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OCCURRENCE AND SIGNIFICANCE OF LYMPHOCYTOSIS IN CATTLE

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Supported by the United States Atomic Energy Commission on research performed under contract AT (11-1)-910. Presented at the National Cancer Institute, National Institutes of Health Conference on the Significance of Lymphocytosis in Cattle, September 1967. This paper is not intended for publication as such. It represents a brief synopsis of some of the results of the leukemia research program at the College of Veterinary Medicine, University of Minnesota. Most of the data is relevant to the occurrence and significance of lymphocytosis in cattle; however, some additional epidemiologic data is included in the introduction as background information. The members of the leukemia research team are D. K. Sorensen, V. Perman, R. K. Anderson, R. E. Shope, Jr., A. F. Weber, V. Larson, J. B. Stevens, D. Goltz, S. K. Dutta, V. Dirks, E. Groth, J. H. Sautter, B. J. Payne and Margaret Snyder.

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INTRODUCTION AND 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 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.

In 1960 we initiated a study on the occurrence and distribution of bovine leukemia in Minnesota and the adjacent part of Wisconsin. These studies included investigation of familial and environmental factors with supporting clinical, hematologic and pathologic studies. This work was supported by the Atomic Energy Commission.

The cooperation of practicing veterinarians in the study area was enlisted and standardized reporting and laboratory procedures were developed. Field studies were conducted on all reported cases, at which time epidemiologic data was collected. In addition to the 2,973 specimens and reports

received from practicing veterinarians, 275 specimens were received from slaughtering plants. Of these 3,248 submissions, 1,434 cases had sufficient clinical and laboratory data to be diagnosed bovine leukemia; however, reliable epidemiologic information could be obtained for only 1,231 of the confirmed cases.

Definitive population statistics acquired through the cooperative efforts of the State Federal Livestock and Crops Reporting Service, The Minnesota Livestock Sanitary Board and the Animal Health Division, USDA were used for determination of specific incidence rates and other epidemiologic evaluations.

A summary of the most significant findings of these and related studies relevant to lymphocytosis and bovine leukemia follows.

1. Geographic Distribution of Cases of Leukemia by Counties and Livestock Reporting Districts in Minnesota

Average annual incidence rates for confirmed cases of leukemia for the period December 1, 1960 through November 30, 1965 are presented for each county in Minnesota in Figure G-1 and for the livestock districts of the state of Minnesota in Figure G-2. In presenting these data the average annual incidence rates are based on the cases occurring in female dairy cattle two years of age and older since reliable population statistics are available for female dairy cattle in this age group, and this group also includes the majority of the confirmed reported cases. The average annual incidence rates for individual counties in Minnesota, varying from 1.3 to 33.4 cases per hundred thousand animals, are presented in Figure G-1.

Incidence rates appear to be less in the predominantly beef cattle areas in Southwestern Minnesota and also are low in the northern areas of

AVERAGE ANNUAL INCIDENCE RATE BY COUNTY OF REPORTED AND CONFIRMED CASES OF LEUKEMIA IN DAIRY CATTLE IN MINNESOTA

DEC. 1, '60 - NOV. 30, '65

FIGURE G-1

*RATE PER 100,000 FEMALE DAIRY CATTLE 2 YEARS AND OLDER

AVERAGE ANNUAL INCIDENCE RATE BY DISTRICT FOR
COUNTIES REPORTING CASES OF BOVINE LEUKEMIA IN
FEMALE DAIRY CATTLE IN MINNESOTA

DEC. 1, '60 - NOV. 30, '65

RATE PER 100,000 FEMALE DAIRY CATTLE 2 YEARS OF AGE AND OLDER

the state as a reflection of fewer cattle and fewer veterinarians in those counties with a predominance of lakes and forest land. The incidence rates were comparably greater in the areas with a predominance of dairy type cattle as shown in the southeast, central and west-central counties of the state. From these data it appears that the geographic distribution of cases in Minnesota is similar to the distribution of herds of cattle with predominantly dairy type animals and to some extent reflects the distribution and availability of veterinary medical services in the state.

2. Age Distribution of Reported Cases of Bovine Leukemia

The age distribution of reported cases of bovine leukemia in Minnesota is summarized in Tables G-1 and G-2 and Figure G-3. It should be noted that the incidence increases with age when one takes into consideration the number of animals at risk in each age group and uses age specific incidence rates. The average annual rate per hundred thousand animals during the last five years shows a 300 per cent difference between the youngest and oldest age groups of animals.

Table G-1

Average Annual Incidence Rate by Age of Reported Cases of Leukemia Among Female Dairy Cattle 2 Years of Age and Older in Minnesota

<u>Dec. 1, '60 - Nov. 30 '65</u>			
<u>Age Group</u>	<u>Average No. of Animals at Risk</u>	<u>Average Annual Number of Cases</u>	<u>Incidence Rate Per 100,000</u>
2-5 years	917,966	72.0	7.8
6-9 years	453,299	77.4	17.5
10+ years	49,735	11.8	23.7

Table G-2

Incidence Rate by Age of Reported Cases in Leukemia Among Female Dairy
Cattle 2 Years of Age and Older in Minnesota
From 1961 through 1965

<u>Age Group</u>	<u>1961</u>	<u>1962</u>	<u>1963</u>	<u>1964</u>	<u>1965</u>	<u>Average Annual Incidence Rate Per 100,000</u>
2 - 5 years	7.8	11.7	5.9	6.1	7.5	7.8
6 - 9 years	17.5	22.7	16.9	10.6	13.0	17.5
10 + years	26.0	37.8	24.9	12.1	14.1	23.7

3. Distribution of Cases Among Breeds of Cattle

Genetic factors appear to influence the occurrence of leukemia or apparent susceptibility to leukemia in other animals such as poultry and mice. It has been suggested that breed characteristics as a reflection of hereditary factors may play a role in influencing the occurrence of leukemia among the cattle population. Table G-3 presents data on cases by breed of animal among 109 cases of leukemia which have been reported in purebred registered animals during this study.

Table G-3

Cases Among Purebreds

Distribution of Leukemia Among Female Dairy Cattle in Minnesota Two Years
Old and Older

<u>Breed</u>	<u>No. of Cases</u>	<u>Percentage of Total Cases</u>
Holstein	89	81.7
Guernsey	5	4.6
Milking Short Horn	3	2.8
Brown Swiss	0	0
Jersey	6	5.5
Other	6	5.5

Figure G-3
Incidence Rate* By Age of Reported and Confirmed Cases of
Leukemia Among Female Dairy Cattle in Minnesota

*Rate per 100,000 female dairy cattle 2 years and over in Minnesota. Dairy animals less than 2 years of age are not included because population data are not satisfactory.

