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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

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I. PERIOD COVERED BY THIS REPORT:

This report covers the period from December 1, 1960 to December 31, 1966. 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 was 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 of 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 past year 157 cases were reported and confirmed. This was a 25 per cent decrease in confirmed cases, but it appears 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 had 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. The data continues to support the previous years data and conclusions 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.) in multiple case herd studies the data continues to suggest lymphocytosis as a pre-clinical manifestation of bovine leukemia. Cohort analysis of index cases, new cases and lymphocytosis in a few herds indicate a high correlation. These trends of age association as a factor in lymphocytosis and leukemia suggest a hypothesis of both vertical and horizontal transmission of a leukemogenic factor. Data obtained from hematologic study of single case herds indicate that 75 per cent of these herds are similar to multiple case herds.

III. PROGRESS REPORT:

A. General Information

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

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

The 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.

The approach to the study and procedures used are partially described in some detail in Progress Report No. 1 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. Three terms perhaps need definition in the general introduction as they will be used in various sections throughout this report. 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. Multiple case data refers to similar information obtained from herds where more than one case of leukemia has occurred in one year. Herds where more than one case has occurred within a year have been selected, and all animals in the herd have been studied. 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 three or more standard deviations (S.D.) above the normal count for animals of that age. The lymphocytes consist of a relatively normal population of cells which are found in normal animals in leukemia herds. The lymphocytosis is not associated with any demonstrable pathologic process or tumor growth.

The data will be presented under several sections as follows:

Epidemiologic Studies

Multiple Case Herd Studies

Hematologic Studies on Single Case Herds and
Normal Herds

Studies on Animals With a Marked Lymphocytosis

Transmission Studies

B. Epidemiology Studies

The original proposal made to the Atomic Energy Commission provided for collection of relevant epidemiological data and the orderly processing, tabulation, and evaluation of such data.

Epidemiological studies were directed at: (1) problems of occurrence and distribution in time and space (2) environmental factors and their possible relationships to incidence of reported leukemia.

It was recognized from the outset that since bovine leukemia was not a "reportable" disease, the collection of data on cases and data associated with cases would require an extraordinary degree of cooperation from practicing veterinarians as well as farmers. Environmental factors were studied on both a retrospective and prospective basis. The data includes the period December 1, 1960 to December 31, 1965 and represent 1170 confirmed leukemia cases in Minnesota and Western Wisconsin out of a total of 2975 reports of possible cases submitted for diagnosis.

Reports of possible cases were logged and assigned a leukemia study number at the time specimens for laboratory examination were received. Comprehensive epidemiological data were obtained on the animals diagnosed as confirmed cases of leukemia including the herd environment by means of interview of the farm operators. The data on the 1170 leukemic animals were put on punch cards. A complete punch card record for each leukemia case consisted of seven cards. Three other bodies of data are either completely punched or in the process of being punched to coincide with completion of the survey. These include (1) the leukemia multiple case herd study with four cards per case; (2) the Steele, Aitkin, Hubbard county samplings

of individual herds, animal by animal, with four cards, and (3) the current Socio-Economic factor survey in progress with a total of ten cards per herd.

Great care has been taken in checking these cards to insure that errors of reporting, transfer and punching are at an absolute minimum. Card sorting procedures utilizing the equipment at the St. Paul Campus Computing Center were used to tabulate these data, and classify them by various categories, in the manner of the tables shown in earlier reports of the project.

Statistical treatment of the data was facilitated by use of standard program routines on the IBM 1401 computer in service at the St. Paul Campus Computing Center.

The data on the leukemia population was subjected to statistical analysis in the summer of 1966. These analyses treated leukemia frequency as the dependent variable. In most cases, analyses were run for each of the five years' figures individually, prior to pooling the data for the entire period. Tests for homogeneity of variances (or permissibility of pooling) were run in all critical instances. Some of the more significant results will be discussed in the section to follows.

1. Statistical Analysis and Evaluation of Data on Occurrence,
Distribution and Environmental Factors

Descriptive statistics (means, standard deviations, errors and confidence limits) were obtained for most of the tabulated results on an annual as well as five year basis. Where appropriate, χ^2 tests were made to check the deviation of observed and confirmed cases from theoretically expected leukemia cases by classes. Correlation and regression coefficients were calculated

for data involving definable levels of an independent variable (e.g. time, herd size) relating to the incidence of leukemia.

Multiple regression and variable analyses were not run in the summer of 1966. Current work in this area is directed towards the maximum effective evaluation of the data during the entire period of this study.

The results of the analyses will be discussed under three categories: Consistency, major findings and supporting evidence.

a.) Consistency

That phase of the epidemiologic study of cases reported by practitioners and confirmed by project personnel was terminated at the end of 1965, so that the distribution map of incidence of leukemia by county and livestock district as shown on the 1966 progress report remains unchanged.

Examination of the year by year figures (Table B-1) for leukemia shows no significant differences in reporting of cases. This consistency of reporting applies regardless of area of the state, the number of cases reported and the inevitable changes in veterinary personnel serving the farm constituency at the local level. Yearly differences exist, but there is no statistical reason not to pool these data. Likewise, year by year type of cattle (dairy vs. beef), sex, breed (purebred vs. grade), age and herd size distributions show a remarkable degree of consistency.

b.) Major Findings

The yearly and overall relationship for increased leukemia incidence with increased age of the animal was shown to be highly significant (Table B-2).

At the same time, the association of size of herd with incidence of leukemia was likewise highly significant, both on an annual basis and on the basis of the total five-year period (Table B-3).

Table B-1

Distribution of Leukemia Cases By
Selected Categories in Minnesota

	1960	1961	1962	1963	1964	1965	Total
<u>Total Cases</u>	91	247	292	212	160	168	1170
<u>Type:</u>							
Purebred		43	56	23	26	25	173
Grade		197	233	186	131	141	888
<u>Age:</u>							
2-5 yrs.		69	108	62	55	65	359
6-9 yrs.		84	100	82	56	53	375
10 & over		13	12	15	5	8	53
X ²		39.80	23.00	55.16	15.34	9.19	127.31
<u>Class:</u>							
Dairy		220	273	202	147	156	998
Beef		12	15	7	9	7	50
<u>All Animals:</u>							
Male (mid steers)		10	12	4	5	3	34
Female		236	279	208	151	163	1037

Table B-2

Age Distribution of Bovine Leukemia Cases, 1961-1965

<u>Age Group</u>	<u>Observed</u>	<u>Expected</u>	<u>Difference</u>
2 - 5 years	359	508	-149
6 - 9 years	375	251	124
10 years and over	53	28	25

$$\chi^2 = 127.31, 2 \text{ d.f.}$$

Table B-3

Classification of Leukemia Cases By Size of Herd, 1961-1965

<u>Herd Size</u>	<u>Observed</u>	<u>Expected</u>	<u>Difference</u>
0 - 9 cows	19	70	-51
10 - 19 cows	66	277	-211
20 - 29 cows	132	241	-109
30 - 49 cows	304	41	163
50 and over	242	34	208

$$\chi^2 = 1,708.08, 4 \text{ d.f.}$$

c.) Supporting Evidence

The differential incidence of leukemia in terms of herd size poses a major problem of interpretation. Tabulated data on farm practices with regard to management, the general farm situation and indications of specialization, as well as breeding and livestock replacement practices suggest instances in which there is a negative association of degree of specialization with incidence of leukemia, including movement of animals and other associated farm animal husbandry and management.

