MAR 18 1968

C00-910-6

BOVINE LYMPHOCYTIC LEUKEMIA: STUDIES OF
OCCURRENCE AND DISTRIBUTION INCLUDING
INVESTIGATIONS OF FAMILIAL AND ENVIRONMENTAL
FACTORS WITH SUPPORTING CLINICAL, HEMATOLOGIC,
PATHOLOGIC, INDUCTION AND TRANSMISSION STUDIES

MASTER

PROGRESS REPORT NO. 8 TO THE U.S. ATOMIC ENERGY COMMISSION
ON RESEARCH PERFORMED UNDER CONTRACT AT (11-1)-910
1967 - 1968

Principal Investigator

D. K. Sorensen, Department of Veterinary Medicine

Co-Investigators

R. K. Anderson, Department of Veterinary Bacteriology and
Public Health
V. Perman, Department of Veterinary Pathology and Parasitology
R. E. Shope, Department of Veterinary Medicine
A. F. Weber, Department of Veterinary Anatomy

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

February 1, 1968

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

fly

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

TABLE OF CONTENTS

	Page
I. Period Covered By This Report	1
II. Background Information	1
III. Progress Report	9
A. General Information	9
B. Epidemiologic Studies	11
1. General	11
2. Socio-economic Influences of Reporting of Bovine Leukemia	12
3. Estimates of the Incidence of Bovine Leukemia in Minnesota	18
4. Five Year Record	24
C. Multiple Case Herd Studies	25
1. General	25
2. Genetic Studies	26
3. Cohort Analysis	34
D. Hematologic Studies on Single Case Herds and Normal Herds	41
1. Analysis of Select Normal Herds	41
2. Analysis of Random Cattle Populations in Three Minnesota Counties	41
3. Hematologic Studies on Single Case Herds of Juvenile and Adult Type Leukemia	41
E. Studies on Animals with a Marked Lymphocytosis Originating From Multiple Case Herds	43
1. Studies on the Promotion of Tumor Growth or Induction in Lymphocytosis Animals	43
2. Immunoresponsiveness of Leukemic Animals	46
3. Cytokinetic Studies of Lymphopoiesis	51
4. Ultrastructure Studies	53
5. Chromosome Studies	57
F. Transmission Studies	59
1. Intrafetal Inoculation	59
2. Foster Nursing Experiments	60

I. PERIOD COVERED BY THIS REPORT:

This report covers the period from December 1, 1960 to December 31 1967. In certain sections only data from the past year is presented.

II. BACKGROUND INFORMATION:

Because of a lack of any definitive knowledge of the epidemiology of this disease in the United States, this current study was initiated to investigate the occurrence and distribution of leukemia in cattle and the relationship of environmental or familial factors to its occurrence in the state of Minnesota and parts of Wisconsin. This was the only comprehensive study of this type being conducted in the United States. Currently more limited studies are being done in Pennsylvania, California, Michigan and Illinois.

During 1964 investigators at Michigan State University reported on epizootiological studies of bovine leukemia. They reported data from 304 laboratory confirmed cases of leukemia during this period of time.

There have been two other studies on the occurrence of leukemia conducted in other countries of the world. The study by Bendixen of Denmark is of a similar nature and is comprehensive in regard to occurrence and distribution but does not include studies on the relationship of environmental factors to the occurrence of this disease. These studies, which were begun in 1952, indicate that in Denmark leukemia in dairy cattle does not show a uniform distribution. The occurrence prior to a control and eradication program was 4.1 cases per 100,000 animals in the country as a whole. In 1956 Olson in Sweden started investigations on the occurrence of leukemia in herds of northern Sweden in which cases had been discovered at slaughter.

Currently, intensive epidemiologic and/or control and eradication

programs for bovine leukemia are underway in a number of countries, namely Great Britain, Denmark, West Germany, Poland, Russia, Czechoslovakia, Yugoslavia, Israel, East Germany and Brazil.

The criteria of lymphocytosis in a herd and individual animal basis is the principal parameter of measurement and is being used to diagnose and identify leukemia on a herd basis. Denmark and West Germany have initiated comprehensive control and eradication programs involving all cattle.

Preliminary unpublished data available indicates that lymphocytosis is not a feature of bovine leukemia in Great Britain, however, the adult form of the disease and multiple case herds with the adult form have not been observed.

In the other countries, the adult form of leukemia and multiple case herds are recognized. Preliminary unpublished and published reports substantiate the previous reports from European countries that lymphocytosis is a feature of bovine leukemia.

Other data on the distribution and prevalence of bovine leukemia in various countries is limited to meat inspection statistics and observations by veterinarians in practice.

Studies to determine the influences of environmental factors on the occurrence and distribution of leukemia in man are in progress at the School of Public Health, University of Minnesota. Cooperation has been established with this group of investigators for purposes of integration and exchange of information. Although it is realized that bovine lymphocytic leukemia and the human leukemias are not known to have identically significant etiologic relationships, it is appropriate to ask if certain common environmental factors in this region are acting on animals and man to influence the occurrence and distribution of the two disease entities to produce a high incidence in both man and cattle.

Results of the first year's study were largely preliminary in nature. The cooperation of practicing veterinarians in the study area was enlisted, standardized reporting and laboratory procedures were developed as well as a criteria for diagnosis. Data on the normal cattle population in the study area was compiled to enable us to evaluate the data compiled on occurrence and distribution. More comprehensive information on the clinical manifestations were accumulated and practicing veterinarians were given this information to better recognize the disease. The data obtained on the occurrence, distribution and possible influence of environmental factors was preliminary and was not evaluated.

Data through the second year of the project revealed that 333 cases of bovine leukemia were reported and confirmed. This number of cases allowed us to study various factors associated with the occurrence of leukemia. These included disposal of cases, age incidence and rate of occurrence of cases according to the size of the herd. We were able to begin to evaluate the hematologic data, clinical manifestations of the disease and pathological and biochemical changes occurring in cases of leukemia.

Data through the third year of the project revealed that 668 cases of leukemia were reported and confirmed in the study area. Last year, 325 cases were reported which is 49% of the total cases for the first 29 months of the study. One significant finding was the increase in rate of occurrence which occurred with age. The rate per 100,000 in the two to five year old animals for the total period was 9.9 cases and this rate increased to 29.9 per 100,000 in the ten year old and over age group. This appears to be similar to the findings for lymphocytic leukemia and allied pathologic states in man. Another interesting finding has been that the data has shown that the rate of occurrence of reported cases

increases greatly as the number of animals in the herd increased. This higher rate in the larger herds may be suggestive of an infectious agent with horizontal rather than vertical transmission of an agent.

Data through the fourth year of the project revealed that 879 cases of leukemia were reported and confirmed in the study area. During the past year 211 cases were reported and confirmed. The data continued to support the previous years data that there was an increase in rate of occurrence with increasing age and that the rate of occurrence of reported cases increased greatly as the number of animals in the herd increased. Studies on a selected group of dairy herds in three counties of Minnesota to determine patterns of lymphocytosis of the peripheral blood were undertaken during the third and fourth year of the project. These studies involved 4,232 adult animals from 233 herds and represented approximately 10 per cent of the adult animals in the three counties. Results of these studies indicated that from 34 per cent to 54 per cent of the herds studied and from 5 per cent to 6 per cent of the animals have a characteristic lymphocytosis. These results were interesting and allowed us to speculate on the theoretical number of leukemia cases which can be expected in a given population. We were also able to increase our data on the other phases of the project and to evaluate some of the data on possible influence of environmental factors on the incidence of leukemia.

Data through the fifth year of the project revealed that 1,036 cases of leukemia were reported and confirmed in the study area. During the sixth year 157 cases were reported and confirmed. This was a 25 per cent decrease in confirmed cases, but it appeared to reflect a decrease in reporting rather than a decrease in actual incidence rates. The data continued to support the previous years data and conclusions in regards to the geographic distribution, and the increased rate of occurrence with

increasing age and in the larger size herds. In the multiple herd studies, 16 cases of leukemia have developed subsequent to the initiation of the study. All 16 of these cases have preclinical lymphocytosis. Approximately 15 per cent of all adult cows in the multiple case herds have a significant lymphocytosis. For the period of study the incidence rate of clinical cases of leukemia in the lymphocytosis group of cows is approximately one case for each 14 cows which certainly makes this a high risk group. It is further important to note that approximately 50 per cent of the cows with a persistent lymphocytosis are sold from the herd for reasons other than leukemia and thus never have an opportunity to develop the clinical disease. Data on the hematologic studies also revealed that multiple case herds had a higher percentage of animals with a lymphocytosis than control herds.

Data through the sixth year of the project revealed that 2,973 specimens and reports have been received from practicing veterinarians and 275 specimens were received from slaughtering plants. Of these 3,248 submissions, 1,434 were confirmed as cases of bovine leukemia, however, epidemiologic investigations were conducted on 1,231 of the confirmed cases. Results of studies of occurrence and distribution have not substantiated any significant increase in reported cases in Minnesota.

Epidemiologic data through the seventh year of the project, in regards to the reporting of bovine leukemia, has not changed from that accumulated through the sixth year of the project. A request from our leukemia research team for review and critical evaluation by a review team was granted. 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 and are as 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 herds and comparison of 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 lymphocytosis animals, to attempt to increase the incidence of tumor formation, to determine the nature and significance of ultrastructural differences in bovine lymphoid cells of normal, preleukemic and leukemic animals.
- 5.) Following suggestions of the Atomic Energy Commission site visit committee to initiate preliminary studies on attempts of transmission of bovine leukemia.

