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BOVINE LEUKEMIA: ETIOLOGIC, PATHOGENETIC AND DIAGNOSTIC STUDIES

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This work is dedicated to them and to my parents.

INTRODUCTION

Bovine leukemia (lymphosarcoma, leukosis, pseudoleukemia, malignant lymphoma, lymphadenosis) is a chronic neoplastic disease of cattle characterized by malignant proliferations of lymphoid cells, progressive cachexia and high mortality. Diffuse or nodular, the neoplastic growths are gray, soft and often multicentric. Once introduced, the disease is usually well-established in a cattle population, it is sporadic and commonly affects mature cattle.

This disease is a neoplasia of lymphoid tissue termed lymphosarcoma or malignant lymphoma. The designation leukemia may be misleading since an increased lymphocyte count (persistent lymphocytosis), while common, is not a constant feature. However, the word leukemia is useful in referring to the bovine leukemia virus (BLV) now associated with lymphosarcoma of cattle so that the virus may be considered along with the other known leukemic viruses of chickens, mice and cats.

Definition

Although the term leukemia seems at first glance to be readily understood, its exact definition is difficult. Leukemia (white blood) implies a condition of the blood characterized by a greatly increased leukocyte count. Furthermore, what is fundamentally abnormal in leukemia is not the blood but rather the tissues - the several tissues which produce the blood cells and the many tissues in which leukemic cells may settle and accumulate. Indeed, blood is of secondary importance in leukemia. Blood may be considered as a "traffic stream" through which cells pass from their site of origin in the blood-forming organs to tissues all over the body, and so to their eventual destruction.

The quantity of leukocytes present in the blood is determined by three factors: rate of entry from the blood-forming organs, duration of sojourn in the blood, and rate of disappearance into the tissues. Of these, the entry and disappearance rates are relatively equal in health. Rises in the leukocyte count may occur, theoretically, from an increased entry or decreased exit rate, or from prolongation of the intravascular life span. The count may fall when there is decreased entry to or increased withdrawal from the blood, or when the intravascular life span is shortened. It should be emphasized that normally the movement of granulocytes is strictly in one direction; from the blood-forming organs, to the tissues via blood. There is no evidence that, having once left the blood, granulocytes can re-enter. In this, they contrast with lymphocytes, many of which circulate from the blood through the lymph nodes and back into blood, via the large lymphatic vessels. In certain forms of leukemia, however, granulocytes or their precursors may well return to the circulation after having passed through an organ such as spleen (Galbraith, 1967).

Although it has been the custom to consider leukemia as primarily and fundamentally a proliferative disorder, the rate of proliferation need not be faster than normal and is often substantially slower. A basic defect of leukemic leukocytes, especially in the acute forms, is lack of normal maturation. So long as these cells remain immature, they retain the capacity for further proliferation, however slow this may be, and their lifetime may therefore be greatly increased. Indeed, many leukemic cells may be regarded as being relatively immortal.

Because leukemic cells may have a prolonged total and reproductive life, they are likely to accumulate in the tissues, and this may be an

essential element in the pathology of leukemia. It is not the rate of cellular proliferation but the overall magnitude of the process which appears to be responsible for the distinctive characters of leukemic cell growth.

Leukemia may be said to represent an abnormal form of proliferation of one of the white-cell-forming tissues, that is, the bone marrow, lymphoid tissues, or the system of plasma cells. Fortunately, not all leukocytic proliferations are leukemic; in fact, benign proliferations of the white cells represent one of the commonest forms of normal body reactions. Thus polymorphonuclear leukocytosis occurs with trauma, exercise, or even with stress or excitement. Leukocytosis is not always the outcome of increased cellular growth, however, as a redistribution of granulocytes, with a shift of "marginated" cells into the circulating pool can lead to a rising granulocyte count. However, the leukocytosis of many pyogenic bacterial infections (streptococci, staphylococci, etc.), in which there is a great increase in the polymorphonuclear cells of the blood, is a proliferation of bone marrow granulocytes in response to a specific bacterial organism. By obscure, though probably humoral mechanisms, the local infection evokes first, a mobilization of existing bone marrow granulocyte reserves and then, proliferation of the bone marrow granulocyte precursors. This proliferation is purposeful, and directly related to a well-defined etiologic agent; in this instance, a bacterial organism. Once the invading organisms have been overwhelmed and there is no longer any need for excess granulocytes, the bone marrow reverts to its normal growth pattern, and the blood leukocytosis gives way to normal blood counts (Hardin, 1960).