The work of analysis was interrupted for several months due to lack of statistically competent personnel associated with the project. It is expected that these preliminary results will be amended within the next few months.

Discussion of Results

Highly significant relationships between incidence of reported leukemia and age of animal affected, as well as herd size, have been demonstrated. In both cases, the relationships are extremely consistent for each of the five years these data were collected.

Increased incidence of clinical manifestation of leukemia with advancing age has been the conclusive finding for the adult form of the disease, not only for this project but also for other investigators. Likewise, increasing incidence with increasing herd size has also been reported by European workers.

The non-random association of leukemia with herd size must be weighed in terms of the known higher average age of animals in small herds based on Steele, Hubbard and Aitkin county studies. On the basis of higher percentage of older animals, small herds

might be expected to actually have a higher frequency of leukemia than large herds. The fact that this expectation is not realized could be interpreted in terms of an hypothesis involving the transmission of a leukemogenic agent, provided that there is no relationship between herd size and diagnostic effectiveness among the factors influencing the reporting of leukemia.

This latter possibility should be resolved, at least in large measure, by the Socio-Economic survey currently in progress on which analyses can be initiated by April. The data available from the herd environment questionnaire taken on the leukemia cases is not without indicators as to the reliability of the small herd sample. These include correlation of presence of other animal species on the farm with leukemia incidence (see Table B-4) and data on origin of the dam of the index case, incidence in breeds other than Holstein and data on movement of animals. All of these would normally be associated with or are analogous to the small mixed farm dairy enterprise.

Assuming a transmissible agent, the problem of a non-uniform level of risk arises. The large size herd involves higher risk and increased exposure potential on two counts: (1) within herd transmission due to greater number of animals (2) introduction, a function of actual finite number of purchases or movement of individuals into the herd. In this study small herds have shown a reduced risk from introduction. Should this be the case, this information may have important implications for the study of the epidemiologic study of leukemia in man. Therefore the Socio-Economic studies and the analysis of control herds and herds with cases is proceeding with all possible emphasis.

Table B-4

Overall Correlations of Other Animal Species Present
on Farms With a Number of Bovine Leukemia Cases, 1961-65

<u>Variable</u>	<u>Correlation Coefficient</u>
No. of Swine	- .3999
No. of Sheep	- .3584
No. of Horses	- .5151
No. of Chickens	- .4004

2. Retrospective Studies on Socio-Economic and Environmental Factors

Relationship of the Increasing Incidence Rate of Leukemia to the Aggregation of Cattle in Larger Herds

One of the most interesting findings of these studies has been the striking increase in the number of cases of leukemia in the larger herds of cattle where the rate, per hundred thousand animals at risk, is six and one half times greater than among the cattle in small herds with less than ten cattle. The incidence rate is not based on the number of herds but rather on the number of cattle contained in the herds when grouped according to size to provide an incidence rate per one hundred thousand cattle.

Table B-5 and Figure B-1 show the percentage of expected cases in relation to the cases actually observed in each herd size group for the period of the study; Table B-6 and Figure B-2 present the average annual incidence rate of reported confirmed cases of leukemia, per hundred thousand cattle at risk, in each herd size group of dairy cattle in Minnesota for the period of the study, December 1, 1960 to December 31, 1965.

Table B-5

Comparison of Observed and Expected Occurrence of Leukemia
in Relation to Aggregation of Cattle*
Dec. 1, '60 - Nov. 30, '65

<u>Size of Herd</u>	<u>No. of Cows at Risk in Each Herd Size Group</u>	<u>Expected Average Annual Number of Cases</u>	<u>Expected Percentage</u>	<u>Observed Average Annual Number of Cases</u>	<u>Observed Percentage of Expected Cases</u>
1 - 9	118,481	17.1	100	8.2	49.7
10 - 19	454,569	65.3	100	31.0	49.1
20 - 29	370,129	53.3	100	47.8	92.3
30 - 49	196,807	28.3	100	52.2	191.2
50+	45,085	6.5	100	20.2	320.6

* Female dairy cattle two years and older in Minnesota.

Table B-6

Average Annual Incidence Rate* of Reported Confirmed
Cases of Leukemia Per 100,000 Dairy Cattle in Minnesota
Dec. 1, '60 - Nov. 30, '65

<u>Size of Herd</u>	<u>Average Annual No. of Cases in Each Group</u>	<u>Number of Cows at Risk in Each Size Group</u>	<u>Average Annual Rate Per 100,000 Cows</u>
1 - 9	8.2	118,481	6.9
10 - 19	31.0	454,569	6.8
20 - 29	47.8	370,129	12.9
30 - 49	52.2	196,807	26.5
50+	20.2	45,085	44.8

* Female dairy cattle two years of age and older in Minnesota

Figure B-1

Percentage of Expected Cases in Relation to the
Cases Actually Observed in Each Herd Size
Group

Dec. 1, '60 - Nov. 30, '65

Figure B-2

Average Annual Incidence Rate of Reported Confirmed
Cases of Leukemia Per Hundred Thousand Cattle
at Risk in Each Herd Size Group of Dairy
Cattle in Minnesota

Dec. 1, '60 - Nov. 30, '65

It has been determined that the large herds have a smaller proportion of cattle in the older age groups as compared with the small herds, and that this is contrary to what one would expect since the incidence rate of leukemia is much greater in older animals. In view of these facts, the increased incidence rate becomes even more significant.

It would appear that the age of animals in large herds is not the factor responsible for the marked increased incidence in the larger herds, and intraherd transmission of an infectious agent is suggested.

3. Socio-Economic Studies

We have also been concerned with other factors such as the amount of veterinary medical services available to the various herds grouped according to size, socio-economic factors, and other factors which may influence reporting and may be unduly influencing the higher incidence in large herds. Following discussion of these factors with staff members and consultants of the Atomic Energy Commission, it was agreed that it was important to conduct an investigation of relevant socio-economic factors to evaluate their influence on the reported increased incidence of leukemia in the larger herds.

In an effort to investigate and evaluate these variables a retrospective study was designed and initiated in the summer of 1966.