Table G-4

Distribution by Predominant Breed Characteristic of Expected Cases and
 Reputed Cases of Leukemia Among Female Dairy Cattle in Minnesota
 Dec. 1, '60 - Nov. 30 '65

<u>Predominant Breed Characteristic</u>	<u>Estimated No. of Cows in Population</u>	<u>Average Annual No. of Cases</u>	<u>Observed Percentage of Total Cases</u>	<u>Expected Percentage of Total cases</u>
Holstein	1,145,340	136.8	90.9	81.0
Guernsey	132,916	6.6	4.4	9.4
Milking Short Horn	41,006	3.4	2.3	2.9
Jersey	28,280	3.6	2.4	2.0
Brown Swiss	36,764	0	0	2.6

Table G-4 presents cumulative data for female dairy cattle two years and older for the five year period of the study. The Holstein breed has a slight excess of observed cases above the percentage expected on the basis of the number of Holsteins in the total population. This difference has remained relatively constant for the five year period of the study. On the other hand, the Guernsey breed of cattle has about one half the observed percentage of cases that would be expected on the basis of total cattle in the population and this also has remained quite constant during the entire study.

Preliminary evaluation and analysis indicates that this difference in reported occurrence of cases may well be related to the size of herds of Holsteins and Guernseys in the population. From our preliminary analysis it would appear that the number of animals in the Guernsey herds is relatively smaller than the number of animals in the Holstein herds. Since it has been shown that the incidence rate increases with an increasing herd size

(number of animals in the herd) one might well expect to find less cases among Guernsey cattle than among Holstein cattle as has been observed in this study.

4. Environmental and Familial Factors

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

One of the provocative findings of these studies has been the rather dramatic increase in the number of cases of leukemia in the larger herds of cattle where the rate per hundred thousand animals at risk, is 6.5 times greater than in the small herds with less than ten cattle. It should be noted that 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.

The increased incidence rate becomes even more significant when one considers the fact that the large herds have a smaller proportion of cattle in the older age groups as compared with the small herds and this is contrary to what one would expect since the incidence rate of leukemia is much greater in older animals than in younger animals from two to five years of age. Therefore it appears that the age of animals in large herds is not the factor responsible for the increased incidence in the larger herds. The increased incidence rates in large herds even though they have a smaller proportion of aged animals may be suggestive of intraherd transmission of an infectious agent.

Accumulative data for the period December 1, 1960 through November 30, 1965 are presented in Table H-1 to show the percentage of expected cases

actually observed in each herd size group. These data indicate that cases are reported in herds with less than 20 cattle only 49 per cent as frequently as should be expected while the frequency of occurrence of cases in herds with more than 50 cattle is 320 per cent of the expected frequency.

Table H-2 presents 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 December 1, 1960 through November 30, 1965. These data indicate that the average annual incidence rate increases from 6.9 per hundred thousand cattle in herds of less than ten cows to an incidence rate of 44.8 per hundred thousand cattle in herds with fifty or more cows. This is more than a six fold increase in frequency of occurrence.

It should be noted that the factors influencing this increase have not been delineated other than the factor of age, where in fact one would expect an increased incidence in the small herds with the increased number of aged cows.

Table H-1

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 H-2

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

b. Movement and Transfer of Animals From One Herd to Another

Cumulative data collected to ascertain whether the leukemia case was born and raised for its entire lifetime in the herd where a diagnosis of leukemia was made, indicates that 71 per cent of all reported leukemia cases were born, raised and spent their entire life on the farm of origin. Data have also been collected on the number of other animals introduced into the herd as well as the time these animals were introduced relative to the time the leukemia case was reported.

On the basis of preliminary analysis it was found that in the past five years only 20 per cent of the leukemia herds had not introduced animals as compared to 35 per cent of the herds with no introduction in the general population. In the past ten years only 12 per cent of the leukemia herds had not introduced animals as compared to 26 per cent of the general population. It was also noted that a high proportion, 71 per cent of the small herds, had not introduced animals by movement or transfer in the last five years.

c. Familial Factors

Cumulative data in this study indicate that among 42 cases of bovine leukemia there was a familial relationship with other leukemic animals. Since adequate data was not obtainable from the case reports, familial factors will be discussed with the data on multiple case herds where adequate records are available.

d. Chemical Insecticides and Feed Additives

The role of chemicals in carcinogenesis is being investigated in view of the popular reaction to articles which seem to over-dramatize the

possible scope and adverse influence of agricultural chemicals on domestic animals and man.

Tabulation of cumulative data presented in Table H-5 shows the results of questions pertaining to the use of hormones, antibiotics and anthelminthics. According to the data, penicillin was the most commonly used medication followed by streptomycin, tetracycline, sulfa drugs and cortisone. No evidence was obtained that any of the cases had received oral pesticides. The regimen of treatment noted here is probably consistent with what one would find in the treatment of any sick animal. Most animals were apparently in good health prior to the onset of leukemia, since we found that only 35 per cent of the animals had been treated for a previous disease condition.

Table H-5

Summary of Data Pertaining to History of Administration of
Drugs and Chemicals During Lifetime of the Leukemia Case

<u>Chemical Agent</u>	<u>Percentage of Cases with History of Use July 1, '60 - Nov. 30, '65</u>
Penicillin	25.8
Streptomycin	21.3
Tetracyclines	9.1
Chloromycetin	.5
Polymyxin	.7
Nitrofurans	1.0
Phenothiazine	.9
Tetrachlorethylene	.0
Cortisone	7.5
Stilbestrol	.7
Oxytocin	.5
Siliconized bloat medicine	.3
Sulfa drugs	6.4
Others (electrolytes, etc.)	10.3

Wood preservatives were used by 29 per cent of the farmers but only 20 percent of the cases had opportunity for direct contact. Approximately 33 per cent of the farmers used lead base paint but only 17 per cent of the cases had opportunity for contact with the paint.

Cumulative data concerning the use of insecticides on these farms indicate that 92.5 per cent of the farms use insecticides to some degree with only 7.5 per cent denying the use of insecticides. The majority,

63 per cent, used insecticides once or twice a day, 28.5 per cent used insecticides less frequently and only a small percentage used insecticides more than twice a day. Insecticides were the most frequently misused chemicals on the farm, indicating that the farmer either did not read the label or ignored the warnings on the label as indicated by Table H-6. Among the farmers using insecticides more than one fourth used prohibitive compounds such as chlorinated hydrocarbons.