Data on the leukemia population was subjected to statistical analysis. These analyses treated leukemia frequency as the dependant variable. In most cases, analyses were run for each of the five years' figures indi-

vidually, prior to pooling the data for the entire period. Tests for homogeneity of variances were run in all critical instances.

These analyses indicate remarkable consistency of reporting of leukemia from year to year allowing for the pooling of data.

Data on the socio-economic factor, as they effect the reporting of leukemia, was obtained, tabulated but not analyzed.

Genetic and cohort analysis of index, new cases and lymphocytosis in a number of multiple case herds are extremely important. Although the role of genetic factors is not completely defined, it appears to be of lesser importance to the natural development of bovine leukemia. In contrast, cohort analyses indicate a remarkable association between index cases, new cases and lymphocytosis in cohort groups and the development of lymphocytosis or bovine leukemia among members of the cohort. The common factor to these cohort groups appears to be colostrum or milk fed commonly to members of the cohort.

The data continues to support the previous year's data and conclusion that:

- 1.) incidence rates of bovine leukemia are much higher than previously estimated.
- 2.) incidence rates increase greatly with increasing age of cattle.
- 3.) incidence rates increase greatly when cases are classified by size of herd of origin.
- 4.) the increased rate based on herd size is not related to the age factor.
- 5.) the incidence rates are consistent with the hypothesis of an infectious disease where the incidence is related to the number of susceptible animals added to the population each year.
- 6.) distribution of cases are not affected by geographical and environmental factors.

- 7.) Multiple case herds studies continue to indicate lymphocytosis as a pre-clinical manifestation of bovine leukemia. Cohort analysis of multiple case herd data suggest that natural transmission of bovine leukemia may be both vertical and horizontal by a leukemogenic factor in colostrum and/or milk from leukemia and lymphocytosis cattle.
- 8.) hematologic data of single case (adult type) herds indicate that 75 per cent or more of these herds are similar to multiple case herds.

III. PROGRESS REPORT:

A. General Information

The approach to the study and procedure used are partially described in some detail in Progress Report no. 1 and as modified in Progress Report no. 7 to the U. S. Atomic Energy Commission (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 through this report.

1. Single case data, submission or reports refer to information on specimens obtained from a case of leukemia in a herd where only one case was reported the past year.
2. Juvenile leukemia - a consistent clinical form of leukemic occurring in young animals usually under 6 months of age but up to 1 year.
3. Thymic-lymphosarcoma- a consistent clinical form of leukemia occurring in young animals from 6 months to 2½ years of age, approximately.
4. Adult leukemia - leukemia with variable clinical and pathologic manifestations occurring in animals usually over 2 years of age. The adult type typifies the type of leukemia found in multiple case herds.
5. Multiple case data refers to data obtained from herds where more than one case of leukemia (adult type) has occurred in one year.
6. Lymphocytosis - is referred to as a preclinical phase of the tumor form of bovine leukemia. The lymphocytosis is characterized by an elevation of the lymphocyte count above three

standard deviations greater than the normal count for animals of 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%) is usual. The lymphocytosis is not associated with any demonstrable pathologic process or tumor growth and appears somewhat analogous to the early stages of chronic lymphocytic leukemia of man.

The data will be presented under several sections as follows:

- B. Epidemiologic Studies
- C. Multiple Case Herd Studies
- D. Hematologic Studies on Single Case Herds and Normal Herds
- E. Studies on Animals with a Marked Lymphocytosis
- F. Transmission Studies

B. Epidemiologic Studies

1. General

Processing, analysis and interpretation of statistical data gathered in epidemiological studies carried out during the contract year was a primary aspect of these investigations. Information based on 1967 surveys was then related to previous surveys and findings.

The work involved the writing of a whole series of programs for the available digital computer facilities, in order to utilize the current and earlier data available on punched cards. Programs were written largely in the Fortran computer language, and a file of them is available. Use was also made of generalized available programs in the University Computing Center Library, where these could be exploited for the project. Library routines and functions in the Computer compiler were found very helpful in programming for the special aspects of the epidemiological problems considered. The programs were used to handle data on cards, transfer data to tapes, establish parameters for the populations under study, check the nature of the distribution of data variables, and find evidence relating various sample attributes and factors to each other and the reported incidence of bovine leukemia in Minnesota.

The study of the socio-economic factors involved in reporting of bovine leukemia in Minnesota is being analyzed and a portion of the data is being written up. This study involved the comparison of a sample of dairy farms reporting bovine leukemia in 1964-65 with a statistically paired series of farms with no reported incidence during this time. Comparisons were made for level of veterinary services,

farm management aspects, and herd environment.

Considerable time was required to monitor and check the punched data and also to edit the reporting forms as received from the interviewing team. Data collected in earlier years and used for comparison with the 1967 sampling were also subjected to careful scrutiny to eliminate probable sources of bias and error.

2. Epidemiology: Socio-economic Influences of Reporting of Bovine Leukemia

a) Veterinary Services.

The major effort was centered on the study of a sample of paired dairy farms, one member of the pair having reported bovine leukemia in the herd in 1964-65, the other having no report. Each pair was matched closely for locality, herd size and the grade of milk produced (A or B). A more detailed description of the procedures used in setting up the study, and the sampling method, is given in the attached copy of the first paper of the series of reports based on this study. The non-leukemia reporting member of each pair was selected randomly from farms falling into the proper herd size and milk grade categories in the same quarter-township. Selection of the non-reporting herd was made without reference to any current, past or future probability of bovine leukemia in the herd. A previous hematologic survey in three counties had indicated that a high probability exists of any particular herd having lymphocytosis and hence a clinical case of leukemia in any point in time.

Herds in this sample that reported leukemia in 1964-65 were shown to differ significantly from the non-reporting herds in the mean level of veterinary service. This support was measured in terms of the number of professional calls made by the veterinarian to the farm,

expressed either on a per herd or per cow basis. Professional service also included the mean level of expenditures per herd or per cow for veterinary services and medicines, and the degree to which the farm operator utilized the veterinarian's services. On the other hand, no difference could be shown between the reporting and the non-reporting herds for the mean cost of drugs administered to the cattle by the farmer himself.

The analysis showed that the differences between reporting and non-reporting herds were greatest in the part of the sample producing grade A milk. Differences were also large for the part of the sample composed of herds with 50 animals or more. (Comparisons here are with grade B milk producers and smaller sized herds, respectively). Since grade A milk production involves primarily the larger herds, the two classifications show considerable communality.

The differences in total animal health care associated with reporting of leukemia in dairy cattle indicate that the presence of a practicing veterinarian on the premises is essential for the diagnosis and reporting of individual leukemia cases. This can be seen in Table B-1.

The leukemia-reporting herds show a greater frequency of the higher levels of veterinary services in comparison to the non-reporting group. Levels of veterinary service may be ranked thus:

- (1) minimum level -- emergency calls only
- (2) intermediate level --
- (3) highest level -- regular preventive medicine program

The differential frequency distribution of the two groups for the three classes is highly significant ($\chi^2 = 15.81$, $P < 0.01$ for 2 degrees of freedom).

TABLE B-1

Comparison of Overall Means for Leukemia Reporting Herds
and Matched Non-leukemia Reporting Herds of Selected
Samples of Dairy Cattle in Minnesota
1966-67

	Reporting Herds *	Non-reporting Herds **
Veterinary calls per farm	16.20	9.63
per cow	0.56	0.34
Veterinary service level		
Emergencies, farms	12	34
Emergencies plus extra services	83	69
Regular preventive program	12	4
Dollar spending on veterinary services and drugs		
Per farm	\$ 235.41	\$ 144.03
Per cow	\$ 7.60	\$ 4.92
Dollar spending on non-veterinary drugs		
Per farm	\$ 26.01	\$ 26.84
Per cow	\$ 1.06	\$ 1.00
Total dollars spent for animal health		
Per farm	\$ 264.42	\$ 170.87
Per cow	\$ 8.66	\$ 5.92

* Bovine leukemia reported in 1964-65.

** Bovine leukemia not reported in 1964-65.

b) Management

Management data comparisons between leukemia reporting and non-reporting herds suggest that significant differences exist in a number of areas. For the leukemia herds, movement of milking animals off the farm was associated with low production, infertility and weight loss in greater measure than for the non-reporting herds. The veterinarian saw a higher proportion of the animals leaving the farm for these and other reasons in the reporting herds than in the comparison group.

In the matter of record keeping, there was a distinct superiority of the leukemic herds over the matching sample; this carried through for the degree to which the farm operator knew his animals by name. More animals were raised on the farms of the leukemic herds as compared to the non-reporting herds. Comparisons of leukemia reporting herds with non-reporting herds are given in Table B-2 for these and some other aspects.

The management data thus show no conflict with the concept of the "affluent cow" herd type being the one most likely to be associated with reporting of leukemia in comparison with herds of "lower economic status".

c) Herd Environment

Summarization of herd environmental data furnished means and frequency tables which are interpreted as supporting the overall hypothesis that leukemia reporting is associated with a level of economic support superior to that shown for the comparable non-reporting sample. Table B-3 shown a few of the more interesting comparisons between the two herd categories with regard to reporting.