A questionnaire was devised; one section relating to socio-economic information, the other section to herd environment data. The socio-economic form covered the quantitative use and cost of veterinary services, management of the herd in regard to

replacement of animals and reasons for removal from the herd, kinds of breeding and production records kept, educational level of the farmer and an assessment of the standard of living and level of management of the farmer. Herd environment information included the number and type of livestock present, movement of animals into and out of the herd, use of possible leukemogenic agents (wood preservatives, insecticides, and herbicides), feeding practices and water sources, and a soil survey if available. This herd environment information is being collected to match data obtained from all of the reported leukemia cases during the period December 1, 1960 to December 31, 1965.

Three population groups were to be surveyed. The first group included a sample of the leukemia cases reported from December 1, 1960 to December 31, 1965. We included all of the farmers in Minnesota reporting two or more cases of leukemia during the period of the study (44 cases); as well as all of those reporting one case of leukemia during the period of the study with a good history of previous leukemia cases in the herd (33 cases). Of the farmers reporting only one case of leukemia and no history, we interviewed 122 owners of herds with cases occurring between January 1, 1964 and December 31, 1965. The last two years were chosen because it was believed that interviews from these farmers would be more readily obtained and more accurate in terms of recall.

The second group was chosen from a sample selected in 1962 for the purposes of obtaining normal hematologic data. This sample included 51 farmers whose herds were known to be free of leukemia whose animals had normal lymphocyte counts.

Interviews for these two groups have been completed, the information coded and punched into IBM cards and some preliminary tabulations and cross tabulations have been made.

The third group was chosen on a geographic basis to avoid regional differences which might influence economic practices, husbandry, and availability of veterinary services.

We chose 100 herds to match a sample of the leukemic herds on the basis of geographic location, herd size and whether the herd produces grade A or B milk. We located the leukemic herd on a township map and applied a grid of random numbers to the quarter township including the farm and drew up a standard procedure to make the matching process within the quarter township as random as possible (instructions in appendix). This series of interviews is currently being made, and will be checked by sequential sampling as the survey progresses until it can be evaluated with some degree of assurance.

How to Use the Quarter Township Randomizations

1. Superimpose the set of random numbers on the actual grid, with the section containing the farm to be matched falling where it will.

1

2

3

Set of Numbers

*Farm to be
matched

3	4	5
7	2	6
9	8	1

3	4	5
7	*2	6
9	8	1

* Farm to be
matched

2. Go to Section 1. Start at the southwest corner and go around the section looking for a suitable matching farm. If none is found, go to Section 2, then 3, until all nine have been tried.
3. Omit from survey any farm sampled before (like the farm to be matched in Section 2).
4. If no farm is found within the quarter township, select an adjacent quarter township (see guide) and repeat the procedure with a new set of random numbers.
5. Use a set of numbers once only; check it off after use.
6. If no matching farm is found, go to the nearest adjacent township, and proceed as before.

C. Multiple Case Herd Studies

1. Epidemiologic Studies

a.) General

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

Table C-1 lists the multiple case herds and information on tumor cases occurring in these herds. The length of time each herd has been under study is dependent upon the date of occurrence of the index cases. Herd MH-20 is an exception to this and has been studied since 1961. This is an institutional herd with adequate records indicating history of leukemia for ten years.

Of the herds listed in Table C-1 only 1, 4, 5, 25, 29 and 31 are presently under intensive hematological and epidemiological study. The remainder of the herds are followed only in respect to reporting new cases of leukemia. The decision to de-emphasize hematologic study of these herds was based upon the general lack of detailed record keeping on individual animals in the herd. Several new high incidence herds with good record systems have been added to the study recently. More herds of this type will also be studied in the future.

Ten of the herds listed in Table C-1 continued to have new cases of leukemia. In all, 20 cases have developed subsequent to the initiation of hematologic study. All of these cases have exhibited persistent preclinical lymphocytosis prior

to the development of tumor growth. Approximately 17.7 per cent of the adult cows in these herds have a persistent lymphocytosis. New cases in different herds developed tumors during the past year.

Table C-1

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

<u>Herd Number</u>	<u>No. of Animals 2 Years Old or Older</u>	<u>Breed</u>	<u>Leukemia No.</u>	<u>Occurrence</u>	<u>Sex</u>	<u>Age</u>
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-2	31	Holstein	L-200	Dec. 1960	Fe	6 yrs.
			L-201	Dec. 1960	Fe	6 yrs.
MH-3	57	Holstein	MH3-46	Feb. 1959	Fe	6 yrs.
			MH-347	Jan. 1960	Fe	6 yrs.
			L-209	Dec. 1960	Fe	6 yrs.
			L-2046	Dec. 1963	Fe	8 yrs.
			L-2397	Aug. 1964	Fe	5 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.
MH-6	29	Shorthorn	L-316	Feb. 1961	Fe	7 yrs.
			L-335	Mar. 1961	Fe	10 yrs.
			L-865	Jan. 1962	Fe	7 yrs.
MH-8	18	Holstein	L-273	Feb. 1961	Fe	10 yrs.
			L-362	Mar. 1961	Fe	9 yrs.
MH-15	30	Red Poll	L-483	May 1961	Fe	4 yrs.
			L-484	Mar. 1961	Fe	10 yrs.
		Holstein	L-485	Feb. 1961	Fe	6 yrs.
			MH15-16	July 1962	Fe	5 yrs.
MH-15-2	41	Red Poll	L-2249	April 1964	Fe	10 yrs.

<u>Herd Number</u>	<u>No. of Animals 2 Years Old or Older</u>	<u>Breed</u>	<u>Leukemia No.</u>	<u>Occurrence</u>	<u>Sex</u>	<u>Age</u>
MH-18	21	Holstein	MH18-22	July 1960	Fe	7 yrs.
			L-517	June 1961	Fe	6 yrs.
MH-20	87	Holstein	0-415	April 1954	Fe	5 yrs.
			0-1385	Sept. 1957	Fe	6 yrs.
			0-1417	Sept. 1957	Fe	6 yrs.
			0-714	Mar. 1959	Fe	5 yrs.
			0-489	Sept. 1959	Fe	9 yrs.
			L-676	Aug. 1961	Fe	12 yrs.
MH-22	49	Holstein	L-43	Aug. 1960	Fe	5 yrs.
			L-587	Aug. 1961	Fe	8 yrs.
MH-23	25	Holstein	L-457	May 1961	Fe	4 yrs.
			L-656	Sept. 1961	Fe	4 yrs.
			L-1018	April 1962	Fe	5 yrs.
			L-1431	Oct. 1962	Fe	5 yrs.
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-27	74	Holstein	L-1340	Sept. 1962	Fe	4 yrs.
			L-1436	Oct. 1962	Fe	10 yrs.
			L-1457	Nov. 1962	Fe	3 yrs.
			L-2102	Jan. 1964	Fe	6 yrs.
MH-28	71	Holstein	L-1614	Mar. 1963	Fe	6 yrs.
			L-1868	Aug. 1963	Fe	9 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-32	18	Shorthorn	D-562	Sept. 1963	Fe	5 yrs.
			D-701	Feb. 1965	Fe	8 yrs.
			D-553	Aug. 1965	Fe	7 yrs.
			L-2887	Sept. 1965	Fe	7 yrs.
			L-2896	Oct. 1965	Fe	4 yrs.