Herbicides are being used with increasing frequency each year. Cumulative data for the last five years are presented in Table H-7 showing the number of farms using herbicides. Seventy-three per cent of the farmers have used herbicides at least once during the last five years and 27 per cent indicated that they had never used herbicides during the five years prior to the occurrence of the leukemia case.

Medicated feeds and possible contamination of feed and water were explored and insignificant results have been reported in previous reports. Generally from these results it appears that no single chemical commonly available to livestock owner and investigated in the course of our studies could be correlated with more than a fraction of the leukemia cases.

We do have data concerning the methods of feeding calves and whether or not the calf was fed the dams milk or milk from other cows. This may provide some evidence concerning the role of milk as a factor if a transmissible agent is involved in leukemia. These data will be further analyzed during the next year to provide an evaluation of this factor.

Table H-6

Summary of Reported Use of Insecticides on Premises and Cattle
in Herds Where Leukemia Cases Occurred

<u>Insecticide Used</u>	Used on Premises	Used on Animals
	July 1960 <u>Nov. 1964</u>	July 1960 <u>Nov. 1964</u>
Organic phosphate	21.3%	10.7%
Chlorinate hydrocarbons	23.0%	17.1%
Pyrethrins	64.9%	60.2%

Table H-7

Number of Times Herbicides were Used on the Farm in the Last
Five Years
July 1, '60 - Nov. 30, '65

<u>No. of Times Used</u>	<u>No. of Farms Using</u>	<u>Percentage of Total</u>
1	256	24.3
2 - 4	279	26.5
5 - 9	212	20.1
10 - 14	18	1.7
15 - 19	8	.8
Never	280	26.6

e. Artificial Insemination and Natural Service

Cumulative data are presented in Table H-8 regarding the relative frequency of artificial insemination and natural service as a method of breeding the dams of the reported cases of leukemia when compared to the method of breeding used for all other dairy cattle in herds where cases have occurred as well as all the dairy cattle in Minnesota. One could conclude that the method of breeding (artificial insemination or natural breeding) is not a factor influencing the occurrence of leukemia.

It is interesting to note that one of our herds with several cases of leukemia (a herd of registered Holsteins) had a bull, used as a herd sire, that was diagnosed as a case of leukemia. Since the records in this herd are quite complete, it may be possible to evaluate the possible role of the male in influencing the occurrence of this disease in a herd under these conditions.

Table H-8

Percentage of Leukemia Cases as Progeny of Artificial
Insemination Compared to Natural Service
July 1, '60 - Nov. 30, '65

<u>Breeding Method</u>	<u>All Minnesota Dairy Cattle</u>	<u>Dairy Cattle in Case Herds</u>	<u>Confirmed Leukemia Cases From These Herds</u>
Artificial	59	46.6	46.4
Natural	41	53.4	53.6

5. Studies on Prevalence of Lymphocytosis Among Cattle in Minnesota

a. Survey Studies

In view of the reports of Bendixen of Denmark, Gotze of Germany and the numerous reports from other investigators associating lymphocytosis with bovine leukemia, we initiated a survey study of our cattle population in Minnesota to ascertain the prevalence of lymphocytosis herds and to identify herds in the general population in which bovine leukemia could be expected to occur with a high degree of probability as opposed to those with a low probability for occurrence.

With the cooperation of the State Federal Livestock and Crop Reporting Service and the Animal Disease Eradication Division of the USDA, we selected four counties for study.

Lake County, a remote low cattle population county, was selected to provide herds of low contact and low animal introduction features. All dairy herds (29) in this county were evaluated.

Hubbard and Aitkin counties are fringe counties to the principle dairy area of the state. A random sample, stratified as to herd size, was selected. Initial hematologic parameters consisted of total leukocyte counts and differential cell counts on adult animals (4 years or older) with leukocyte counts greater than 10,000. Since approximately eight hours intervened between collection of blood specimens and preparation of blood smears lymphocytic and monocytic were not differentiated, but classified as lymphoid cells.

Steele County, a major dairy cattle county, was studied. A random sample, stratified as to herd size, was selected. Blood specimens were

obtained from all dairy cattle two years of age or over present in the milking line. Blood smears were prepared from EDTA blood approximately eight hours after the samples were collected. Differential cell counts and quantitative data were obtained on all animals. Since lymphocytic and monocytic could not be differentiated with certainty in all cases, these cells were classified as lymphoid cells.

In addition to hematologic data, vital data and environmental data was obtained for each animal and for the farm. A summary of these studies is present in Table L-1.

The data on lymphocytosis was evaluated using Bendixen's criteria for normals. Subsequent evaluation of selected, apparently leukemia-free herds from the same population, reveal normal lymphocytic values very similar to that reported by Bendixen allowing for meaningful interpretation by methods followed.

Subsequent to those studies (two and one half years later) 60 Holstein herds were selected from Hubbard, Aitkin and Steele counties for further evaluation. During the intervening period a number of selected herds had been dispersed and others eliminated for various reasons, four herds because of occurrence of bovine leukemia.

Of the 60 herds re-evaluated, 37 herds were selected for further analysis to provide base-line hematologic values, particularly in reference to lymphocytosis. The herds selected are composed of grade and purebred Holstein cattle. These herds were considered free of leukemia on the basis of history and had been in the study program over two years. Only female animals two years of age and over were sampled. Complete hematologic data including differential cell counts from blood smears

Table L-1

Summary of Lymphocytosis Studies
on Cattle Populations

Population Group	Number of Herds	Number of Animals	Number of Observations	Number of Herds with Lymphocytosis	Per Cent of Herds with Lymphocytosis	Number of Animals with Lymphocytosis	Per Cent of Animals with Lymphocytosis
Hubbard County	49	704	704	23	47	32	4.5
Aitkin County	70	1189	1189	23	33	56	4.7
Steele County	113	3106	3106	43	38	77	2.4
Lake County	29	148	148	2	7	2	1.2

made from freely flowing blood, was obtained. The population data is summarized in Table L-2. The leukocyte data obtained is summarized by age group for all animals and depicted in Table L-3. A comparison of the data on selected Holstein herds was made with the data obtained on the random sample of Steele County dairy cattle made in 1963. The tabulated data for the leukocytes are given in tables L-4, L-5, L-6, L-7 and L-8.