The age differential with regard to animals of age 10 years and

TABLE B-2

Comparative Mean Status of Leukemia Reporting Herds in Relation to Matched Non-leukemia Reporting Herds of Selected Samples of Dairy Cattle in Minnesota, 1966-67, for Selected Management Attributes

Attribute of Leukemia Reporting Herds	Relationship > or < Non-leukemia Reporting Herds
Movement of animals off farm:	
For low production	greater frequency
Infertility	greater frequency
Weight loss	greater frequency
For use as milking animals in other herds	greater frequency
Record Keeping:	
Breeding history	more complete
Individual animal record	better
Production records	more detailed
Age of animals when moved off farm:	
4 years	more
between 5 and 9 years inclusive	lower frequency
10 years and over	higher frequency
Educational level of farm operator	slightly higher
Veterinary inspection at time animal moved off farm	greater frequency
Frequency of mastitis in herd	lower frequency
Animals known by name	greater frequency
Animals raised on farm	greater frequency

TABLE B-3

Comparative Mean Status of Leukemia Reporting Herds in Relation to Matched Non-leukemia Reporting Herds of Selected Samples of Dairy Cattle in Minnesota, 1966-67, for Selected Herd Environment Categories

Attribute	Relationship
Presence of non-bovine species	More frequent
Presence of chickens on farm	More frequent
Use of chlorinated hydrocarbons	More extensive*
Use of herbicides	More extensive*
Use of woodlot pastures	More frequent
Feeding of alfalfa brome hay	More frequent
Use of ponds as source of water	More frequent
Frequency of animals aged 10 or older	More frequent

* Highly significant

older seemed to be concentrated in the herd size class with 20 to 29 animals in the milking herd. This needs to be evaluated in relation to the findings of an earlier survey of Steele County which showed an association of older animals with smaller herds of less than 20 animals. The sample involved here had a low frequency of very small sized herds, so that a degree of reasonable doubt must be allowed here. Previous work has shown evidence of higher degree than expected of misuse of the chlorinated hydrocarbon type of insecticides on leukemia reporting dairy farms.

The socio-economic data have not been completely analyzed with respect to age of the animals, herd size and their possible interaction in relation to the reported incidence of leukemia. In earlier progress reports, a linear increase of incidence rate with age of animal at risk was noted. Likewise, a marked increase in incidence ratio was found to be associated with increased herd size. The relationship of age and herd size to other socio-economic factors is under study to determine the possible influences of these two independent variables on leukemia reporting.

3. Epidemiology: Estimates of the Incidence of Bovine Leukemia in Minnesota.

a) Based on the 1967 Socio-economic Study.

This study, already discussed from the standpoint of findings relating to the reporting of bovine leukemia, may be useful in obtaining an estimate of the true incidence of the disease in the state. The 107 pairs of herds studied were confined to three of the dairy oriented livestock districts of the state, namely F, J, and I. These may be identified from previous progress reports, and include a large part

of the main dairy farm area of Southern and Central Minnesota. If the entire sample taken is considered representative of general dairy practice and management in Minnesota, the results suggest that the herds that report leukemia are significantly different from the general population of dairy herds in the state with regard to the frequency of veterinary service, the nature of the service, and the amount of money spent on the health care of the dairy herd. In addition, the reporting herds differ from the non-reporting ones in areas of management. This includes good record keeping, well-timed removal of low producing animals from the herd, and general farm practice and specialization.

If we accept the assumption that reporting of bovine leukemia is limited to that part of the population of dairy animals in the state that has a mean level of 0.56 times (per cow) that the veterinarian makes a professional visit to the farm, we need to establish how large this population may be for the entire state, with its 1.4 million dairy cows aged 2 years and older. The socio-economic survey has given us a mean of 0.34 professional visits by the veterinarian per cow in the non-reporting population of animals. The two years in which the reports were taken, 1964 and 1965, saw a total of 242 reported leukemia cases for the state. If these cases are attributed to the "reporting" part of the total population, we would get these cases applying to 10.5% of the total sample of animals.

If it is assumed that the incidence between the reporting sample and the rest of the dairy cattle of Minnesota is similar, then extending the incidence in the reporting fraction to the entire state gives an incidence of 155 per 100,000 animals at risk.

The method used to estimate the size of the leukemia reporting population was to assume a normal distribution of veterinary calls for the state about the mean of 0.34 calls per animal, based on the 1967 survey. The mean for the leukemia reporting group, 0.56 calls, would fall towards the upper end of the distribution. To be on the safe side, the lower limit of this mean of 0.56 was set at 2 standard deviations below its calculated value. The fraction of the general population represented by this mean was then estimated by statistical procedures. The use of this low limit for the reporting group mean estimate rather than the actual value implies that this estimate will be a conservative one.

b) Based on the 1962 Steele County Survey.

This survey was made to establish hematologic parameters of a random sample of Minnesota dairy cattle, and was stratified for herd size to make it representative of the general population of animals. A total of 103 lymphocytotic animals (based on a single sample, but classified by a deviation of three standard deviations from the age-adjusted lymphoid cell mean) was found in that sample of herds.

A high correlation between lymphocytosis and the subsequent incidence of bovine leukemia has been reported in present and previous work under this contract. The incidence was 19 out of 210 animals. Cohort studies have indicated that lymphocytosis and leukemia are expressed in 80% of the animals in the cohort groups. This work supports the work of Bendixen, Gotze and others, relating lymphocytosis in "normal" animals to the occurrence of leukemia in multiple case herds.

The incidence of leukemia reported for the population of lymphocytic animals in the multiple case herds that have been under study suggests

that 4 of the 103 lymphocytotic animals found in the Steele county survey might become leukemic per year at risk. The Steele County survey involved a total of 2157 animals. Assuming that only lymphocytotic animals become leukemic, we would have a calculated incidence of 1.8 animals per 1,000 for a sample in which all herds were drawn at random, and stratified for representative purposes. The narrow basis of definition of a lymphocytotic animal and the assumption that leukemia is confined to this group only, again makes this a conservative estimate. Even so, this gives an estimated incidence of 180 per 100,000 animals at risk for dairy cows two years old and older.

c) Variability of reporting by veterinarians.

The implication that the base of dairy cattle from which leukemia cases are reported is much smaller than the entire population of dairy animals in the state, must also be related to the veterinarians themselves. Reporting of the disease is not obligatory for practicing veterinarians, as is the case for some other diseases. The veterinarian's acuity in diagnosis and his interest in the disease will therefore be major factors in the level of his reporting. Thus, Minnesota in the years 1961-65 had a total of 437 veterinarians in large animal practice and 835 cases of bovine leukemia were reported during this period. However, 15 veterinarians reported a third of these cases.

If the 1960-65 reporting summary is examined, it is seen that the 18 leading veterinarians in terms of total reported cases furnished a total of 92 individual yearly records, for an average of 3.8 cases per record per year. A sampling of the top 5 men in terms of reporting would give a much higher rate of cases per practice.

It is known (personal communication) that even the members of this

select group do not report all the cases of leukemia they diagnose. In addition, it has been shown that even with the cooperation of the farmer and veterinarian, 50% of bovine leukemia cases will not be diagnosed.

If this average number of cases reported per year were applied to the entire number of large animal veterinarians in the state, the total number of animals reported per year would be 1674 ± 127 . This would give an incidence of 136 ± 33 cases per year per 100,000 animals at risk. Such an extrapolation ignores the differences in area and the type of practice of the veterinarian, even within the large animal field. The estimate of incidence by this method must therefore be considered as a first approximation, subject to refinements of methodology. Since this is an admittedly conservative estimate, it is slightly lower than the other two estimates (155 and 180 respectively) but certainly close enough to be considered of the same general order as these other two estimates based on entirely different approaches to the problem.

More accurate leukemia incidence estimates based on the reporting of selected veterinarians representative of the dairy areas of the state, would require establishing the average number of dairy cattle examined in these practices.

Information on the number of cases that have not been reported previously is also essential. Comparisons could then be made with other, near-by veterinary practices to establish any differences and similarities that might lead to the development of methods to utilize this approach to get another independent estimate of incidence of bovine leukemia. Such a survey would also be useful in relating some

of the results of the 1967 socio-economic survey to factors of veterinary reporting, diagnostic acuity and type of practice. From there, a more accurate figure could perhaps be extrapolated to the entire population of dairy cattle in the state.

d) Comparison of projected incidence estimates.

The use of the 1966-67 socio-economic sampling and the 1962 Steele County survey give two completely independent approaches to the estimation of the true incidence of bovine leukemia in Minnesota. The values obtained are remarkably similar to each other, as can be seen in Table B-4.

TABLE B-4

Estimates of the Incidence of Bovine Leukemia in Minnesota Based on Three Different Methods of Estimation Using Independent Data Obtained in 1966-67 and Previous Years.

<u>Method</u>	<u>Estimated Incidence per 100,000 animals at risk*</u>
Extrapolation from socio-economic survey, 1966-67	155
Steele County Hematological Survey, 1962	180
Extrapolation from mean annual reporting of 18 veterinarians, 1960-65.	136 $\pm$ 33

* Refers to dairy cows, 2 years old and older

The economic, agricultural, animal health and possibly public health implications suggested by an apparent incidence of 150 per 100,000 animals at risk or greater, are outside the scope of epidemiological estimates. Should these implications dictate a refinement of these estimates, which exceed the reported statewide incidence by

12 to 15 times, other approaches, such as intensive samplings drawn at random, might well be tried.