Total cases: 66

2. Natural Occurrence of Leukemia

Analysis of leukemia cases occurring in multiple case herds

with respect to date of birth, indicates a high incidence of leukemia case clusters in certain cohort groups. There is also a relatively high incidence of persistent lymphocytosis exhibited in nonleukemic members of these cohorts. Table C-2 illustrates this clustering in some of the herds where birth dates are available for the leukemia cases. These cases are tabulated by month and year of birth. In several clusters, dates of birth of leukemia cases are within a one week period.

In addition to the strong association of leukemia cases and persistent lymphocytosis in cohort studies, other clusters of cows with lymphocytosis were found. These clusters, although not containing cases of leukemia do have in common a close proximity of birth dates. Many of the cohorts containing clusters of leukemia cases and/or clusters of cows with persistent lymphocytosis contain members which are daughters of leukemia cases or dams with persistent lymphocytosis.

It is interesting to speculate on all the common exposures which cohort groups experience. The major consideration in exposure experience for a group of calves born within a month of each other and other calves born during the same year would be in the early neonatal period. During this period, they are segregated into very limited age groups. On all farms where clusters can be documented, there is a common practice of pooling of colostrum. More precisely, calves born at the same time can potentially receive any colostrum available at that time. This is true not only of calves which should be receiving colostrum during the first few days of life but also other calves which are two to three weeks of age. These slightly older calves consume

Table C-2

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

<u>Herd Number</u>	<u>Month of Birth</u>	<u>Number Studied</u>	<u>Number Developing Leukemia</u>	<u>Number Developing Lymphocytosis</u>	<u>Percentage With Leukemia & Lymphocytosis</u>
MH-1	Aug. 1955	7	3	2	71%
	July 1958	9	1	7	89%
MH-2	* 1954	2	2	-	100%
MH-4	Sept. 1956	8	1	6	88%
	Sept. 1957	5	1	3	80%
MH-5	* 1953	3	2	1	100%
MH-15	Oct. 1955	4	1	3	100%
MH-20	* 1952	2	2	-	-
	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	Nov. 1958	<u>3</u>	<u>3</u>	<u>0</u>	100%
Total		66	27	31	

* Precise month of birth is not available, however, at least two cases of leukemia developed in animals of the same age during the same year, and in two herds during the same month.

colostrum which is in excess of the needs of the very young calf. We have recorded cases where some of the clusters tabulated in Table C-2 received colostrum from cows in the early clinical stages of leukemia.

3. Genetic Studies

Geneological studies have been undertaken in eight of the high incidence herds which keep pedigree records. There have been 15 cases of leukemia in these herds which have had 22 mature daughters under study. Three of these dam-daughter pairs have died of leukemia. There have also been eight occurrences of paternal half-siblings developing leukemia in herds where natural breeding is practiced. These bulls had been used for breeding on these farms for several years and therefore possibly are more significant as a representative of a period in time rather than for their possible genetic effect on leukemia.

The number of cases of leukemia in closely related animals is too small for definitive evaluation. If we accept the hypothesis of persistent lymphocytosis as a pre-clinical state of leukemia, the number of animals available for analysis is greater. Table C-3 presents the numerical relationship between 312 dam and daughter pairs in respect to leukemia-lymphocytosis and those animals not exhibiting leukemia or lymphocytosis.

Table C-3

The Relationship of Leukemia and Lymphocytosis in
312 Dam-Daughter Pairs Studied in
Multiple Case Leukemia Herds

<u>Pair Group</u>	<u>Description of Dam-Daughter Pair</u>	<u>Number of Pairs</u>	<u>Percentage</u>
I	Dam-Daughter: Leukemia, Lymphocytosis Neg.	154	49
II	Dam-Daughter: Leukemia, Lymphocytosis Pos.	39	13
III	Dam: Leukemia, Lymphocytosis Pos. Daughter: Leukemia, Lymphocytosis Neg.	48	15
IV	Dam: Leukemia, Lymphocytosis Neg. Daughter: Leukemia, Lymphocytosis Pos.	71	23
		312	100

It is seen that 45 per cent of the dams with leukemia or lymphocytosis have daughters similarly affected, while only 32 per cent of the unaffected dams produce daughters with lymphocytosis or leukemia. Analysis of this data by the X^2 method is presented in Table C-4. It can be seen at the usual .05 level of significance that the difference in the ratios is significant.

Table C-4

Comparison of 312 Dam-Daughter Pairs For Observed Leukemia and Lymphocytosis With the Expected Occurrence of Leukemia and Lymphocytosis In Multiple Case Leukemia Herds

<u>Pair Group</u>	<u>Description of Dam-Daughter Pair</u>	<u>Number of Pairs</u>	
		<u>Observed</u>	<u>Expected</u>
I	Dam-Daughter: Leukemia, Lymphocytosis Neg.	154	146
II	Dam-Daughter: Leukemia, Lymphocytosis Pos.	39	31
III	Dam: Leukemia, Lymphocytosis Pos. Daughter: Leukemia, Lymphocytosis Neg.	48	56
IV	Dam: Leukemia, Lymphocytosis Neg. Daughter: Leukemia, Lymphocytosis Pos.	71	79
		312	

$$X^2 = 4.46$$

$$P < 0.038$$

$$1 \text{ d.f.}$$

It would be difficult to draw any definite conclusions from limited data of this type but there is an indication that vertical transmission (prenatal or neonatal) of causal factors may be possible. The lymphocytosis observed in daughters of unaffected dams may be accounted for by the possible horizontal spread within cohort groups during the neonatal period.

D. Hematologic Studies on Single Case Herds and Normal Herds

1. Analysis of Selected Normal Herds

Data was presented in the sixth progress report on the selection of normal control herds for establishment of hematologic normal values and as a control group for some epidemiologic studies. The data obtained on a stratified sample of 37 herds from three counties was subjected to further analysis. Although the statistical studies are incomplete, data is available on the relationship of age to lymphocyte and total leukocyte count. Single regression analysis indicate a highly significant drop in lymphocytes and in total leukocyte counts with increased age of animal studied. In previous reports, the age effect on lymphocyte counts of animals over four years of age was not considered significant. To more accurately evaluate previously obtained data on single and multiple case herds as well as the county survey studies, the data is being reviewed in light of the significance attached to the age effect on the lymphocyte counts in cattle.

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

Previous reports from Danish workers indicated that juvenile leukemia is not associated with lymphocytosis in normal animals in the herd of origin. Unpublished data from Great Britain, where only juvenile and thymic-lymphosarcoma of young animals are reported, indicate that lymphocytosis in normal animals was not observed in over 700 herds examined. These workers also reported that juvenile leukemia has a sporadic distribution.

