/ Lymphocyte Thin Section Profile	H193	12	1.4	1.2	28	7
	H220	5-1/3	4.3	2.8	30	4
	H223	7-1/4	1.4	0.6	12	3
	H237	2-1/2	1.3	0.4	17	6
	H238	2	3.5	2.3	27	5
	H239	3	2.8	3.6	23	3
	H327	4	1.0	0.6	2	1
	H335	3	2.6	2.2	48	2
Averages			2.3	1.7	23	4

* 50-75 cells observed

2. A Correlative Study of the Occurrence of Nuclear Pockets in Peripheral Blood Lymphocytes in Cattle Classified According to the Bendixen Key

A cooperative study of the relationship between an increased incidence of nuclear pockets in lymphocytes of peripheral bovine blood and lymphocytosis was initiated by the Department of Veterinary Anatomy and the Danish Serum Laboratory in cooperation with Dr. Hans Bendixen of Copenhagen. The objective of this study was to investigate the feasibility of using the increased incidence of nuclear pockets in the peripheral blood lymphocytes of cattle as a criterion for the recognition of the prodromal phases of bovine leukemia available.

The pilot study was conducted on 70 animals in seven herds of cattle. The nuclear pocket counts were conducted in such a manner that the observer was not aware of the herd of origin of the individual cows nor of the hematologic status of either the herd or the individual cows.

Based upon previous studies of normal, lymphocytotic, and leukemic cattle arbitrary classifications were given to animals on the basis of the percent of lymphocytes which showed nuclear pockets. If less than 1% of the lymphocytes had nuclear pockets the animals were classified as negative, between 1.0 and 2.0% of the lymphocytes had nuclear pockets the animals were classified as positive. The results of the study are summarized in the table below.

Correspondence of results of lymphocytic nuclear pocket and persistent lymphocytosis evaluations in a blood study of 80 cows selected from 8 Danish dairy herds.

<u>Lymphocytic Evaluation</u>		<u>Nuclear Pocket Evaluation</u>		
<u>Bendixen Group</u>	<u>No. of Animals</u>	<u><1%</u>	<u>1-1.9%</u>	<u>2% and></u>
Group I	$\boxed{53}$	$\boxed{43}$	7	3
Group II	$\triangle 8$	4	$\triangle 1$	3
Group III	$\odot 19$	1	0	$\odot 18$
% correspondence		$\square 43/53=82$	$\triangle 1/8=12\%$	$\odot 18/19=95\%$

Eight of the animals in Bendixen's Group I had nuclear pockets in peripheral blood lymphocytes. Six of the seven suspicious were found in herds considered leukemia free by Bendixen. The other suspect animal was found in a herd in which several of the animals were lymphocytotic. The single Group I animal classified positive on the basis of nuclear pockets was in a leukemia free herd in an area of Denmark known to be free of leukemia. The animals classified Group II by Bendixen showed no correlation with the incidence of nuclear pockets. Eleven of 12 animals in Bendixen's Group III were scored positive on the basis of nuclear pocket incidence. The other animal was negative on the basis of the nuclear pocket count.

The concordance of lymphocytosis and the incidence of nuclear pockets in peripheral blood lymphocytes is striking but not absolute. The reasons for the discrepancies are not obvious. Certainly leukemia is not the only cause of the anomalous nuclear configuration and it seems probable that leukemia is not the only

cause of lymphocytosis in cattle. These results, in addition to those cited above, indicate that nuclear pockets have considerable potential as a diagnostic tool. Refinement of the technique will be dependent upon further investigation of the occurrence of nuclear pockets in peripheral blood lymphocytes of cattle and upon basic virologic and cytologic study of these anomalous structures.

3. Nuclear Pockets in Lymphocytes of Peripheral Blood and Lymph

A comparison of the incidence of nuclear pockets in lymphocytes of the efferent lymph from the prescapular lymph node and of the peripheral blood of preleukemic cattle was studied in four lymphocytotic cows which were under constant hematologic and clinical observation in our isolations facilities. Buffy coat leukocytes were obtained from electron microscopy by simple centrifugation of whole bovine blood drawn from the external jugular vein. Immediately following the collection of the blood sample, the efferent duct of the prescapular lymph node was cannulated and 10 ml. of efferent lymph was taken for ultrastructural study. Separate samples of blood and lymph were taken at the same time to determine the capacity of the lymphocytes to produce C-type virus in PHA stimulated cultures. The results are summarized in the table below.