Estimates of incidence making use of differential reporting among veterinary practices located in proximity, should be made. These could establish the number of animals sampled or examined by each veterinarian. Related to accurate estimates of the total cases of leukemia diagnosed, these should give yet another independent estimate of the true incidence of the disease.

A fourth possibility involves the relation of single case herds to multiple case herds in the matter of incidence over time. The frequency with which a herd in the first category moves into the second (multiple case herd) category would give a measure of long-time incidence. This would require continuation and possible expansion of some of the present studies in this field.

4. Five year Record.

Over the years 1961 to 1965, records on 835 cases of bovine leukemia among female dairy cattle 2 years old and older were collected for Minnesota. Preliminary analyses of these records have been begun. There are problems associated with some of these data which need to be resolved and clarified, so that two and three way tabulations of variables can be made. These should then be tested for possible association with each other. Interpretation of the results in the light of the findings of the recently conducted socio-economic study may be very feasible, and offer helpful indications for further study. This body of data should also serve as a check for the postulated incidence of the disease in the state.

C. Multiple Case Herd Studies

1. General

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.

Table C-1 is a tabulation of the herds presently under study and some of the information of these herds. Six of these herds (MH 1, 4, 5, 25, 29, and 31) have been under study for several years. Thirteen herds studied in previous years are not presently under study due to herd dispersals or they were eliminated from the study group due to lack of detailed record keeping. During the past year 5 new herds (MH - 33, 34, 35, 36, and 38) have come under study. Several other new multiple case herds were studied but they have been dispersed and are not listed in Table C-1.

TABLE C-1

List of Multiple Case Study Herds and Cases Reported From
These Herds in Minnesota and Wisconsin

Herd Number	No. of Animals 2 Years Old or Older	Breed	Case No.	Occurrence	Sex	Age
MH-1	48	Holstein	L-1	Feb. 1960	Fe	4 yrs.
			L-2	Mar. 1960	Fe	4 yrs.
			L-4	Mar. 1960	Fe	4 yrs.
			L-135	Nov. 1961	Fe	7 yrs.
			L-764	Nov. 1961	Fe	10 yrs.
			L-984	Jan. 1962	Fe	3½ yrs.
			MH1-35	July 1963	Fe	14 yrs.
			L-2236	April 1964	Fe	7 yrs.
MH-4	100	Jersey	L-287	Feb. 1961	Fe	4 yrs.
			L-288	Feb. 1961	Fe	5 yrs.
MH-5	50	Holstein	L-32	Aug. 1960	Fe	7 yrs.
			MH5-50	Mar. 1960	Fe	7 yrs.
			MH5-76	Sept. 1966	Fe	6 yrs.

Table C-1 (cont.)

Herd Number	No. of Animals 2 Years Old or Older	Breed	Case No.	Occurrence	Sex	Age
MH-25	35	Holstein	L-418	Feb. 1961	Fe	4 yrs.
			L-1179	June 1962	Fe	10 yrs.
			MH25-33	Sept. 1964	Fe	4 yrs.
			MH25-40	June 1966	Fe	5 yrs.
MH-29	80	Holstein	L-2357	July 1964	Fe	4 yrs.
			L-2500	Dec. 1964	Fe	4 yrs.
			MH29-9	Nov. 1965	Fe	4 yrs.
			MH29-76	June 1966	Fe.	3½ yrs.
MH-31	47	Holstein	L-839	Dec. 1961	M	2 yrs.
			L-2893	Sept. 1965	Fe	6 yrs.
MH-33	42	Holstein	L-1942	Sept. 1963	Fe	8 yrs.
			L-2090	Jan. 1964	Fe	10 yrs.
MH-34	36	Holstein	L-1177	May. 1962	Fe	5 yrs.
			L-2932	Nov. 1965	Fe	13 yrs.
			L-3030	Jan. 1966	Fe	11 yrs.
			L-3155	May 1966	Fe	6 yrs.
MH-35	27	Holstein	L-2566	Jan. 1965	Fe	12 yrs.
			MH-35-28	Dec. 1965	Fe	5 yrs.
			L-3800	Sept. 1967	Fe	5 yrs.
MH-36	43	Holstein	L-1834	July 1963	Fe	4 yrs.
			L-2367	July 1964	Fe	5 yrs.
			L-3288	July 1967	Fe	6 yrs.
MH-38	60	Holstein	L-703	Oct. 1961	Fe	3 yrs.
			B-961	Oct. 1965	Fe	7 yrs.
			M-1161	Oct. 1966	Fe	5 yrs.
			L-3270	Apr. 1967	Fe	5 yrs.
			T-1161	July 1967	Fe	6 yrs.

2. Genetic Studies

Familial Relationship of Leukemia Cases

In the study of 68 cases of bovine leukemia in 18 high incidence herds studied for several years there were noted close familial relationships between some cases. In 4 herds there was an occurrence of both a dam and her adult daughter which died of leukemia. In one herd, 3 full siblings died of leukemia and also their dam had died of an undiagnosed chronic

disease. There were 4 instances of 2 paternal half siblings which died of leukemia. On one farm, 4 of the reported cases of leukemia had a common sire. Of the 68 leukemia cases studied 29 had a close familial herd member which also had died of leukemia. Eleven of these 29 related cases had a close maternal relationship and 18 cases were related by a common sire with another case.

Close paternal relationship in herds using natural breeding may not be too significant because all animals born in a 2 to 3 year period may have the same sire.

Familial Studies on Leukemia and Chronic Lymphocytosis

The number of leukemia cases are small for a significant study of possible genetic effects on the occurrence of leukemia. Since chronic lymphocytosis is commonly observed in leukemic herds and if it is assumed to be characteristic of the preclinical stage of leukemia, then studies of animals exhibiting chronic lymphocytosis may give meaningful results in genetic or natural transmission investigations. The heritability effects of sires and dams on their daughter's adult lymphocyte counts were undertaken to study the possibility of genetic predisposition to lymphocytosis.

The analysis of variance results used in the calculation of the heritability estimate for paternal effects on lymphocyte counts of paternal half siblings in 9 high incidence leukemia herds are presented in Table C-2. A total of 522 adult daughters from 52 sires were used in this calculation. A significant heritability of 0.412 was calculated in this paternal half sibling analysis. Since these 52 sires were used in natural breeding there is no way to separate the genetic effect implied by this estimate from a possible infectious factor's differential time effect or the effect of other environmental factors on lymphocyte counts. If there is an

infectious cause of chronic lymphocytosis and leukemia, then a sire group's lymphocyte counts may be due more to the presence or absence of this agent than to their genetic predisposition. The sire effect may be coincident with a time effect in which case lymphocyte counts would be due to differences in the group exposure potential and exposure experience.

TABLE C-2

Analysis of Variance for Average Lymphocyte
Counts of Paternal Halfsibblings in
Leukemia Herds

Source of Variation	d.f.	Sum of Squares	Mean Squares
Between herds	8	226,493,005	28,311,625
Between sires	51	895,054,515	17,550,088
Within	462	4,094,552,652	8,862,668
Total	521	5,216,100,172	
K = 8.54		Sire component of variance = 1,017,262	
Heritability = 0.412		S. E. _H = .074	

The heritability estimates resulting from regression analysis of average adult lymphocyte counts of dam-daughter pairs in 8 high incidence leukemia herds are tabulated in Table C-3. The variation between the heritabilities of the 8 herds is large but the lower values may be due to the small sample sizes. The 2 herds with the smallest number of dam-daughter pairs have the only negative heritability estimates and both have large standard errors. Herd MH-4 has the largest number of dam-daughter pairs but has a non-significant heritability estimate. This is the only herd of the group which is under "loose housing" management.

TABLE EC-3

Heritability Estimates of Average Lymphocyte
Counts of Dam-Daughter Pairs in High
Incidence Leukemia Herds

Herd Number	Number of Dam-daughter pairs	Heritabilities	SE _H
MH-1	60	0.738	0.271
MH-4	85	0.168	0.183
MH-6	7	0.034	0.774
MH-20	52	0.844	0.374
MH-24	22	0.214	0.268
MH-25	36	0.170	0.510
MH-27	16	0.282	0.396
MH-28	32	0.764	0.336
All 8 herds	310	0.388	0.110

The heritability of all dam-daughter pairs in all 8 herds when calculated together is 0.388 ± 0.110 and is significant. Whether this estimate of maternal heritability is due to genetic effect or to vertical transmission of some other causative factor remains unanswerable.

To gain more objectivity in the analysis of the familial relationship in the 310 dam-daughter pairs the continuous quantitative lymphocyte counts were transformed into 2 qualitative groups on the basis of presence or absence of chronic lymphocytosis. Bendixen's "Key" was used to dichotomize the groups, i.e. the cows classed as "chronic lymphocytosis" animals were those with lymphocyte counts consistently greater than 2 standard deviations above the mean for cattle from nonleukemic herds

and the "nonlymphocytosis group" with lymphocyte counts below this level. Although a Minnesota Key is available for normalcy, the Bendixen Key used to dichotomize the groups is a more stringent evaluation, since the upper limits of the 95% confidence level for normal lymphocyte counts is consistently outside the upper limits of the 99% confidence level for Minnesota cattle. The dams and daughters which had died with leukemia were classed with the chronic lymphocytosis groups. Twenty-four dams of the 80 dams in this group had died of leukemia.