In the adult form of leukemia, the Danish workers indicate a sporadic distribution for a small portion of the cases, while the

majority are endemic in distribution.

In the previous progress report hematologic data was reported on 20 single case herds of the adult type. During this contract period 22 additional single case herds of the adult type and ten single case herds of the juvenile type were studied. The herds were selected from the index single case herds in Minnesota. In order to assure comparability with other data, fifteen counties were picked from which to draw the samples on the basis of the geographic distribution of the multiple case herds and county population studies. Only Holstein herds were chosen. The herds considered were stratified according to herd size and selected on the basis of herd size distribution for the multiple case herds and county studies. A further stratification was necessary in order to take into account the date the L-case was reported. Only a few cases from 1960 and 1964 were chosen since the 1960 records were considered below the reporting standards achieved later, and because of the possibility that the 1964 cases might turn out to be multiple case herds.

The data obtained on the 32 additional single case herds has been tabulated but not fully evaluated. Inspection of the tabulated data on the second sample of single case (adult type) herds indicate similar findings to the previous sample where 75 per cent of the herds were characterized as of the multiple case herd type. The ten single case (juvenile type) herds were more characteristic of the normal herds sampled. Three of the ten herds were characterized as lymphocytosis herds, however, it is not possible to exclude simultaneous occurrence of the adult form of leukemia in these herds. Statistical evaluation of this data is in progress.

3. Comparison of Single Case and Multiple Case Herds

Comparison of the single case herd multiple case herd hematologic data has not been completed. Since over 65 of the single case herds have become multiple case periods on the basis of retrospective and prospective study the probability of similarity is great.

E. Studies Pertaining To Animals With a Marked Lymphocytosis
Originating From Multiple Case Herds

1. Studies On The Natural Outcome of Animals With Lymphocytosis

Although one of the objectives was to accumulate animals from multiple case herds to determine the natural outcome of lymphocytosis animals, this objective was not fully completed. Selected animals were acquired from these herds through normal culling practices and additional lymphocytosis animals were obtained from other leukemia herds for the studies reported in part E 2.

Since it was not feasible to acquire a sufficient number of lymphocytosis animals for this particular objective, these animals were studied and evaluated on the basis of their natural herd environment. The number of tumor cases and lymphocytosis animals was summarized for ten multiple case herds and is depicted in Table E-1.

Of the 920 animals evaluated for periods of time ranging from 2-7 years, 4.9 per cent of the animals developed tumors. Animals with persistent lymphocytosis accounted for 17.7 per cent of the 920 animals studied. Twenty new cases in addition to the index tumor cases developed during this period. All 20 new tumor cases had a persistent lymphocytosis prior to tumor growth. No tumor cases were observed in animals that had normal lymphocyte counts. During the period of study, 12.3 per cent of the lymphocytosis animals developed tumors.

Table E-1

Summary of Tumor Cases and Lymphocytosis Animals In Ten
Selected Multiple Case Herds

<u>Herd No.</u>	<u>Average Herd Size</u>	<u>Total Animals Studied</u>	<u>No. of Years Studied</u>	<u>No. of Lymphocytosis Animals</u>	<u>No. of Tumor Cases</u>
1	44	115	7	26	8
3	54	107	4	19	4
5	45	113	6	6	3
6	26	45	3	7	3
15	26	74	4	11	4
20	80	147	3	25	7
23	25	32	2	9	4
25	28	74	5	14	4
27	67	108	2	23	4
29	<u>72</u>	<u>105</u>	2	<u>23</u>	<u>4</u>
Total	470	920		163	45

2. Studies on Lymphocytosis Animals and Promotion of Tumor Growth

a.) General

This study was undertaken in an attempt to clarify the role of lymphocytosis in cattle which is associated with leukemia cases. Numerous data accumulated in Minnesota and throughout the world indicate that there is a consistent appearance and persistence of lymphocytosis in cattle in leukemic herds. Furthermore in herds which have undergone long periods of hematological study, there develop additional cases of leukemia which invariably exhibited a pretumor lymphocytosis. Due to the usual production practices in these dairy herds most of the cows exhibiting lymphocytosis are

normally culled from the herds before they attain the ages at which high incidences of leukemia are observed. Therefore, most of these cattle do not develop leukemia while under study. Approximately 7 to 12 per cent of animals with a persistent lymphocytosis develop frank tumor growth.

It is postulated that cows with lymphocytosis are the result of induction by a viral agent and may require a promoting factor in order to manifest tumor growth. Accepting an Inducer-Promotor theory of leukemogenesis, it is theorized that treatment with low level radiation, hormones or chemical carcinogens may shorten the time required for these cows to develop into clinical leukemia cases.

There is also the possibility that these animals are maintained in a protracted induction state due to a highly competent immune mechanism which controls aberrations as they appear. Alteration of the immune capability of these animals by treatment with radiation, adrenal corticosteroids, chemical carcinogens or other lymphodepressant agents may promote a leukemic stage.

Regardless of the mechanism of action of the treatment it is essential to determine the true role of lymphocytosis as seen in these cattle. If lymphocytosis is proven to be a pre-tumor state of leukemia much progress could be made in etiological studies of bovine leukemia. The normal long induction period of bovine leukemia requires such extended periods of study which results in very small numbers of cases appearing in study herds. Further, if the induction period is shortened, the transmission studies would be simplified.

b.) Radiation Studies

Methods. The animals in this study were given sublethal total body irradiation from a Cesium 137 deep well source. Based on previous studies in this laboratory, a midline dose of 75 r of total body irradiation from a gamma source was selected as a sublethal dose. The exposure was made separately to each side of the standing animal for a combined exposure time of approximately 18 minutes. Dose rate was established by attachment of radiation counting chambers to the near and far sides of the hip and shoulders. Entrance dose was approximately 11 r/minute and exit dose approximately 1.75 r/minute. This resulted in approximate midline doses of 4.3 r/minute. Doses were calculated and measured for individual animals. The total midline dose for each animal was calculated to be $75 \text{ r} \pm 1$ per cent.

Experimental Animals. Five Jersey cows originating from high incidence herds and exhibiting persistent lymphocytosis were used for this experiment. Four of these cows have been followed hematologically by semiannual bleedings for six years. The other cow has been under study for two years. Their ages ranged from 5 to 8 years. Pretreatment absolute lymphocyte counts on these cows averaged 11,000/cmm. and the lymphocytes represent 75-85 per cent of the total leukocytes on differential examination.