<u>Cow No.</u>	<u>% of Lymphocytes with Nuclear Pockets</u>		<u>C-type Virus Production</u>	
	<u>Blood</u>	<u>Lymph</u>	<u>Blood</u>	<u>Lymph</u>
L3497	6.6	0	+	-
20	1.7	0	+	-
2	3.3	0	+	-
11	2.8	0.2	+	Occasional C-type particles observed

The data indicate that lymphocytes with nuclear pockets do not recirculate through the peripheral lymph nodes from the blood and only rarely are they present in the efferent lymph from such nodes. All the peripheral blood lymphocyte cultures produced virus while only the sample of lymph which contained very few nuclear pockets produced C-type virus.

4. The Ultrastructure of Peripheral Blood Agranulocytes in Apparently Normal Cattle Selected from Leukemia and Leukemia-Free Herds

The ultrastructural study of the agranulocytes of lymphocytotic cattle from multiple-case herds and nonlymphocytotic cattle from leukemia-free herds was based on sampling of buffy coat leukocytes obtained by simple centrifugation of whole bovine blood.

The peripheral blood agranulocytes of 5 normal and 12 lymphocytotic cows were scored for the presence, absence, or number of selected ultrastructural components visible on the viewing screen of an electron microscope. The agranulocytes were placed in one of 5 morphological categories on the basis of their ultrastructural characteristics. The resulting data was analyzed statistically to determine if there were any significant differences of numbers of lymphocytic and monocytic cell types between normal and lymphocytotic cattle. Tests were also performed to find if there were significant differences in the ultrastructural characteristics within each cell type between normal and lymphocytotic cows.

The differences in agranulocytic cell numbers between normal and lymphocytotic cows were as follows:

1. The proportion and absolute number of inactive lymphocytes was higher in lymphocytotic cows as compared to the normal.
2. The number of active lymphocytes, while higher in absolute number, were present in equal proportion in both groups of animals.
3. The proportion and absolute number of monocytes were decreased in lymphocytotic cows as compared to normal animals.
4. An increase in the absolute number of monocytes per cmm of normal bovine blood was observed when the computation was done with the differential count results obtained with the electron microscope as opposed to the light microscope.

Significant differences between the inactive lymphocytes of normal and lymphocytotic cows were found to involve the following ultrastructural features:

1. The nuclei of the inactive lymphocytes of lymphocytotic cows were less pachychromatic than those of normal animals.
2. Nucleolar profiles were visible in a greater proportion of the inactive lymphocytes from lymphocytotic animals than from normal cows. The number of inactive lymphocytes containing long, flat sacs of granular reticulum were increased in the lymphocytotic cows compared to the normal animals.
3. The number of cytoplasmic, or azurophil granules, were reduced in the inactive lymphocytes from lymphocytotic animals.

4. Mitochondrial profiles were present in increased numbers in the inactive lymphocytes from lymphocytotic animals.
5. The artifact characterized as membrane separations were found only in inactive lymphocytes of lymphocytotic cattle.
6. Cell to cell contacts occurred with increased frequency between lymphocytes in the lymphocytotic animals compared to the normal cows.

The observed differences of the active lymphocytes between normal and lymphocytotic cattle were nearly identical to those observed between the inactive lymphocytes. There was the additional observation that the nuclear bodies were observed more frequently in the lymphocytic nuclei of lymphocytotic cows.

The only significant difference between the monocytes of normal and lymphocytotic cows was that the mitochondrial profiles per cell were fewer in the lymphocytotic animals.

The general conclusions which can be drawn from the data are listed below.

1. The ultrastructural differences observed between the agranulocytes of normal and lymphocytotic cows tend to reflect morphologic changes which are consistent with changes generally associated with neoplasia.
2. The increased number of mitochondrial profiles in the lymphocytes from lymphocytotic cows could reflect the metabolic shift to aerobic glycolysis characteristic of leukemic lymphocytes.
3. There are morphologic indications of increased immunologic activity in lymphocytotic cattle.

E. Transmission Studies

It was proposed last year that transmission studies would be initiated utilizing defined cell free and virus rich inoculum. The experimental animals in these studies are of two groups 1) immunologically incompetent (60-90 day old) fetuses of defined parentage and 2) immunologically immature colostrum deprived newborn calves of defined origin. These studies have been initiated during the past year and have progressed within the limitations of the availability of viral inoculum, equipment for viral concentration and experimental animals.

The following report on these studies will include dosage considerations and progress.