The results of analyzing the 310 dam-daughter pairs on the basis of chronic lymphocytosis and/or leukemia or not having leukemia or chronic lymphocytosis is as follows:

1) Nonlymphocytotic dam and daughter	164
2) Nonlymphocytotic dam and lymphocytosis or leukemia in daughters	66
3) Chronic lymphocytosis or leukemia in dams and daughters	39
4) Chronic lymphocytosis or leukemia in dams and nonlymphocytosis in daughters	<u>41</u>
TOTAL	310

It is noted that 49% (39/80) of the affected dams have daughters with chronic lymphocytosis. The nonaffected dams had only 29% (66/230) of their daughters which exhibited chronic lymphocytosis. In Table C-4 a chisquare test shows there is significant difference between the ratios of the two groups ($P = .001$).

Even though the above statistical analyses indicate there is a significant genetic effect or vertical transmission from dam to daughter as concerns lymphocytosis there still remains the 29% (66/230) of the

daughters of nonaffected dams whose state is unsolved. Examination of the 66 daughters in this group with respect to time of birth revealed that 53 (88%) of them were born near the time at which other cows with lymphocytosis were born. In fact these 53 daughters were all born within a 4 week period and in many cases within days of the time that leukemia and lymphocytotic dam's progeny were born. Examination of lymphocyte counts of this group's paternal half siblings revealed that the lymphocytosis seen in this group was not due to sire affect since the majority had normal lymphocyte counts. The neonatal cohort analysis seen in this group of animals and other studies reported would indicate the possibility of horizontal transmission in very young calves.

In this group of dam-daughter pairs it is evident that vertical transmission or genetic susceptibility of leukemia and lymphocytosis was not demonstrated as a constant finding since 41 (51%) of the daughters of affected dams were not similarly affected. It was noted in the herds that many adult cows which exhibited persistent lymphocytosis did not become lymphocytotic until they were 5 years of age. There were 24 (59%) animals of the group of 41 daughters of affected dams, 4 years of age or younger at the time the analysis was done. Perhaps a portion of the variation between these dams and daughters was due to this lack of age adjustment. But it was also noted that of 24 daughters of leukemic dams only 12 were affected i.e. 9 exhibited persistent lymphocytosis and 3 others died of leukemia. Several of the nonlymphocytotic daughters of leukemia cases were less than 4 years of age but also several were older than 5 years of age and did not exhibit lymphocytosis. There-

TABLE C-4

Analysis of the Difference Between Presence of
Lymphocytosis and Leukemia in
Dam-Daughter Pairs

	Dams		
	<u>Non- lymphocytotic</u>	<u>Lymphocytotic or Leukemic</u>	<u>Total</u>
<u>Daughters</u>			
Nonlymphocytotic			
Observed	164	41	205
Expected	152	53	
Lymphocytotic or Leukemic			
Observed	66	39	105
Expected	<u>78</u>	<u>27</u>	<u>—</u>
Total	230	80	310
<hr/>			
$\chi^2 = 10.84$ 1 d.f.	$P = .001$		

fore, it must be concluded that vertical transmission or genetic predisposition of lymphocytosis is not constant for all cases. Other unknown factors may be required for lymphocytosis to be acquired vertically.

Genetic Studies Summary

Close familial relationships were noted between cases of bovine leukemia in some of the high incidence herds. Leukemia cases were noted in dams and daughters, in full siblings, and in paternal half siblings. Twenty-nine of the 68 leukemia cases occurring in 18 herds had a close

familial herd member which had also died of leukemia. Eleven of the cases had a close maternal relationship and 18 cases a paternal relationship to another case of leukemia.

The familial clusters of leukemia cases indicate a possible genetic predisposition on the occurrence of leukemia. Familial clusters of leukemia have been reported by several investigators and the clusters have been shown to occur in statistically significant numbers (Croshaw et al., 1963). If bovine leukemia is caused by an infectious agent then the occurrences of leukemia in animals with close maternal relationships could be the result of non-genetic vertical transmission with or without genetic susceptibility playing a role.

The clustering of leukemia cases in sire groups, reported here and in the literature, occurred in herds using natural breeding. It is not unusual for all animals born in a 2 to 3 year period to have the same sire in this type of herd. Therefore, a group of leukemia cases which have a common sire also have other mutual relationships such as common exposure potential and experiences which are the result of environmental conditions for a period in time. Nonrandom distribution of "leukemia virus" in respect to time could differentially affect the sire groups of which the herd is comprised. Therefore, sire groups which were not at risk due to absence of an agent or sire groups with a lower mean age cannot be meaningfully compared to affected sire groups. In this study the number of leukemia cases and the duration of study were not sufficient to make meaningful comparisons possible.

The heritability estimates calculated from lymphocyte counts of paternal half-siblings ($0.412 \pm .074$) and dam-daughter pairs (0.388 ± 0.110) indicate a significant genetic effect (Table C-3 and C-4). Dams

and daughters were additionally demonstrated to be significantly similar when dichotomized into lymphocytotic or nonlymphocytotic groups (Table C-4). Drawing the conclusion that lymphocyte counts in progeny are due only to genetic effects, on the basis of these data, would be erroneous if lymphocytosis is caused by an infectious agent. Similar results could be expected if an agent was transmitted vertically from dam to daughter and by the presence or absence of an agent, during a period of susceptibility, in sire groups.

In summary, the role of genetic susceptibility on the occurrence of leukemia and lymphocytosis could not be determined by observations and analyses done on the data from these high incidence herds. Statistical analyses, by other investigators, of familial leukemia case and lymphocyte data were also unable to demonstrate whether familial similarities are due to genetic effect or nongenetic vertical transmission, (Henricson and Olsen, 1961) (Croshaw et al., 1963) (Bendixen, 1965). Definitive studies to determine the role of genetic predisposition would require experiments designed with all factors, with the exception of genetic constitution, being orthogonal.

3. Cohort Analysis

The analysis of leukemia cases occurring in multiple case herds with respect to date of birth, indicated a high incidence of leukemia clusters in certain neonatal cohort groups. This was reported last year and another neonatal cohort cluster was noted in one of the new study herds (MH-38). Additional cohort analysis of data for all adult animals in 10 herds, where complete animal records were available, revealed a significant clustering, by date of birth, of animals with

persistent lymphocytosis. Many of these clusters involved animals sharing a common neonatal period with one or more animals which later, as an adult, developed leukemia. The results of these analyses of neonatal cohorts which contained leukemia cases are presented in Table C-5.

The dates of birth indicated in Table C-5 are all documented by registry or equivalent records. The month indicated is the predominant month recorded for each cohort and the birth date interval, for each cohort, was a maximum of 28 days. The actual birth date interval for many leukemia cases was much less than 28 days and in some cases only several days.

Animal records in 7 high incidence leukemia herds revealed that 2 or more animals which had died of leukemia, had been born within a 4 week period (Table C-5). Four of these herds had 2 leukemia cases, 2 herds had 3 cases and one herd had 4 leukemia cases born within a 4 week period. A total of 37 leukemia cases occurred in these 7 herds and 18 of these cases were born in neonatal cluster groups.

A total of 64 adult animals were studied in 11 herds. These animals were born in 14 neonatal cohorts. Twenty-five (39%) of these animals developed leukemia as adults. Additionally 31 (48%) animals, in these neonatal cohorts, exhibited persistent lymphocytosis during adult life. The 61 adult animals, in these groups may represent only a fraction of the entire cohort groups since nearly all male animals and some female animals are normally culled before reaching maturity.

In addition to the above cohorts which contained leukemia cases, there were 31 neonatal cohorts which had high incidences of lymphocytosis but no leukemia cases.

TABLE C-5

Relationship of Tumor Cases and/or Lymphocytosis to Time of Birth
In Multiple Case Herds

Herd Number	Month of Birth	Number Studied	Number Developing Leukemia	Number Developing Lymphocytosis	Percentage With Leukemia & Lymphocytosis
MH-1	Aug. 1955	7	3	2	71%
	July 1958	9	1	7	89%
MH-2	Sept. 1954	2	2	-	100%
MH-4	Sept. 1956	8	1	6	88%
	Sept. 1957	5	1	3	80%
MH-15a	Oct. 1955	4	1	3	100%
MH-20	Aug. 1960	3	1	1	67%
MH-23	Sept. 1957	6	4	2	100%
MH-25	Aug. 1959	5	1	2	60%
	Nov. 1960	4	1	3	100%
MH-27	Jan. 1958	3	2	1	100%
MH-31	Oct. 1959	2	2	0	100%
MH-32	Oct. 1958	3	3	0	100%
MH-38	Nov. 1961	3	2	1	100%
Total		64	25	31	87%

The appearance of the neonatal cohort clusters of leukemia cases and/or lymphocytotic animals was very spectacular since many other neonatal groups born during the same years usually had very few animals that exhibited lymphocytosis. Investigations to determine the possible common exposure experiences that leukemia and/or lymphocytotic groups had and which other calves born in the same year did not have resulted in the following findings:

1. The leukemia and/or lymphocytotic cluster groups contained progeny of leukemia case dams and/or dams with persistent lymphocytosis. Several of the leukemic dams were in the early clinical stages of leukemia when these progeny were born.
2. In the herds, where the cluster groups were born, newborn calves were kept in age segregated communal type pens. Therefore, most newborn calves had direct contact with other calves of similar ages during neonatal life.
3. In these herds calves were fed colostrum and milk during the neonatal period.
4. Colostrum and milk, fed to calves, was usually from a pooled source, so it was possible for a newborn calf to receive milk or available colostrum from a source other than its own dam. Additionally, the same calf feeding utensils were used for all milk-fed calves and these could act as fomites in the transmission of milk born infections.
5. Following the above period of common neonatal environment and sources of food, the calves lived in a common environment with other older calves. Calves lived in a common

There may be many other possible common environmental factors which the cluster groups experienced and other groups of calves born during the same year did not, even though they are raised in the same barn. Analysis of leukemia and/or lymphocytosis clusters on the basis of seasonal influence and size of neonatal cohorts did not result in any significant differences between these groups and other groups of animals studied in the herds.