Response to Radiation. Twenty-four hours post irradiation hemograms indicated decreases in lymphocytes to 25-50 per cent of pre-treatment levels. Maximum reductions of lymphocytes were attained by the third post irradiation day with average

reduction to 28 per cent of pre-treatment levels. Neutrophils and blood platelets were similarly reduced with maximum reductions occurring at three and four weeks respectively. The time sequence of response is similar to results of previous studies on effects of ionizing radiation on calves reported from this laboratory. It is significant in relation to the lethal dose of radiation for cattle to indicate that the neutropenia was marked in all animals. One cow exhibited acute signs of total body radiation related to the severe neutropenia. This animal has recovered from the severe infection following intensive antibiotic therapy. The radiation studies will be repeated when hematologic values return to pre-irradiation values or stabilize. A reduction in subsequent doses of ionizing radiation is planned.

Apparently, hemic tissue of the cow is unusually sensitive to the effects of total body irradiation.

c.) Adrenal Corticosteroid Studies

Methods. Treatment for this portion of the study consisted of administration of prednisolone (Sterane^R PFIZER) intramuscularly. Various dosages were used in establishing the continuous level of the drug necessary for maximum lympho-depressant effect. We are presently administering 6 mg./lb./day to each animal for a period of six days followed by four days of diminishing withdrawal doses. This dose regimen apparently does not cause overt signs of hypercortico-steroidism with the possible exception of slight increase in water and sodium retention.

Experimental Animals. Four Holstein cows originating

from high incidence herds were used in this study. These cows range in age from four to seven years of age. All of these cows had been under hematological study for more than one year previous to the experiment. Absolute lymphocyte counts ranged from 12,000 to 32,000 per cmm. during the pretreatment period. Lymphocytes represented from 70 to 90 per cent of the leukocytes in differential smear examination.

Response. Due to the fact that these animals were used intermittently in establishing base line data for lympho-depressing doses the results of this treatment are probably not representative of potential response in cows of this type. Generally the lymphocytes were depressed to at least 50 per cent of pretreatment levels when 6 mg. per pound of body weight of prednisolone was administered. Maximum lymphocyte response was noted during the six day course of treatment. At the time of writing this report, which is three weeks after the last treatment, lymphocytes still have not returned to pretreatment levels. Neutrophils increased by three to five times during the prednisolone treatment period but generally returned to pretreatment levels by three days post treatment.

Eosinophils disappeared from the peripheral blood during treatment with the higher levels of prednisolone but reappeared a few days after cessation of therapy. Stimulation of the erythron was a general observation and will require further evaluation. Blood platelets were apparently unaffected by the treatment regimen used in this study.

Other Procedures. Experimental animals used in these

studies are acquired as they become available through normal culling from the study herds. This results in limited numbers of cattle available each year. Additional animals are being accumulated for other proposed treatments.

3. Cytokinetic Studies of Lymphopoiesis

The nature and mechanism of lymphocytosis in normal animals originating in multiple case herds is poorly understood. Cytokinetic studies of known model systems in which lymphocytosis is a prominent feature were undertaken to ascertain whether similarities exist.

A lymphocytosis associated with heparin administration has been reported to occur in calves. The blood lymphocyte count increases rapidly then falls following intravenous heparin administration. A similar pattern has been reported to occur in the thoracic duct lymphocyte output. It is generally accepted that lymphocytes recirculate from blood to lymph and that the recirculation time may be short. The possibility that heparin may block the lymphocyte recirculation route was considered. Such a block could account for a peripheral lymphocytosis due to accumulation in the blood. In addition, rapid mobilization due to drug action directly and/or possibly hormone release or binding was considered.

To study the above question and to develop a simple system for study of a discrete mass of lympho-reticular tissue in vivo, the efferent lymph vessel of the bovine prescapular lymph node was cannulated. Successful fistulas have been prepared in seven calves with a total patency time of 77 days.

The results from 29 experiments with these animals indicated

that the mean white blood cell content of lymph from the prescapular fistula is 11,600 cells per mm^3 . (Range 6,800-21,500.) Less than 1 per cent of the white blood cells are granulocytes. The rest being lymphoid cells.

The mean lymph flow rate for the 29 experiments was 8.7 ml. per 15 minutes (34.8 ml./hr.) with a range 3.6 to 16.1 ml. per 15 minutes.

The cell output calculated on the basis of the above figures indicate 10.1×10^7 white blood cells per 15 minutes (40.4×10^7 per hr.) are lost via the prescapular fistula.

Following the injection of 300 USP anticoagulation units or more of heparin there was an increase in the white cell content of the prescapular lymph vessel effluent. Following drug administration and after a 15 minute lag period a sharp increase in lymph white cell content was recorded. The peak response occurred usually at $1\frac{1}{4}$ hour post drug injection. Following the peak response the lymph cell count fell rapidly to a value below the pre-injection lymph white cell count.

Concurrent with the increase of cell content, the lymph flow rate began to increase. The flow rate would remain above pre-injection values for three to four hours at which time the experiments were usually terminated.

Because of the increased flow rate the cell output remained above the preinjection values for three to four hours even though the cell count of lymph was diminished.

Observations have been made on 64 experiments during which the absolute lymphocyte content of peripheral blood was monitored following intravenous heparin injections. The peak response

generally occurred between two and one half and three hours post drug injection. The lymphocytosis persisted for up to six hours.

Super imposition of the curves obtained for both lymph and blood lymphocyte counts (Figure E-1) shows the relationship as a percentage change from preinjection values.

The degree of lymphocytosis appears to be dose dependent. The dose response curve suggests a threshold value may exist below which no lymphocytosis could be detected. There was marked variability between calves and in the same calf from day to day in the response to equal doses of heparin.

Heparin of beef lung origin was more effective than hog mucosa heparin in producing lymphocytosis in calves.

At this stage, correlations or similarities between heparin lymphocytosis and lymphocytosis associated with bovine lymphocytic leukemia have not been evaluated.

The establishment of chronic fistulas of isolated lymph nodes gives a suitable experimental model for evaluation of tissue graph (leukemic tissue) experimentation. Since tissue skin grafts may be maintained on regions of the body drained by the thoracic duct in which the thoracic duct effluent was subjected to continuous ionizing radiation, the possibility of adapting leukemic tissue as a graft to the region drained by the single isolated lymph node is a distinct possibility. This would in effect result in continuous bombardment of the host lymph node tissue with neoplastic cells and/or possible virus. Additional study of this system is in progress.

Figure E-1
 Blood and Lymph
 Cell Content per $\text{mm}^3 \times 10^3$
 Per Cent Change

L.F.E. 28

Peripheral blood and efferent prescapular lymph node efferent lymphocyte counts are depicted as a percentage of change over base line values following intravenous heparin administration.

4. Ultra Structural Studies

a.) Studies of the Ultrastructure of Leukocytes of the Normal Cow

See appended protocols for tissue preparation techniques.

This is a study of the agranulocytic leukocytes of cattle. Five non-pregnant, cycling cows will be sampled during the interval of 12-16 days post-estrus. Five pregnant cows will be sampled during the seventh to eighth month of gestation. Where possible, four samples will be collected from each animal. Differential counts and quantitative characterization studies will be made. Special attention will be paid to the monocyte and lymphocytes with nuclear bodies for information useful when studying these cells in pre-leukemic animals.