Table L-2

Summary of Events Pertaining to Steele, Hubbard and Aitkin County Holstein Herds reevaluated During 1965. Herds Selected on Basis of Normal Hematologic Values, Holstein Breed and No History of Leukemia

Size of Herd	<u>Steele County</u>		<u>Hubbard County</u>		<u>Aitkin County</u>	
	# of Herds	# of Animals	# of Herds	# of Animals	# of Herds	# of Animals
1 - 9	2	17	0	0	0	0
10 - 19	11	167	4	67	1	17
20 - 29	6	147	1	28	3	79
30 - 49	4	122	0	0	3	125
50 +	1	58	1	50	0	0
Total	24	511	6	145	7	221

Table L-3

Hematologic Data on Holstein Cattle From Selected Herds in
Steele, Hubbard, and Aitkin Counties, Minnesota, Fall 1965

Leukocytes

Age	No. of Animals	White Blood Cells Per Cumm		Lymphocytes Per Cumm		Monocytes Per Cumm		Lymphoid Cells Per Cumm		Neutrophils Per Cumm		Eosinophils Per Cumm		Basophils Per Cumm	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
2	36	10,330	2,120	4,990	1,480	280	190	5,200	1,470	3,820	1,160	1,240	780	10	30
3	136	9,280	1,850	4,650	960	330	240	4,970	990	3,040	1,050	1,260	700	20	40
4	129	9,240	1,920	4,470	1,020	300	210	4,760	1,070	3,250	1,280	1,300	680	10	40
5	122	8,530	1,730	4,140	1,000	250	180	4,380	1,010	2,960	1,200	1,190	670	10	30
6	141	8,230	1,644	3,940	960	270	210	4,200	1,000	2,730	890	1,270	670	10	40
7	115	7,970	1,480	3,670	820	240	160	3,900	830	2,750	970	1,320	670	10	30
8	68	7,780	1,650	3,600	900	260	200	3,880	920	2,530	950	1,370	730	10	40
9	36	7,360	1,680	3,380	1,180	210	130	3,590	1,150	2,540	1,120	1,230	490	20	50
10 yrs. & over	56	7,480	1,660	3,290	890	230	160	3,480	870	2,580	1,320	1,370	580	10	30

Table L-4

Comparison of White Blood Cell Values on the Selected Holstein Herds from Aitkin, Hubbard and Steele Counties with the Randomly Selected Herds Representing the Dairy Cattle Population of Steele County

Selected Herds				Steele County Herds				
Age	No. of Animals	Mean	SD	Age	No. of Animals	Mean	Median	SD
2	36	10,330	2,120	2	322	9,560	9,200	2,440
3	136	9,280	1,850	3	384	9,210	8,800	2,380
4	129	9,240	1,920	4	361	9,040	8,700	2,360
5	122	8,530	1,730	5	333	8,660	8,200	2,300
6	141	8,230	1,644	6	257	8,430	8,000	2,330
7	115	7,970	1,480	7	169	8,220	7,700	2,340
8	68	7,780	1,650	8	119	7,950	7,500	2,180
9	36	7,360	1,680	9	55	8,010	7,300	2,330
10 yrs. & Over	56	7,480	1,660	10 yrs. & Over	30	8,010	7,950	2,190

Table L-5

Comparison of Lymphoid Cell Values on the Selected Holstein Herds From
Aitkin, Hubbard and Steele Counties with the Randomly Selected
Herds Representing the Dairy Cattle Population of Steele County

Selected Herds				Steele County Herds				
Age	No. of Animals	Mean	SD	Age	No. of Animals	Mean	Median	SD
2	36	5,200	1,470	2	322	5,780	5,400	1,860
3	136	4,970	990	3	384	5,230	4,990	1,670
4	129	4,760	1,070	4	361	4,930	4,660	1,640
5	122	4,380	1,010	5	333	4,680	4,280	1,710
6	141	4,200	1,000	6	257	4,430	4,140	1,580
7	115	3,900	830	7	169	4,270	3,840	1,600
8	68	3,880	920	8	119	4,040	3,650	1,480
9	36	3,590	1,150	9	55	3,900	3,710	1,350
10 yrs. & Over	56	3,480	870	10 yrs. & Over	30	3,890	3,460	1,250

Table L-6

Comparison of Total Neutrophil Values on the Selected Holstein Herds From
Aitkin, Hubbard and Steele Counties with the Randomly Selected Herds
Representing the Dairy Cattle Population of Steele County

Selected Herds				Steele County Herds				
Age	No. of Animals	Mean	SD	Age	No. of Animals	Mean	Median	SD
2	36	3,820	1,160	2	322	2,850	2,580	1,410
3	136	3,040	1,050	3	384	2,950	2,760	1,280
4	129	3,250	1,280	4	361	3,020	2,800	1,450
5	122	2,960	1,200	5	333	2,930	2,760	1,310
6	141	2,730	890	6	257	2,960	2,730	1,290
7	115	2,750	970	7	169	2,910	2,750	1,470
8	68	2,530	950	8	119	2,870	2,700	1,220
9	36	2,540	1,120	9	55	3,090	2,750	1,770
10 yrs. & Over	56	2,580	1,320	10 yrs. & Over	30	3,070	2,680	1,590

Table L-7

Comparison of Total Eosinophil Values on the Selected Holstein Herds From
Aitkin, Hubbard and Steele Counties with the Randomly Selected Herds
Representing the Dairy Cattle Population of Steele County

Selected Herds				Steele County Herds				
Age	No. of Animals	Mean	SD	Age	No. of Animals	Mean	Median	SD
2	36	1,240	780	2	322	930	800	720
3	136	1,260	700	3	384	1,010	820	760
4	129	1,300	680	4	361	1,060	910	730
5	122	1,190	670	5	333	1,010	910	620
6	141	1,270	670	6	257	1,040	920	810
7	115	1,320	670	7	169	1,030	860	750
8	68	1,370	730	8	119	1,010	900	650
9	36	1,230	490	9	55	990	840	740
10 yrs. & Over	56	1,370	580	10 yrs. & Over	30	1,020	880	720

Table L-8

Comparison of Basophil Values on the Selected Holstein Herds From
Aitkin, Hubbard and Steele Counties with the Randomly
Selected Herds Representing the Dairy Cattle
Population of Steele County

Age	No. of Animals	Mean	SD	Age	No. of Animals	Mean	Median	SD
2	36	10	30	2	322	20	0	50
3	136	20	40	3	384	30	0	60
4	129	10	40	4	361	30	0	60
5	122	10	30	5	333	30	0	50
6	141	10	40	6	257	30	0	60
7	115	10	30	7	169	30	0	50
8	68	10	40	8	119	20	0	50
9	36	20	50	9	55	30	0	40
10 yrs. & Over	56	10	30	10 yrs. & Over	30	20	0	50

It is readily apparent that the mean leukocyte values on the selected herds with the low standard deviations compare favorably to mean and median blood values on Steele County animals in the 1963 study. The white blood cell count, means of lymphoid cells and the means of neutrophils are essentially the same. The mean eosinophil levels in 1965 which are slightly higher than the mean values in 1963 may be related to the time of the year (fall versus spring) at which the animals were bled. Of particular significance is the fact that monocytes constitute such a small proportion of lymphoid cells (6 per cent) and are so constant with age, that for purposes of evaluation, lymphoid cells are essentially lymphocytes. This observation lends greater significance to the hematologic data obtained in the random sample on Steele County animals. Thus, this provides a basis for determining baseline parameters for leukocytes which are representative of the dairy cattle population on which our data has been obtained.