Cohort Analysis Summary

The high incidence of leukemia cases and/or lymphocytotic animals in certain neonatal cohort groups (Table C-5) indicate a definable age susceptibility or a time-limited environmental hazard in the causation of leukemia and persistent lymphocytosis.

It is apparent in these studies that some common exposure experience was specific for certain cohort groups of calves during the neonatal period and not for other animals born during the same year. There were possibly many common experiences which the leukemia cohort groups of calves had, including direct contact with each other and common sources of food. Age segregation practices in these herds were such that the animals in each neonatal cohort were in direct contact during neonatal life. Calf-feeding practices in the herds also made it possible for calves to receive colostrum and milk from a pooled source during the first month of life.

There should be little doubt about the significance of the close proximity of birth in the 7 clusters of leukemia cases observed in these herds. Neonatal exposure experience with calves which develop leukemia as adults, evidently is also in some way associated with the occurrence of persistent lymphocytosis. If the persistent lymphocytosis seen in

cattle of leukemia herds is a prodromal stage of leukemia, then these associations have added significance.

The association of leukemia and/or lymphocytotic dams with the neonatal cohorts is consistent with the concept of a transmissible agent. In view of the husbandry practices in these herds, these dam's progeny, milk, and colostrum could have been factors which all members of the cohorts had in common.

The higher incidences of leukemia in larger herds also is consistent with a concept of neonatal transmission since the milk and colostrum pools in these herds come from more sources. Also, in larger herds there are larger numbers of newborn calves at risk in all neonatal cohorts. Therefore, newborn calves in larger herds have greater exposure potential.

In view of the data on familial leukemia case clusters, lymphocyte heritability estimates from dam-daughter pairs, and the cohort analyses, a hypothesis of both vertical and horizontal transmission of bovine leukemia can be explained. Familial similarities, seen in dam-daughter leukemia cases and dam-daughter lymphocyte counts, suggest the possibility of vertical transmission in leukemia and lymphocytosis. Dissimilarities between "normal dams" and some of their daughters can largely be explained on the basis of possible horizontal transmission of an agent from affected dams or their progeny during the neonatal period. In this hypothesis the role of neonatal exposure as supported by the cohort analyses suggests that infection is the most significant possible factor in the natural occurrence of leukemia and lymphocytosis.

Genetic aspects, under this hypothesis and in view of the data, would primarily involve a possible degree of susceptibility rather than hereditary transmission.

D. Hematologic Studies on Single Case Herds and Normal Herds

1. Analysis of Select Normal Herds

Data was presented in the sixth progress report on the selection of normal control herds for the establishment of hematologic normal values as a control group for some epidemiologic studies. The data obtained on a stratified sample of 37 herds from three countries is being subject to further analysis. Additional hematologic and epidemiologic data was obtained on these herds, since the initial sampling gives data only for one point in time. The data has been tabulated and is being coordinated with previous data for analysis.

The data will form the basis for final evaluation of single case herd and multiple case herd studies.

2. Analysis of Random Cattle Populations in Three Minnesota Counties

Final analysis of this data will involve comparison and analysis to the hematologic data obtained on selected normal herds. No progress was made since the analysis of studies on selected normal herds has not been completed.

Manuscripts concerning epidemiologic and hematologic data of the three-county study are in preparation. Significant findings from these studies have been reported in previous progress reports.

3. Hematologic Studies on Single Case Herds of Juvenile and Adult Type Leukemia

In the previous progress report preliminary observations were summarized for 42 single case herds of the adult type and 10 single case herds of the juvenile type. Inspection of the tabulated data on the adult type single case herds indicate 75 per cent to resemble

multiple case herds in respect to the occurrence of lymphocytosis.

The ten single case (juvenile type) herds were more characteristic of the normal herds sampled. Three of these ten herds were characterized as lymphocytosis herds; however, it is not possible to exclude the simultaneous occurrence of the adult form of leukemia in these herds.

Final statistical analysis to normal herds and multiple case herds will be made as soon as analyses of the selected normal herd hematologic data is completed.

E. Studies Pertaining to Animals with a Marked Lymphocytosis Originating From Multiple Case Herds

The initial objective to determine the natural outcome of animals with lymphocytosis by assembly of these animals was altered as indicated in the seventh progress report. Since it was not feasible to acquire sufficient numbers of lymphocytosis animals within the framework of the contract, these animals were studied and evaluated on the basis of their natural herd environment. This data is reported in the section on multiple case herds.

In instances, where lymphocytosis animals were to be normally culled from multiple case herds, these animals were purchased for other studies.

1. Studies on the Promotion of Tumor Growth or Induction in Lymphocytosis Animals

a) General

It is postulated that lymphocytosis is a prodromal phase of bovine lymphocytic leukemia. This protracted induction state may be due to a highly competent immune mechanism which controls aberrations as they occur.

Further, accepting a Promotor-Inducer theory of leukemogenesis, it is theorized that treatment with carcinogenic factors may shorten the time required for these cows to develop clinical leukemia.

b) Radiation Studies

Methods - The animals in this study were given sublethal total body irradiation from a Cesium 137 deep well source. Details of radiation procedures are reported in the seventh progress report.

Initial exposure to the midline dose of 75 r was made in January of 1967. Following consultation with Brookhaven National

Laboratory staff, it was decided to give additional exposures to 25r of total body radiation after hematologic recovery to the initial exposure or stabilization of hematologic parameters was attained. Additional exposure to 25r of total body radiation was made in November of 1967.

Experimental Animals - Five Jersey cows from multiple case herds and with persistent lymphocytosis and under study for 2 to 6 years were selected. The animals ranged in age from 5 to 8 years. These animals were normal on repeated physical examination and no evidence of tumor growth was apparent. Three additional Jersey cows added to the group and were given 25r of total body radiation as their initial and only exposure.

Response to Radiation - Details of the immediate response to 75r of total body radiation were reported in the previous progress report. Maximum reductions of lymphocytes were attained by the third post radiation day with an average reduction to 28 per cent of pre-treatment levels. Granulocyte and blood platelet levels were similarly reduced with maximum reduction occurring at three and four weeks respectively. Marked hemopoietic depression was evident in all cows, with one animal exhibiting acute signs of total body radiation related to severe neutropenia. This animal required intensive antibiotic therapy for extended periods as a result of systemic and local infection. The animal died 9 months following initial radiation of an exacerbation of acute gangrenous mastitis. No evidence of neoplastic growth was observed at necropsy or on histopathologic examination of selected tissues.

In general myeloid regeneration including platelets, was rapid and near base line values were attained by the 60th post-irradiation day.

Lymphocyte regeneration appears linear over a 5 to 6 month period. In some cases 100% lymphocyte counts were attained and with 2 animals, 80% of baseline lymphocyte levels were reached.

The second exposure to 25r of total body radiation was associated with significant hematologic depression of lymphocytes and measureable depression of granulocytes. No significant change occurred with platelet counts.

The regenerative response of lymphocytes after the second radiation (25r) does not appear linear nor as rapid. With some animals the counts are stable at the lowest level reached following radiation.

The response to 25r of total body radiation for the three cows not previously radiated was significant with respect to lymphocyte depression and in one animal a mild neutropenia existed. No significant effect was noted with blood platelets.

No evidence of tumor induction is evident in the seven animals under study at this time.

c) Adrenal Corticosteroid Studies

Methods - Prednisolone (Sterane^R PFIZER) was chosen as a lymphocyte depressant and possible inducer or promotor of leukemogenesis (seventh progress report). Two treatment periods with prednisolone, administered at the dose of 6 mg./lb./day for six days followed by a four day diminishing withdrawal dose, have been studied in 4 lymphocytosis animals. The lymphocytosis animals had absolute lymphocyte counts ranging from 12,000 to 32,000 prior to the initial treatment.

Results - Similar responses were observed on all animals following steroid administration. A rapid drop in circulating lymphocytes (30 - 45% of baseline levels) followed each regime of steroid

administration. The regenerative period was approximately 1 month i.e. for return to baseline or stabilized lymphocyte counts. This period is much shorter than that following radiation treatment, suggesting that the effect on lymphocytes may be associated with difference in lymphocyte populations affected.

The regenerative response following the initial treatment with steroids was similar in all animals with baseline or near baseline levels attained. Following the second treatment, a similar return to near baseline levels was observed, however, all 4 animals experienced a gradual decline in circulating lymphocytes during the following 7 month period with 3 of 4 animals at 50% of baseline levels at the present time.

Whether this decline is indicative of impending tumor growth needs to be determined. In naturally occurring bovine leukemia, a decline in lymphocyte counts occurs frequently simultaneously with tumor development. No further steroid therapy will be attempted until lymphocytes counts stabilize or return to baseline levels.

2. Immunoresponsiveness of Leukemic Animals

a) Fluorescent Antibody

Pilot studies to investigate some immunological aspects of bovine leukosis have been undertaken in collaboration with scientists at the Glenwood Hills Research Laboratories in Minneapolis, Minnesota.