Preliminary work has been done on normal pregnant and non-pregnant cows in order to develop adequate techniques of sampling and processing blood samples and methods of evaluation. The following is a summary of some incomplete quantitative data obtained to date in studies to characterize monocytes in normal cattle:

Measurements of Mitochondrial Cross Sectional Areas In Sections of Lymphocytes and Monocytes In "Normal" Bovine Peripheral Blood

	Lymphocytes (12 cells)	Monocytes (12 cells)
Range of number of mitochondria per cell section	1 - 8	9 - 29
Average number of mitochondria per cell section	4.6	14.8
Range of sizes of mitochondrial profiles	$0.055u^2 - 0.435u^2$	$0.03u^2 - 0.247u^2$

	Lymphocytes (12 cells)	Monocytes (12 cells)
Range of average sizes of mitochondria per cell section	$0.116\mu^2 - 0.352\mu^2$	$0.050\mu^2 - 0.130\mu^2$
Average size of mitochondrial profiles	$0.206\mu^2$	$0.097\mu^2$

On the basis of this data it can be tentatively concluded that the monocyte of cattle, like that of the human, has ultrastructural characteristics which are unique.

An attempt is being made to develop a technique for the reliable differential counting of agranulocytes in electron microscopic preparations, using the Anderson buffy coat plug technique. No results are available at the present time.

b.) Studies of the Ultrastructure of Leukocytes of Pre-Leukemic Cows

See appendix for protocols of tissue preparation techniques.

It is planned to obtain at least four blood samples from pre-leukemic cows for electron microscopic studies. To date it has been possible to obtain one pretreatment sample from each of five pre-leukemic cows. An attempt will be made to obtain four samples from each pre-leukemic cow after any given treatment described in studies on the promotion of tumor growth, section E 2.

Too few observations have been made to allow for any definite conclusions regarding the occurrence of definitive early characteristic morphological changes which could be used in predicting the development of the leukemic state.

Nevertheless, two structural changes of possible significance were noted in lymphocytes. First, it was noted that nuclei of medium lymphocytes of pre-leukemic cows fairly frequently contain slender tongue or half-moon shaped evaginations which were not observed in cells of so-called normal cattle. Second, nuclear bodies of lymphocytes are either absent, or present in ghost or bizarre forms. They are very prominent in lymphocytes of normal cattle.

c.) Studies of the Peripheral Blood and Lymph Nodes of the Leukemic Cow.

See appendix for protocols of tissue preparation techniques.

Samples of peripheral blood were studied from leukemic animals brought to the University.

Too few samples have been studied to provide a basis for generalizations concerning the structural characteristics of neoplastic agranulocytes. It can be said, however, that nuclear changes of the kind mentioned for pre-leukemic cattle are abundant in lymphocytes of leukemic cattle.

d.) Studies of the Peripheral Blood and Lymph Nodes of Antigen-Stimulated Calves

See appendix for protocols of tissue preparation techniques.

A study which is somewhat ancillary to our main efforts concerns macrophage and immunocyte relationships in lymph nodes of antigen-stimulated calves. To date, two calves have been inoculated with Brucella abortus strain 19 antigen twice at intervals of 11 days in sites distal to the popliteal lymph node. Samples were taken of cortical and medullary lymph node 11 days post-inoculation for studies of reticular

macrophage-immunocyte cytokine activities. Blood samples were also obtained from these animals to gain further information concerning the response of monocytes to the presence of antigen.

Studies of tissues from the lymph nodes show that fixed macrophages make many contacts with pyroninophilic-type cells. The latter show the presence of much ergastoplasm, which is characteristic of developing immunocytes. Of interest is the fact that these pyroninophilic-type cells contain prominent nuclear bodies. It leads to the questions as to why plasma cells are never seen to contain nuclear bodies, and also the relation of nuclear bodies to immunogenetic activities.

F. Transmission Studies in Bovine Leukemia

1. Intrafetal Inoculation

Past experiences by this study group and others in the area of attempted transmission of leukemia dictates a need for re-evaluation of procedures. Therefore most of the progress we have accomplished in transmission work has been in a basic "tooling up" phase.

During 1960 transmission of leukemia was attempted by inoculation of six animals with material from adult leukemia cases. Two of these were calves of one week of age which received intravenous injection of tumorous lymph node suspension. Two other calves less than one week of age received intravenous injections of thoracic duct lymph from a terminal case of leukemia. The last two animals which were approximately nine months of age each received 500 cc. of plasma and "buffy coat" from two cases of leukemia. These animals were followed clinically and hematologically until they were six years of age at which time they were slaughtered.

Five of the animals remained clinically normal and without evidence of persistent lymphocytosis throughout the six year study period. One neutered male animal developed a marked lymphocytosis at two and one half years of age which persisted until the time he was slaughtered. All of his lymph nodes were found to be apparently normal both grossly and histologically at the time of slaughter. This animal was one of the calves which received intravenous thoracic duct lymph at an age of less than one week.

There are indications hematologically that in the one animal

there was possibly a successful transmission. This is based upon data indicating the association of lymphocytosis with leukemia in study herds and evidence of preclinical lymphocytosis in new leukemia cases arising in these herds. Definite proof of transmissibility would require development of clinical leukemia if it had been possible to follow the animal longer. The decision to terminate the study of this animal was forced by the lack of facilities for housing a fractious 2200 pound animal.

For the new transmission studies it was thought that in view of the present theories on the importance of immunological tolerance that results could be improved if transmission was attempted before the age of immune maturity. This is known to be true in transmission of murine leukemia. Since the calf differs from the mouse in the respect of its being immunologically competent at birth, we undertook studies to determine at what time in fetal development that antibody production begins.

The study to determine the age at which the fetus is able to produce antibody against viral agents was approached in two ways. The first of these was accomplished by checking slaughterhouse fetal blood for the presence of antibody to two very common bovine viruses, i.e. Bovine Viral Diarrhea (BVD) and Infectious Bovine Rhinotracheitis (IBR) viruses. Over 100 paired maternal and fetal sera were collected and examined. The fetal blood was checked for serum neutralizing (SN) titer to both viruses. The normal bovine placenta does not allow passage of antibody from the maternal to the fetal blood supply. Therefore if the fetal blood had SN antibody to one of the viruses then the dams blood was checked to see if it contained SN antibody to both viruses which would rule out the

possibility of passive immunity due to a faulty placenta.

Six serums from fetuses with ages from 105 days to 260 days showed BVD neutralizing antibodies and not for IBR. The maternal sera from two of the six dam fetal pairs showed SN antibodies for IBR virus. This would indicate that BVD neutralizing antibodies in bovine fetal serum are due to active antibody production rather than to passive immunity since it is unlikely that BVD neutralizing antibodies could pass the placental barrier while IBR neutralizing antibodies could not. In addition, allowing ten days from infection to detection of SN antibodies it would seem that the bovine fetus can produce antibodies by at least 95 days of age.