The data on the selected herds (Table L-1) thus provides the basis for a MINNESOTA KEY to evaluate lymphocytosis. The mean lymphocyte values and lymphoid values are illustrated in Figures J-1 and J-2 with two and three standard deviations indicated to illustrate the range of values. Figure J-3 compares the mean lymphocyte value and the three standard deviation level with the Bendixen Key to which we had previously referred our data. It is apparent that the three standard deviation level is below the level of significant lymphocytosis on the basis of the Bendixen Key. THUS, THE DATA BASED ON LYMPHOCYTOSIS AS EVALUATED BY THE BENDIXEN KEY IS GIVEN SIGNIFICANCE IN TERMS OF NORMAL VALUES SINCE THE CATTLE POPULATION IN WHICH OUR DATA HAS BEEN OBTAINED IS NOW

Mean and Two Standard Deviation Levels for Lymphoid Cells on Normal Minnesota Cattle in Selected Herds from Steele, Hubbard and Aitkin Counties

LYMPHOID CELLS VERSUS AGE OF ANIMAL
 LYMPHOID CELLS ARE DECODED BY MULTIPLYING BY 100
 AGE OF ANIMAL IS DECODED BY DIVIDING BY 2

NUMBER OF PIECES OF DATA
 ENTERED
 REJECTED
 OUT OF ORDER

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SYMBOL TABLE

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Figure J-1

Mean and Three Standard Deviation Levels for Lymphoid Cells on Normal Minnesota Cattle in Selected Herds from Steele, Hubbard and Aitkin Counties

LYMPHOID CELLS VERSUS AGE OF ANIMAL
 LYMPHOID CELLS ARE DECODED BY MULTIPLYING BY 100
 AGE OF ANIMAL IS DECODED BY DIVIDING BY 2

NUMBER OF PIECES OF DATA
 ENTERED
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 NUMBERS INDICATED

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SYMBOL TABLE

Figure J-2

Comparison of Mean and Three Standard Deviation Lymphoid Cell Levels
on Minnesota Cattle to the Normal Lymphocyte Values for
Danish Cattle as Depicted in the Bendixen Key

Figure J-3

DEFINED. THE MINNESOTA KEY OBTAINED ON MINNESOTA CATTLE AND USED FOR LYMPHOGRAM ANALYSIS HAS LIMITS OF SIGNIFICANCE WITH THOSE INDICATED BY THE BENDIXEN KEY.

Confidence intervals for mean lymphoid cell counts on selected normal herds and for the random sampled herds on Steele County (1963) are given by age of animal in Tables L-9 and L-10.

b. Prevalance of Lymphocytosis (County Survey Studies)

With the establishment of a MINNESOTA KEY for lymphogram analysis, a preliminary reevaluation was made of the county survey studies on Steele, Hubbard and Aitkin counties. The results are summarized in Table L-11. Although the results are similar to the initial evaluation on the basis of the Bendixen Key, greater significance is given to the calculated rate of bovine leukemia based on lymphocytosis.

Table L-9

Mean Lymphoid Cell Counts with Confidence Intervals of the
Mean for Selected Normal Herds

N	Age	Mean	<u>Confidence Intervals</u>	
			5%	1%
36	2	5300	4700 to 5800	4500 to 6000
136	3	5000	4800 to 5100	4700 to 5200
129	4	4800	4600 to 4900	4500 to 5000
122	5	4400	4200 to 4600	4100 to 4600
141	6	4200	4000 to 4400	4000 to 4400
115	7	3900	3700 to 4000	3700 to 4100
68	8	3900	3700 to 4100	3600 to 4200
36	9	3600	3200 to 4000	3100 to 4100
56	10	3500	3200 to 3700	3200 to 3800

Table L-10

Mean Lymphoid Cell Counts with Confidence Intervals of the
Mean for Steele County Animal Examined in 1963

N	Age	Mean	<u>Confidence Intervals</u>	
			5%	1%
322	2	5800	5600 to 6000	5500 to 6000
384	3	5200	5100 to 5400	5000 to 5400
361	4	4900	4800 to 5100	4700 to 5200
333	5	4700	4500 to 4900	4400 to 4900
257	6	4400	4200 to 4600	4200 to 4700
169	7	4300	4000 to 4500	4000 to 4600
119	8	4000	4800 to 4300	3700 to 4400
55	9	3900	3500 to 4300	3400 to 4400
30	10	3900	3400 to 4300	3300 to 4500

Table L-11

Dairy Herds in County Indicated Characterized by Animals with Lymphocytosis
on the basis of the MINNESOTA KEY

<u>Source of Herds</u>	<u>No. of Herds</u>	<u>Herds Characterized by Lymphocytosis</u>	<u>Normal</u>	<u>Per cent of herds with Lymphocytosis</u>
Lake County	29	3	26	10
Steele County	113	50	63	44
Aitkin County	70	27	43	39
Hubbard County	48	25	23	52
Single Case Herd	20	15	5	75
Multiple Case Herds	19	19	0	100

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

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 essentially only juvenile leukemia and thymic-lymphosarcoma of young animals are reported, indicate that lymphocytosis in normal animals was not observed in over 700 herds examined.

We have studied 42 single case herds of the adult type and 10 single case herds of the juvenile type. The herds selected had reported a case of leukemia during the early study period and a history of no additional cases during the past two years. Only Holstein herds were selected and female cattle two years of age and over were evaluated. Preliminary evaluation of the data on 20 single case herds of the adult leukemia type is summarized in Table L-11 relative to other population data.