Results of these studies have been encouraging. The use of fluorescent labeled antibody to bovine gamma globulin produced in rabbits has been used in an attempt to detect the presence of globulin coating on leukemic lymphocytes. Normal, lymphocytotic, leukemic and lymphosarcomatous animals have been studied using the fluorescent labeled antibody. To date globulin coating on leukemic lymphocytes was found

in the blood of two animals with extremely high blood lymphocyte counts (L3101 and L3133 with 83,600 and 88,400 lymphocytes / mm.³ of blood respectively). One hundred per cent of the leukemic cells in these two animals were positive. A third animal was found which had 50 per cent positivity of the blood mononuclear cells. Blood smears prepared from 9 normal animals were negative, as were those prepared from 4 lymphocytosis animals (without leukemic blood pictures) were negative for fluorescences of tissue imprint material prevented critical evaluation of such preparations. All other classical immunological procedures gave questionable or negative results. These tests included complement fixation, cytotoxicity, agglutination and immunodiffusion techniques. Efforts are now being directed toward obtaining and studying animals with the leukemic form of bovine leukosis.

b) Attempts to Elicit Antibody Formation in Bovine Animals

Iodoacetate has been reported to enhance the antigenic potential of certain transmissible murine neoplastic cells. Studies were initiated to investigate the possibility of using this technique in the search for antigen-antibody relationships peculiar to bovine leukemia.

Three normal and three leukemia cows were used in this portion of the study. One adult bovine steer which exhibited a persistent lymphocytosis was also used. Two of the normal cows received 3 weekly injections of 70×10^9 neoplastic cells treated with iodoacetate. The treatment consisted of incubation for 30 minutes at 37° C. in a standard tissue culture media containing 10^{-2} molar iodoacetate. The third normal animal received 3 weekly injections of 70×10^9 untreated leukemic lymphocytes. The lymphocytosis steer received two i.v. injections of iodoacetate treated leukemic cells. The source of cells used for these treatments was the buffy coats of two adult cows which had a

leukemic form of the disease.

In addition to the normal and lymphocytosis animals, the two source animals received injections of their own buffy coat cells following iodoacetate treatment. An animal which had an aleukemic (lymphosarcomatous) leukemia also received 2 intravenous injections of iodoacetate treated leukemic cells.

Evaluation of hemograms of the 3 normal animals did not indicate any significant alteration in blood lymphocyte levels even after 8 months of observations. The male lymphocytosis animal prior to treatment had a white blood cell count which ranged from 13,100 to 18,400 cells/mm.³ with 77 to 90 per cent lymphocytes. After three treatments, the white blood cell count ranged from 13,100 to 17,800/mm.³ however the per cent lymphocytes ranged from 62 to 78 per cent for two months. The post treatment decline in blood lymphocyte was considered to be significant but not definitive. This animal was a steer and reached such a size it was no longer possible to maintain the animal in the facilities available. No significant alteration was found in gross or histopathological examination of the tissues of this animal.

One animal with a lymphosarcomatous form and one with a leukemic form of bovine leukemia died before evaluation was possible. The third leukemia animal (L3133) died three weeks after the initial treatment. During this three week period the white blood cell count did not continue to increase as it had prior to treatment. However this finding was of questionable significance because the white count had reached a level of 330,000 by this time (from 88,000 one month prior to the initial treatment), the animal was used as a source of large amounts of blood for collection of leukemic lymphocytes.

c) Attempts to Elicit Antibody Formation in Mice.

Because of the interesting finding in cattle an attempt was made to verify the original work on the induction of tumor immunity with tumor cells "delethalized" by treatment with a sulfhydryl alkylating agent (iodoacetate). For this phase of the study a spontaneous lymphatic neoplasm of Balb/c mice is being used. This neoplasm was characterized by Dr. E. Groth at this laboratory as a rapidly developing (7 day by cell implant and 21 day by cell free filtrate) lymphoid neoplasm. Intraperitoneal implantation results in an ascitic form with concurrent organ infiltrate. This neoplasm was selected because of the rapid growth, the availability of the tumor and mice, and because of the tissue of origin.

A 20% tissue suspension was prepared and divided into two equal portions. One portion was treated with 10^{-2} molar iodoacetate for 10 minutes at room temperature and pH 7. The other portion was treated in the same manner only without iodoacetate. Five adult and 10 baby mice received iodoacetate treated tumor suspension intraperitoneally. Seven days later all baby mice died with massive neoplastic involvement of vital organs and at 18 days all adults were found to be positive for neoplastic growth. Five adult mice receiving the same tissue suspension but not treated with iodoacetate also died with neoplastic involvement.

The next attempt at immunization with iodoacetate treated cells involved five groups of mice. Four groups received intraperitoneal injections of 0.1 cc (3.5×10^5 cells) ascites fluid tumor cells which had been treated with iodoacetate (10^{-2} molar for 30 minutes at 37° C. at pH 7.2). The fifth group of mice received a similar injection subcutaneously. The results of this experiment is presented in Table

E-1. The results suggest the tumor cells were "delethalized" by the treatment with iodoacetate because of the lack of tumor development even up to 120 days post injection. However, the mice could not resist challenge following a single injection of delethalized isologous tumor cells.

Continuation of this phase of study will be to determine the minimum effective dose of cells needed to induce this neoplasm, the effect of hyperimmunization with iodoacetate treated cells on challenge with isologous tumor cells and the effect of treatment of tumor bearing mice with iodoacetate treated cells.

TABLE E-1

Results of Attempts to Immunize with Iodoacetate Treated Isologous Tumor Cells of Balb/c Mice.

Exp. #	# Mice	Route of Inoculation	Post Injection Day of Challenge	# Mice Challenged	Mice Developing Tumors
50	6	I.P.	14 day	6	6/6
51	6	I.P.	90 day	6	6/6
52	7	I.P.	90 day	3	2/3*
53	3	I.P.	120 day	3	3/3
54	5	S.Q.	90 day	2	2/2

* One mouse is still living from this group of challenged animals.

3. Cytokinetic Studies of Lymphopoiesis

a) Cytokinetic studies of a known model system in which lymphocytosis is a prominent feature were undertaken. Information gained would be applied to the study of the nature and mechanism of lymphocytosis in normal animals originating in multiple case herds. The original working hypothesis, which was based on the thesis that heparin induced lymphocytosis resulted from blockage of routes of recirculation of lymphocytes has been discounted. The concept of a biological ion exchange mechanism has been adopted as a working hypothesis to explain the phenomenon observed during the course of these experiments. The impressions gained from the work done is that heparin induced lymphocytosis results in a release of lymphocytes from within the substance of organized lympho-reticular tissue.

The previous progress report contained data from 29 experiments using bovine calves prepared with prescapular efferent lymph duct fistulas. The results of these experiments are the subject of a paper written to describe the preparation and characterization of the prescapular lymph node fistula. A preprint of this paper is appended to this report and is being submitted to the American Veterinary Medical Association Research Journal for publication. In addition another paper in preparation, describes the magnitude and pattern of heparin induced lymphocytosis in both blood and lymph.

b) During the course of this work it has been found that a difference exists between heparin of beef lung origin and heparin of hog mucosa origin. Heparin of beef lung origin was more effective in the induction of lymphocytosis than heparin of hog mucosa origin. In a search for a cause of this difference, molecular weight of heparin was considered.

Molecular gel filtration was used. Sephadex was used in 30 cm. x 1.5 cm. columns. No discernable difference between these two types of heparin could be found. The column effluent pattern for both types of heparin was quite similar in that a small large molecular and a large small molecular component was found to be of equal proportion.

The next approach taken was based on the supposition that a "contaminant" existed in different relative amounts in the two types of heparin. Heparin is a mucopolysaccharide with a high negative charge density. It was thought a strong cation could bind to the highly charged anionic heparin molecule. Such a hypothetical compound could either bind and gain its effect through blocking the negative charges on heparin or bind to heparin and be released by displacement within the body. The implication being that the phenomenon of lymphocytosis associated with heparin would be attributed to the hypothetical contaminant. The later alternative would require the hypothetical compound to be a vasoactive substance. The work done with the chronic fistula preparation of the bovine prescapular efferent lymph duct suggests an enhanced lymph flow following heparin injection. This finding suggests a vasoactive effect related to heparin injection. The marked increase in lymph flow was observed concurrently with the induced lymphocytosis. Because both tissue sources of heparin are reported to be high in histamine content and because histamine has a vasoactive effect and because histamine is a strong cation it was chosen for investigation. Using the method for fluorometric assay of histamine in tissues published by Shore et al. in 1959 an attempt was made to detect and measure histamine in commercial heparin. Heparin of pig mucosa origin was studied. Preliminary data suggests the presence of a very small amount of histamine. The level found in the

preparation examined was 0.0029 μ gm. histamine per mg. heparin or in terms of USP anticoagulation units 0.0029 micrograms per 144 units. These results are not considered definitive as yet nor has histamine been measured in heparin of beef lung origin. This line of investigation is continuing.

c) Preparations have been made for further cytokinetic studies of lymphoproliferation and only awaits the finding of cases of bovine leukosis with large numbers of lymphoblasts in the peripheral blood. These animals will be used to evaluate the lymph arising from a discrete mass of lymphoreticular tissue by method already developed. Such studies will allow a statement to be made relative to leukemic lymphocyte recirculation and production in the bovine.

d) Preparations have been made to study the functional capabilities of lymphocytes obtained from normal, lymphocytotic and leukemic animals. These studies will center around evaluation of the response of lymphocytes from different sources particularly lymphocytosis animals to phytohemagglutinins in cell culture.