The other approach to the study of establishing the age at which a fetus becomes immunologically competent was by injecting fetuses of various ages with various antigens. Fetuses of seven pregnant cattle have been inoculated with antigens and 14 to 30 days later the animals have been slaughtered and fetal serum obtained. Six fetuses were inoculated with BVD virus which has a physical structure and size similar to the murine leukemia viruses. Four of the six inoculated between 55 and 75 days of gestation aborted apparently due to infection with the virus. One fetus inoculated on the 84th day of gestation did live but did not develop detectable SN antibodies against BVD virus. The other fetus which was 180 days old was alive at time of slaughter 30 days after inoculation and surprisingly had a viremia of BVD virus although it appeared grossly normal. This fetus apparently was immunologically tolerant to the inoculated BVD virus. A 70 day old fetus was inoculated with L. pomona bacterin and at the time of slaughter was alive but did not develop detectable antibodies.

It is apparent from this data that there is no clear cut time at which immune competence occurs in the bovine fetus. The earliest age for immune competence indicated here is at 95 days but can be more than 180 days.

During this past year several successful attempts have been made to establish a tissue culture line from buffy coats or solid tumor tissue from leukemia cases. One successful attempt was from buffy coat from a leukemia cow with a count of 200,000 lymphocytes/cmm. These cells have been successfully passed six times. Two ml. of cells and culture media from this sixth passage was inoculated into two 120 day old fetuses. One of these fetuses was promptly aborted. The other fetus lived and developed into an apparently normal bull calf which was born this past month. This calf will be followed clinically along with other future calves from fetal inoculation studies.

Future fetal inoculations will be conducted on fetuses which are less than 95 days of age. The inoculum will consist of material used above, i.e. lymphoblast tissue culture material or some presently available tumorous lymph node tissue culture material. Presently electron microscope work is being undertaken to determine if viral particles are found in large numbers in pancreatic secretions obtained from pancreatic duct cannulation and collection from leukemia cases. There are reports that this is true in avian leukemias and if it proves fruitful in the bovine cases then viral pellets from this source will also be inoculated into bovine fetuses.

Additional attempts to concentrate infective material, virus from plasma and pancreatic effluent have been made or are currently in progress. Pellets of plasma and pancreatic effluent are

examined by electron microscopy for the presence of characteristic virus particles observed in other murine and feline leukemias.

2. Immunoresponsiveness of Leukemic Animals

During the past year pilot studies have been initiated to investigate some immunological aspects of bovine leukosis. These studies have been undertaken in collaboration with scientists at the Glenwood Hills Research Laboratories in Minneapolis, Minnesota. A general approach is being taken since only limited data is available on the immunological factors of this disease in cattle. The experimental design is intended to allow study on classical immunology as well as on autoimmunity and immunotherapy.

Experimental Design

Tissues, including whole blood, tumor, white blood cells and serum, from tumor cases of leukemia were obtained from living animals. Leukemic cells from these tumor cases were injected as untreated or iodoacetate treated cells into normal and lymphocytosis animals. Iodoacetate has been reported to conjugate or alter tumor cells in such a manner as to elicit antigenicity.

The leukemic animal from which the cells were obtained were studied in respect to autoimmunity, presence of viral antigens, viral induced tumor antigens, chemical induced tumor antigens and active immunization.

The normal animals receiving untreated leukemic cells were evaluated for evidence of transmission, presence of viral antigens, viral induced tumor antigens and active immunization.

The normal animals injected with iodoacetate treated leukemic cells were evaluated for evidence of tumor antigenicity, presence

of viral antigens, viral induced tumor antigens, chemical induced tumor antigens and active immunization.

The evaluation procedures included clinical evaluation, hematologic studies, spontaneous agglutination tests for cold and warm antibody, slide agglutination tests, fluorescent antibody techniques, complement fixation, cytotoxicity and immune diffusion. The tests are being applied to both heterologous and homologous antisera.

Table F-1 indicates the preliminary experiments undertaken. No conclusions are possible at this state of investigation.

Table F-1

Status to Date of the Animals Involved In the
Immunological Experiments

<u>Animal No.</u>	<u>Designation</u>	<u>No. Doses of Leukemic Cells Received 70×10^9/cells/dose</u>	<u>Status</u>
L 3133	leukemic leukemia	2 - iodoacetate	died
L 3238	leukemic leukemia	1 - iodoacetate	died
L 3245	aleukemic leukemia	2 - iodoacetate	under study
4	normal	3 - iodoacetate	under study
14	normal	3 - untreated	under study
5	lymphocytosis	2 - iodoacetate	euthanitized
60	normal	2 - iodoacetate	under study

Immunotherapy

Normal animals repeatedly injected with both iodoacetate treated and untreated tumor cells are now available for massive transfusion into leukemic animals. It is thought advisable to use massive transfusions following sublethal radiation in selected animals. The transfused material will be whole blood, serum and possibly concentrated thoracic duct lymphocytes. The response of the recipient leukemic animals will be monitored by histologic, hematologic and chemical studies.

Protocol for Mitochondrial Measurements in Lymphocytes
and Monocytes of Bovine Blood

Exposures are made of centrally cross-sectioned monocytes and lymphocytes on Kodak Contrast Projector Slide Plates at T4 (x4000), T5 (x5600), or T6 (x8000). The developed plates are then projected in a Leitz Wetzlar slide projector so that the final magnification of all cells studied is x150,000. Tracings are made of the mitochondrial profile images found in each cell section. The area of these enlarged profiles is determined using a Keuffner and Esser Compensating Polar Planimeter. These results are transposed from square centimeters to square microns and the actual unmagnified mitochondrial profile area is calculated using the formula

$$A_{u^2} = \frac{A_{cm^2} \times 10^8}{(1.5 \times 10^5)^2}$$

Attention is also paid to the number of these mitochondrial profiles per cell section.

Protocol for Buffy Coat Pellet Preparation and
Processing for EM Studies of
Bovine Peripheral Blood

Anticoagulated blood is centrifuged 2500 r.p.m., at 20° C. for 20 minutes in an International Refrigerated Centrifuge. The serum is carefully removed from the centrifuge tube or culture tube using a Pasteur pipette. Fixative (modified Karnovsky's) is added slowly, down the side of the tube, with a pipette so as not to disturb the buffy coat. After two or more hours of fixation the buffy coat is removed using a thin spatula. The size of the pellet is determined by the amount of blood used or total leukocyte count of the particular blood sample. The pellet is washed in buffer (0.1 M phosphate) and sliced with a razor blade so as to maintain a three-layer orientation. These pieces are washed in buffer, postfixed in Osmium and processed through ethyl alcohol, propylene oxide into Maraglas. Thin sections are stained with Lead Citrate or Uranyl Acetate and Lead Citrate.