Seventy-five per cent of the single case herds are characterized by animals with lymphocytosis. When the frequency of lymphocytosis within multiple case herds (12.5% of the adult animals) and herd size of single case herds (average 27 animals) is considered, the incidence of lymphocytosis in single case herds compare favorably with multiple case herds. Of significance is the fact that the single case herd mean lymphoid cell values for all groups lie well outside the 1 per cent confidence interval for the mean lymphoid cell count of the selected normal herds. Furthermore, the mean lymphoid cell count for the single case herds for all age groups of

animals lies well outside the 1 per cent confidence interval for the mean lymphoid cell count of the random selected Steele County sample.

Preliminary analysis of the 22 additional single case herds of the adult leukemia type reveal similar data.

Preliminary analysis of the 10 single case herds of the juvenile type reveal 3 of these herds to have animals with lymphocytosis. This is not surprising since in the county population studies from approximately 30 to 50% of the herds have animals with lymphocytosis.

7. Observations on Multiple Case Herds

During the early study period, 66 of the single case herds have become multiple case herds on the basis of retrospective and prospective study. Nearly half of the multiple case herds were subjected to clinical and environmental study. For various reasons such as dispersal, inadequate records, or lack of cooperation, data from ten multiple case herds will form the basis for certain critical evaluations.

As indicated in Table L-11, multiple case herds are characterized by normal animals with lymphocytosis Figure J-4 and J-5. Figure J-6 illustrates a typical single case herd lymphogram and Figure J-7 illustrates a normal herd lymphogram.

During the period of study, 20 cases of leukemia have developed subsequent to the index cases and initiation of the hematologic study. A detailed discussion of the pretumor or preclinical stage of bovine leukemia is given in the following section.

Lymphogram on Multiple Case Herd Four with Minnesota Key Illustrated
by the Mean and Three Standard Deviation Lymphoid Cell
Levels Based on Accumulative Results on Multiple Observations

NUMBER OF PIECES OF DATA
ENTERED 804
REJECTED 6
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SYMBOL
NUMBERS INDICATED

1 2 3 4 5 6 7 8 9 10 OR MORE
1- 1 2- 2 3- 3 4- 4 5- 5 6- 6 7- 7 8- 8 9- 9

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Figure J-4

Lymphogram on Multiple Case Herd Twenty with Minnesota Key Illustrated
by the Mean and Three Standard Deviation Lymphoid Cell
Levels Based on Accumulative Results on Multiple Observations

Figure J-5

Lymphogram on a Typical Single Case Herd with Minnesota Key Illustrated
by Mean and Three Standard Deviation Lymphoid Cell Levels
Based on a Single Observation

Figure J-6

NUMBER OF PIECES OF DATA
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a. The Preclinical (Pretumor) Stage of Bovine Leukemia

The preclinical stage of bovine leukemia is generally characterized only by a persistent lymphocytosis in apparently healthy cattle. This lymphocytosis, when observed, may be present for variable periods of time but is generally present from one to several years prior to the appearance of tumors.

Leukemia cases to be discussed here had preclinical hematological data available and developed in herds under investigation. Eighteen herds of cattle with a history of two or more confirmed index cases of leukemia have been under continuous study from one to seven years. These studies included semiannual clinical, epidemiological and hematological investigations. A total of 20 "new cases" of leukemia developed in ten of these study herds.

All twenty "new cases" of leukemia had at least one preclinical blood examination and the majority had several examinations during periods up to four years. Blood examinations were usually made semiannually on all animals which are two years of age or older in the study herds. Nineteen of the twenty "new cases" of leukemia exhibited significant lymphocytosis (significant by Bendixen's Key and Minnesota Key) prior to the appearance of clinical leukemia. The one animal (L-984) which did not exhibit lymphocytosis had only one blood examination. This examination was made when the cow was $2\frac{1}{2}$ years of age, which was 6 months prior to the appearance of tumors.

Absolute lymphocyte values of all semiannual preclinical observations for the 20 cases of leukemia are plotted against age of each observation in Figure J-8. "Bendixen's Key" is superimposed on this graph. It is noted that most of the lymphocyte counts on these 20 cases are significantly elevated. Ten observations do fall in the lower ranges of the "key." Seven of these low counts are on animals in the younger groups, i.e., two to four years. With the exception of the one observation (4,100 lymphocytes) on L-984 six of these low counts are on animals which developed significant lymphocytosis with advancing age. It has been observed in the study herds that some of the older cows with persistent lymphocytosis did not exhibit high lymphocyte counts at ages less than 5 years. The three lower lymphocyte counts in the 5 and 6 year age groups (Figure J-8) represent the last preclinical observation prior to the appearance of tumors in three cases. Bendixen, in Denmark, and Winquist, in Sweden, have also noted a marked reduction of absolute lymphocyte counts in the late preclinical stage of some of their new cases which developed in herds under study.

Four representative cases with numerous preclinical and clinical blood examinations are illustrated in Figures J-9 to J-12. Absolute values of total leukocytes, lymphocytes and neutrophils are plotted. In general, lymphocytes fluctuate independent of neutrophils and are persistently elevated. Fluctuations of total leukocyte values are primarily the result of changes in the number of lymphocytes. The composite picture of the preclinical and clinical lymphocyte counts on these four cases is presented in Figure J-13. It is noted in this graph that the clinical lymphocyte

PRECLINICAL ABSOLUTE LYMPHOCYTE VALUES
OF 20 CONFIRMED CASES OF LEUKEMIA

Figure J-8

Figure J-9. Leukemia case 135. Leukocyte values for a period of two years prior to the development of tumors and three months of clinical leukemia.

Figure J-10. Leukemia case 764. Leukocyte values for a period of two years prior to the development of tumors and three months of clinical leukemia.

Figure J-11. Leukemia case 2236. Leukocyte values for a period of four years prior to the development of tumors and one month of clinical leukemia.

Figure J-12. Leukemia case 3101. Leukocyte values for a period of three years prior to the development of tumors and one month of clinical leukemia.

Figure J-13. Preclinical and clinical absolute lymphocyte values on four cases of bovine leukemia.

- A. Cow 135 (born July 14, 1953, dead March 6, 1962)
- B. Cow 764 (born April 22, 1952, dead March 1, 1962)
- C. Cow 2236 (born July 29, 1953, dead April 22, 1964)
- D. Cow 3101 (born November 28, 1960, dead June 14, 1966)

values are not necessarily related to the magnitude of preclinical lymphocytosis. This is evident in comparing preclinical and clinical data on cases 3101 and 764. Case 3101 exhibited "borderline" preclinical lymphocytosis but developed very markedly elevated lymphocyte counts during the clinical stage. The opposite of this is noted in the data on 764.