1. Dosage for Inoculated Calves

In our cell free transmission studies it is highly desirable to meet the following dosage criteria.

- a. The dose should be large enough to produce leukemia in 100% of the calves inoculated.
- b. The dose should be large enough to produce leukemia with a reasonably short latent period, yet be small enough to make it feasible to inoculate at least ten newborn calves.
- c. The dose should be defined to the extent that it can be reproduced in this laboratory and 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 article (1) 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 mouse to give a 100% incidence of leukemia. (1) Maintaining

this per gram dosage level in newborn Holstein calves would require 1.82 gram equivalents.* We have decided to increase this to 2.10 gram equivalents as additional insurance in reaching a 100% incidence level.

Latent Period

From Moloney's Table 1, the average latent period** in a mouse receiving the above calculated dosage is 4.0 months. If the life span of the laboratory mouse is taken as 2.5 years and that of Holstein calves at 20 years, then with this dosage level we would expect a latent period of 32.0 months in the calves. However, we feel that one gram of tissue from our mitogen stimulated lymphocyte cultures contains 10 to 100 times as many C-type particles as a gram of the tumorous lymph node tissue used by Moloney. From Moloney's Table 1, our latent period calculated at this 10X to 100X level of dosage becomes 24.0 to 27.0 months, which is quite reasonable.

Feasibility of Inoculating Ten Newborn Calves

In our 72 hour culture system, 100 cc of media produces 0.07 grams of tissue. Therefore, to obtain the 2.1 gram equivalents needed to inoculate one calf, we must have 3000 cc of culture. The 30.0 liters of culture needed to inoculate 10 calves is readily within our present production capabilities.

- * For this calculation the weight of a newborn mouse was taken as 1.25 grams, that of a newborn Holstein calf as 45.5 Kg.
- ** Latent period equals the time from virus inoculation to death from leukemia.

Defining the Dose

The number of gram equivalents per calf is a very rough measurement of the viral dose because, in culture, viral production is extremely dependent upon the source of viral containing lymphocytes. Thus, to better define the dose, we are using the following procedure. After 72 hours of incubation, the total number of viable cells is determined. At the same time a pellet is made for examination in the electron microscope. During the electron microscopic examination the percentage of viable cells in close association with C-type particles is determined. The results are expressed in terms of:

$$\frac{(\text{Total \# of viable cells}) \times (\% \text{ of cells producing C-type particles})}{\text{Total \# of cells producing virus}}$$

Usually this formula the 2.1 grams of cultured cells processed for the inoculation of a newborn calf contains on the average 166×10^6 virus producing cells. For the fetal inoculations we use a dosage level calculated to give the same amount of virus per gram of body weight as given to the calves.

2. Progress in Terms of Material for Calf Inoculation

At the present time we have enough viral producing lymphocytes cultured and processed by zonal centrifugation to inoculate two additional calves. We also have cultured, but not processed, enough material for the inoculation of five additional calves. Three calves have already been inoculated, thus with the material now on hand we can reach our ten calf goal.

3. Newborn Calf Inoculations

The newborn calves for these studies are obtained from defined control herds which are discussed later in this report. Additionally, lymphocyte cultures from the dams of these calves have been negative for C-type virus particles.

During the past year three newborn calves have been inoculated at the above stated dosages. Two additional calves will be inoculated in the very near future since two additional calf doses have been prepared recently. Additional calves will be inoculated as suitable calf doses are prepared.

The inoculated calves are presently all in apparent normal health. Numerous hematological examinations of these calves have also, in general, been within normal limits. Lymphocyte cultures from the two earliest inoculated calves have not been found to contain C-type virus particles.

4. Intrafetal Inoculation Studies

The cows used in the intrafetal inoculation studies are also obtained from the defined control herds. These cows were additionally demonstrated to be negative for C-type virus particles on examination of several lymphocyte cultures. These cows are artificially bred to bulls which also were negative for C-type virus particles on examination of lymphocyte cultures.

Presently, four of the above defined cows are bred or are being bred at the college in preparation for intrafetal inoculations. These inoculations will be made when the fetuses are 60 to 70 days old. Additional pregnant cows will be available from the source herds and their fetuses inoculated as fetal viral doses are prepared.

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F. High Incidence and Control 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. The present 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. Two "new cases" occurred in three of the herds. During the past ten years of herd studies a total of 32 "new cases" of leukemia have occurred in herds which had been under study. Twenty-nine (90%) 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 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 studies.