4. Ultrastructure Studies (Manuscripts and Preprints Attached)

a) Cell Morphology

Ultrastructural studies were undertaken on lymphoid cells from normal preleukemic (lymphocytotic) and leukemic cows in attempts to discern early differences, if any, in the blood of animals with a lymphocytosis which might foretell the development of the leukemia state.

Previous light microscopic studies of the peripheral blood of normal cattle revealed an initial problem, i.e., the distinct

differentiation of the agranulocytic cells. At the electron microscopic level, it was noticed that the bovine monocyte lacked some of the usual characteristic structural and tinctorial properties of other monocytes, and that considerable numbers of large lymphocytes and monocytoïd lymphocytes were also present in bovine blood.

A two-pronged approach to this problem was used:

- 1.) Quantitative determinations were made on peripheral blood lymphocytes and monocytes from five adult, healthy cows. The results were as follows:
 - a.) Mitochondrial profiles of monocytes vs. lymphocytes showed that those of monocytes were highly significantly ($P < 0.001$) more numerous and smaller than those of lymphocytes (4.7 ± 0.357 vs. 13.0 ± 0.769 and $0.090 \pm 0.002 \mu^2$ vs. $0.219 \pm 0.007 \mu^2$ respectively).
 - b.) Ergastoplasmic profiles were highly significantly more numerous in monocytes than in lymphocytes (6.57 ± 0.737 vs. $0.96 \pm .231$); the circumferential size of the ergastoplasmic cisternae profiles was larger for lymphocytes than for monocytes ($0.99 \mu \pm 0.14$ vs. 0.81 ± 0.03).
 - c.) Membrane-bound, lysosome-type granular forms were found to be highly significantly more numerous in monocytes as compared to those of lymphocytes (10.5 vs. 0.56).
- 2.) Another method of differentiating monocytes from lymphocytes was undertaken by studying differences in physico-chemical and/or functional properties of these cells.

- a.) It had been reported that monocytes and lymphocytes from human blood could be separated on glass bead columns. This procedure was applied to the bovine blood but the results were disappointing, in that insufficient quantities of cells were obtained and contamination of the cells with glass beads interfered with further processing of the cells. It was also found that certain chemicals, e.g., "Plasmagel" and "Hypaque", which were used successfully to separate leukocytes from whole human blood, did not work for bovine blood.
- b.) The functional approach involved the study of the differential phagocytic capabilities of the two cell types. Reports in the literature had indicated that various substances, e.g., bacteria, viruses, thorotrast, latex particles, India ink, and mycoplasma (PPLO) had been used in these experiments with some success. Our studies involved the use of latex particles, India ink, and mycoplasma. Experiments with the PPLO (*Mycoplasma gallisepticum*), incubated with bovine peripheral blood leukocytes at 37° C., suggested that monocytes took up and degraded the mycoplasma much more readily than did lymphocytes.

b) Nuclear Bleb Studies

Further investigations on the peripheral blood of normal and lymphocytotic cows led to the discovery of an unusual "bleb-like" structure on the nucleus of lymphocytic cells. It appeared that the

bleb wall was composed of two surfaces of nuclear membrane separated by a layer of heterochromatin, about $450 \text{ \AA}$ in thickness. This bleb occurred with much more frequency in the lymphocytes from cattle showing a persistent lymphocytosis in comparison to non-lymphocytotic animals from normal herds. A preliminary study was undertaken to determine the quantitative differences and in incomplete studies to date it was found that an average of 6.8% (1.0 - 10.4%) of the lymphocytes from the blood of multiple case leukemic herd animals had these blebs compared to only 0.5% from animals in the leukemia-free herds. This increased incidence of the bleb in "preleukemic" animals suggests that this abnormality may be an early indication of cellular pathology which could be used to advantage in further studies of bovine leukemia.

c) Hemal Nodes

Studies of the bovine subcutaneous hemal node were undertaken because of a report indicating the hemal node to be a primary site of involvement in leukemia. This work was also done to provide background information prior to initiating some leukemia-provocative studies in cattle with lymphocytosis. The hemal node is a lymphatic tissue which is readily accessible to the environment and to experimental manipulation. Experiments included *Brucella* antigen uptake by lymph nodes and particulate matter uptake by hemal nodes in young cattle. Microscopic examination of subcutaneous hemal nodes obtained from calves following intradermal injection of India ink around the hemal node revealed that carbon particles were engulfed by macrophages within the hemal node. Aggregates of particles were noted between the

cells of the hemal node. Following intradermal injection, bovine virus diarrhea particles were noted in macrophages of the hemal nodes. Due to the small amount of injected material and the concentration of particles contained within the hemal node, it is felt that the injected material reaches the hemal node by either an ill-defined lymphatic channel, or a partial system of veins.

5. Chromosome Studies

Buffy coat cultures of blood from leukemic and lymphocytotic cows were prepared for chromosomal study. For buffy coat culture, blood was collected in 2% EDTA in saline and maintained at 4° C. for $\frac{1}{2}$ hour. The blood was centrifuged and the white blood cells were separated. The white blood cells were washed twice in Hanks BSS and finally suspended in Eagles Medium, containing 40% autologous serum and 1% phytohemagglutinin, to contain 2×10^6 cells per ml. Several passages were carried out on every 5th day.

For chromosome study the "Air Dried" method was used. White blood cell cultures (5th day) were treated with colchicine, and then treated with hypotonic solution, fixed with a glacial acetic acid and methanol mixture, washed twice with 45% glacial acetic acid and finally smears were prepared on glass slides. Slides were stained with stain and examined.

The results of these limited chromosomal studies with the white blood cells from lymphocytotic cows reveal some abnormalities, particularly in the number of chromosomes, which might be of some significance. However, further detail analysis is being carried out to determine its significance.

It is of extreme interest that similar findings on cells obtained from lymph nodes of animals during the early period of tumor onset, have been observed by Hare at Pennsylvania.

F. Transmission Experiments

1. Intrafetal Inoculation

Bovine leukemia transmission experiments have been initiated. Bovine fetuses have been inoculated in utero following the protocols outlined in Progress Report no. 7 i.e., injecting leukemia tumor cells into the fetus prior to the age of immune competence or in the first trimester of pregnancy.

Materials and Methods

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

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

Results

During the past year, 5 fetuses ranging in age from 90 to 120 days were inoculated by the above procedures. One fetus was aborted 7 days following inoculation. At the time of this report the 4 remaining fetuses are alive and apparently normal, as indicated by rectal palpation. They are anticipated to be delivered during the next several months.

At the present time 5 additional cows have been bred and are in the

early stages of pregnancy and their fetuses will be injected. The source of inoculum for this group will consist of the virus-rich material described elsewhere in this report.

2. Foster Nursing Experiments

Epidemiological data on multiple case herds and in particular the high incidence of leukemia in neonatal cohorts, indicates a strong possibility of horizontal transmission of bovine leukemia via colostrum or milk. The evidence for this hypothesis has been discussed previously, in this report, under the heading of "Cohort Analysis".

During the past year studies have been initiated to determine the role of colostrum and milk transmission in the occurrence of leukemia and persistent lymphocytosis.

Materials and Methods

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

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

Progeny of "normal" cows were removed away from their dams after birth and are also housed separately. These calves are fed either colostrum

from lymphocytotic cows or milk from clinical cases of bovine leukemia.

The 2 groups of calves will be housed separately until they become adults.

Results

Presently, the following number of calves are in each experimental group:

- 1.) 4 - progeny of lymphocytotic cows which received colostrum from "normal" cows.
- 2.) 5 - progeny of "normal" cows which received colostrum from lymphocytotic cows.
- 3.) 3 - progeny of "normal" cows which received milk from clinical cases of bovine leukemia

The calves in these 3 groups range in age from 1 to 7 months.

Lymphocyte values are normally quite variable in the cow until one year of age or older. Therefore, it would be difficult to draw any conclusions on lymphocyte counts obtained on these calves to date. At the present time there are no significant differences between the lymphocyte counts of the 3 groups of calves.

3: Electron Microscopy Studies of Viral Particles

Buffy coat cultures from leukemic and lymphocytotic cows were examined for the presence of any virus particles. The method for isolation on culture is described in section E-5 of this report.

For electron microscopy, white blood cells from the 5th day culture were pelleted. Also, pellets were obtained from plasma of leukemic and lymphocytotic cows by ultracentrifugation. The pellets were fixed with gluteraldehyde, post fixed with osmium tetroxide, dehydrated with graded alcohol and embedded in Maraglas. Ultrathin sections were stained with

uranylacetate and lead acetate and examined.

Electron microscopy of white blood cell cultures and plasma pellets from three cows with persistent lymphocytosis reveals typical C-type viral particles in preparations from two of these animals. These C-type viral particles consist of a central dark nucleoid, surrounded by an outer membrane. The particles are intracytoplasmic and the size varies from 90-100 mu. It is observed that the white blood cells containing the viral particles are usually disrupted and are not present in any of the intact cells in culture. Further studies are in progress to confirm this very important observation.

Thus far, electron microscopy of white blood cell cultures and plasma of one leukemic cow have been made in this particular study. No virus particles have been found and, further studies are in progress.