A composite absolute lymphocyte graph of the data from four additional cases for several preclinical years is presented in Figure J-14. These four cases were not studied during the clinical stage of the disease but the diagnosis of leukemia was confirmed by necropsy examination. Case 1878 died at the age of 4 years and exhibited lymphocytosis on the two final preclinical blood examinations.

Some vital data on the herds in which the 20 new cases of leukemia occurred are presented in Table L-12. These 20 cases represent 2% of the total number of animals studied in these herds. Since nineteen of these cases exhibited preclinical lymphocytosis, probably a more appropriate denominator for calculation of the risk of leukemia for these cases would be the total number of animals with persistent lymphocytosis. In which case, the incidence rate for 19 new cases is 9% of those animals with persistent lymphocytosis during the period of study. Since all animals in these herds were under similar observation then the group of animals which did not exhibit lymphocytosis should have had many more than one case of leukemia occurring in it if chance alone were governing the occurrence of leukemia. The 9% incidence of leukemia in the animals with persistent lymphocytosis is very significantly different from the 0.1% incidence for the animals with normal lymphocyte counts.

Figure J-14. Absolute lymphocyte values prior to the development of tumors on four cases of bovine leukemia.

- E. Cow 1-35 (born November 22, 1951, dead July 8, 1963)
- F. Cow 2046 (born September 12, 1955, dead December 20, 1963)
- G. Cow 1878 (born August 23, 1959, dead August 15, 1963)
- H. Cow 2249 (born January 4, 1955, dead April 7, 1964)

Table L-12

Summary of Leukemia Cases and Lymphocytosis Animals
in Ten Selected Multiple Case Herds

<u>Herd No.</u>	<u>Ave. Herd Size</u>	<u>Years Studied</u>	<u>Total Animals Studied</u>	<u>Total Animals With Chronic Lymphocytosis*</u>	<u>Index Cases</u>	<u>New Cases</u>	<u>Total Cases</u>
1	44	7	115	26	3	5	8
3	54	4	107	19	3	2	5
5	45	6	113	8	2	1	3
6	26	3	45	7	2	1	3
15	56	4	137	56	2	2	4
20	80	3	147	25	5	2	7
23	25	2	32	9	2	2	4
25	28	5	74	14	2	2	4
27	67	2	108	23	3	1	4
29	<u>72</u>	2	<u>105</u>	<u>23</u>	<u>2</u>	<u>2</u>	<u>4</u>
Total	500		983	210	26	20	46

*Chronic Lymphocytosis = 2 + Observations for over a year

Numerous reports on age specific incidence rates of bovine leukemia have indicated that the incidence of leukemia increases with age. This is especially true for animals over 5 years of age. Of the 20 cases presented here, 16 (80%) of them were 5 years of age or older. It has been noted in study of high incidence leukemia herds in Minnesota that animals with persistent lymphocytosis are "culled" at an average age of 5 years due to normal production practices. Thus half of these cattle are not kept in the herds until the ages of higher risk of leukemia. The majority of the animals are "culled" because of low milk production or infertility. Under conditions other than those of dairying, these animals would have many years of additional expected longevity during which to develop leukemia.

b. 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.

Studies of animal records in six high incidence leukemia herds revealed that 2 or more animals which died of leukemia had been born within a four-week period. Three of these herds had two leukemia cases, two herds had three cases and one herd had four leukemia cases born within a month of each other. A total of 35 leukemia cases was seen in these six herds and 16 of these cases were born in neonatal cluster groups.

Cohort analysis of data for all adult animals in ten herds revealed a significant clustering by date of birth of animals with persistent lymphocytosis. This clustering involved animals sharing a common neonatal period with one or more animals which later developed leukemia as an adult (Table L-13). Sixty-one (61) adult cows were studied, which were born in 13 neonatal cluster groups (cohort groups). Twenty-three (37%) of these animals developed leukemia and an additional 30 animals (50%) of the animals in these clusters exhibited persistent lymphocytosis during adult life. Each cohort group contained progeny of leukemic 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. Needless to say, these 61 animals represent only a fraction of the entire cohort group since nearly all male animals and some female animals are normally culled before reaching maturity.

Table L-13.

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-4	Sept. 1956	8	1	6	88%
	Sept. 1957	5	1	3	80%
MH-15	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	Nov. 1958	3	3	0	100%
MH-39	Nov. 1961	<u>2</u>	<u>2</u>	<u>0</u>	100%
Total		68	23	30	

*All dates of birth are documented; maximum interval is 28 days and the predominant calendar month is listed for convenience.

It is interesting to speculate on all the possible common exposure experiences of these groups of young calves including direct contact with each other and common sources of food. All ten farmers did pool fresh colostrum and also it was possible for any young calf, fed milk, to receive some of excess colostrum. Cohort clustering of leukemic and lymphocytotic animals may be related to such practices.

Analysis of hematological data on 310 dam-daughter pairs in high incidence leukemia herds revealed familial similarities (Tables L-14 and L-15). Of the dams which developed leukemia or a persistent lymphocytosis, 49% had daughters which exhibited persistent lymphocytosis in contrast to 29% of the daughters of cows with normal lymphocyte counts. Examination of birth dates of the daughters on this 29% group revealed that 88% of them to be members of neonatal clusters with high incidence of lymphocytosis.

In summary there should be little doubt about the significance of the close proximity of birth in the six clusters of leukemia cases observed in this sample. Neonatal exposure experience with calves which do develop leukemia as adults evidently is also in some way associated with 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 leukemic or lymphocytotic dams with these clusters is consistent with the concept of a transmissible agent. In view of these data the hypothesis for both vertical and horizontal transmission of bovine leukemia can be explained. Familial similarities of lymphocyte counts in dam-daughter pairs 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. It is apparent in these studies that some common exposure experience was specific for certain groups of calves during the neonatal period and not for other animals born during the same year. The role of neonatal exposure as supported by the cohort analysis work suggests that infection is the most significant factor in the natural occurrence of leukemia. Genetic aspects would primarily involve the degree of susceptibility rather than hereditary transmission. The role and significance of groups of lymphocytotic animals is demonstrated by the epidemiologic pattern and association of lymphocytotic animals with multiple occurrence of cases in leukemia herds and the clustering of leukemia and lymphocytosis among cohort animals.

Table L-14

The Results of Analyzing the 310 Dam-daughter Pairs
On the Basis of Persistent Lymphocytosis and Leukemia
Or Not Having Leukemia or Persistent Lymphocytosis Follows:

Non-lymphocytosis dam and daughter	164
Non-lymphocytosis dam and lymphocytosis or leukemic daughters	66
Persistent lymphocytosis or leukemic dams and daughters	39
Persistent lymphocytosis or leukemic dams and non-lymphocytosis daughters	<u>41</u>
TOTAL	310

49% affected dams have affected daughters

29% nonaffected dams have affected daughters

Table L-15

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

<u>Daughters</u>	<u>Non-Lymphocytosis</u>	<u>Lymphocytosis or Leukemia</u>	<u>Total</u>
Non-lymphocytosis			
Observed	164	41	205
Expected	152	53	
Lymphocytosis or Leukemia			
Observed	66	39	105
Expected	<u>78</u>	<u>27</u>	<u> </u>
Total	230	80	310

$$\chi^2 = 10.84 \text{ 1 d.f.}$$

$$P = .001$$

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

a. Pathologic studies on normal, leukemic and lymphocytotic animals

Extensive pathologic studies were conducted on eight juvenile leukemia cases, 44 adult leukemia cases and 14 normal animals with lymphocytosis from multiple case herds. In addition, lymphoid tissue and selected organs from 30 normal dairy cattle without significant lymphocytosis were studied. Studies on the normal animals included correlation of the lymph node tissue weight (L. N.) with the state of nutrition (N), the weight of the cow (W), the circulating lymphoid mass (L), and the age of the cow (A) according to the formula:

$$L. N. = 136 + 150N + 0.61W + 8.67 L - 17.4LnA$$

The measured parameters account for 69% of the variability in L. N. The average deviation of the predicted L. N. from the observed L. N. is 17.8%.

In studies on the 14 cattle with lymphocytosis, one animal had incipient leukemia. Relative to lymphoid tissue no significant quantitative, histologic or cytologic differences were noted between animals selected as "normal" and animals with lymphocytosis.

The juvenile form of lymphocytic leukemia in cattle resembles closely lymphocytic leukemia of children. In the eight animals studied extensively, leukemic proliferation was constantly observed in bone marrow, liver and all lymph nodes. Leukemic proliferation in other organs and tissues were inconsistent.

The adult form of bovine leukemia does not have the consistent pattern of involvement as seen in the juvenile cases. A symmetrical enlargement of lymph nodes was the most common lesion. The heart, abomasum, bone marrow and spleen were involved in most cases. In the 44 cattle examined the increase in lymphatic tissue was from 0.27 to 74.4 times the normal lymphatic weight, with a median of 8.31 and an average of 14.43. If a vital organ was not infiltrated, the adult cow could support up to about 10% of the body weight before death associated with emaciation.

b. Morphological studies of agranulocytes and lymphatic tissue of cattle.

Ultrastructural studies of the cellular effluent of the thoracic duct in the calf have been completed and a manuscript has been published in "Blood". In this study 89% lymphocytes, 1.3% plasmacytes, 4% preplasmacytes, 4.8% reticular lymphocytes, and 0.7% mitotic forms of all cell types observed were found to comprise the agranulocytic cellular constituents of thoracic duct lymph.

Ultrastructural studies of lymphocytes of lymph nodes and peripheral blood indicate that these cells contain a fragile cytoplasm in leukemic cattle. In many preparations virtually nothing was left beyond the nucleus surrounded by shreds of cytoplasm after the application of techniques leading to embedment in plastic.

Light microscopic studies of hemal nodes of 100 dairy cattle showed that those of cutaneous location are significantly larger and contained vastly greater numbers of lymph nodules than visceral hemal nodes.

Germinal centers of cutaneous hemal nodes were found to be significantly larger than those of other lymphatic tissue. In descending order of size, germinal centers of tonsils, lymph nodes, and spleen were smaller than those of hemal nodes. The pathway of blood through hemal nodes and the ultrastructure of these organs are currently being investigated.

In previous work on other tissues the ultrastructure of the so-called "nuclear body" was described in detail. This body, which is spherical and about 0.8-1.2 Microns in diameter, is characteristically located adjacent to the nucleolus. It consists of a filamentous outer form, and a central core which is made up of ribosome-like particulates.

Studies of the nuclear body in bovine agranulocytes showed that they were present only in interphasic lymphocytes. In these cells nuclear bodies were usually disassociated from the nucleolus. Ultrastructural studies of lymphocytes from leukemic cattle showed that lymphocytes frequently contained bizarre nuclear body forms.

c. Studies of the Ultrastructure of Leukocytes of Pre-Leukemic Cows

It is planned to obtain at least four blood samples from each pre-leukemic cow for electron microscopic studies. To date it has been possible to obtain samples from each of 30 pre-leukemic cows.

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. These nuclear extrusions have been shown to be present in lymphocytes of Burkitt's Lymphoma and in some trisomy syndromes of man. Second, nuclear bodies of lymphocytes are either absent, or present in ghost or bizarre forms. They are very prominent in lymphocytes of normal cattle.

d. 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.

Superimposition 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.

e. Studies on Lymphocytosis Animals and Promotion of Tumor Growth

This study was undertaken in an attempt to clarify the role of lymphocytosis in cattle which is associated with leukemia cases. 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.

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 $75 \text{ r} \pm 1 \text{ per cent}$.

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 bleeding 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. During the first eight months of the post-irradiation period there has been a gradual return of lymphocyte numbers to pre-irradiation levels. The rate of return is similar in all animals and 80% of baseline pre-irradiation values have been reached.

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 lymphodepressant 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 lymphodepressing 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 depression was noted during the six day course of treatment. Regenerative time or time to attain pretreatment levels of lymphocyte values was approximately one month. A similar response was noted on repeat trials on the same animals. 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. No evidence of tumor induction by radiation or corticosteroid therapy has been demonstrated.

SUMMARY

Based on data from our laboratory and similar data from other laboratories, there is sufficient evidence to indicate:

That lymphocytosis in normal animals in herds of cattle with adult leukemia is associated with and a part of the development of naturally occurring adult bovine leukemia.

The lymphocytes in these animals may be morphologically abnormal cells. No functional aberrations are known at this time.

Lymphocytosis and naturally occurring adult bovine lymphocytic leukemia is probably transmitted vertically in prenatal or neonatal life and maybe transmitted horizontally in the early neonatal period by an infectious agent.

The role of genetic susceptibility for lymphocytosis and naturally occurring adult bovine lymphocytic leukemia appears to be less significant in view of the evidence for vertical and horizontal transmission of the disease.