A similar mechanism, but one involving a different blood-cell

forming system, is that found in response to certain viral agents which can be seen clearly in infectious human mononucleosis. It is conceivable that the generalized proliferative reaction which takes place in this disorder is actually immunologic in nature, that is, that immunocytes of the lymphoid variety are proliferating with eventual development of an immune response. In mononucleosis, the infective organism, induces an intense, perhaps purposeful proliferation of lymphoid tissues (lymph nodes, spleen, tonsils, Peyer's patches of the intestines, etc.). This results in generalized lymph node enlargement, both peripherally and centrally, in splenomegaly, and in many biochemical abnormalities, chiefly of the serum globulins. The leukocyte count is generally elevated to twice, three, or four times normal with the presence of many abnormal lymphocytes. In a few weeks, however, certainly in a few months, the lymphoid proliferation returns to normal, and the blood picture reverts to its normal features, with perhaps a few abnormal lymphocytes present to indicate the previous disease. Similarly, other infections are characterized chiefly by proliferation, at times quite intense, of the marrow cells (probably early members of the myeloid series) responsible for monocytosis in the blood. This is noted in such conditions as tuberculosis, and in similar, rather indolent or chronic, disorders.

The polymorphonuclear responses of diverse origin, the lymphoid proliferative disorder known as infectious mononucleosis, monocytic proliferation tuberculosis, are all generalized proliferations of one of the white-cell-forming tissues, but they are self-limited, that is, reactive. They are associated with a more or less well-defined etiologic agent to which there is a purposeful and self-limited response. Leukemia is not like this: it is not a normal self-limited reactive process, but

an abnormal, generalized self-perpetuating proliferation of one of the leukopoietic tissues in response to a well-defined, presently discernible agent; and apparently without purposeful or utilitarian value to the body.

This abnormal growth process appears to have an innate hardiness which gives it an ecologic advantage over cells of normal tissue, thereby creating a successful population. The principles of population dynamics have been applied by Burnet (1958), Gorman and Chandler (1963) and others to various types of cellular proliferative activities: immunologic, lymphoid, etc. They may have particular relevance in the case of leukemia, where the principle of "competitive exclusion," as stated by Hardin (1960) can also be invoked. Biological competition is acute between the most similar populations, that is, between white cells which are almost, but not quite, similar, or perhaps even between the white and red cell precursors which are derived from a common stem cell. The most successful population tends to displace all others from a given ecologic niche, although a stable coexistence of two or more populations within a niche can occur. As Gorman and Chandler (1963) wrote, "One population need have but an infinitesimally small advantage over another to displace it completely from the niche if enough time is allowed. The reasons for superiority of one population over another are often extremely subtle and are often not clear even after extensive study of a competitive situation." The inexorable course of leukemia is a demonstration of competitive population dynamics: its most graphic expression is seen in the clonal evolution which is featured in many cases of chronic granulocytic leukemia as they enter their terminal stage.

In brief, leukemia may be defined as an abnormal, neoplastic, generalized, self-perpetuating proliferation of one of the leukocytic tissues,

often associated with abnormal white blood cell counts and an abnormal increase in leukocytic mass.

This definition of leukemia has its limitations. It gives no hint as to the cause of the proliferation. Why should one of the leukocytic tissues suddenly develop this "obsessive" proliferation? In proliferations of infectious origin there is an inciting agent, usually a bacterium or a virus or an antigen. It is, of course, established that a viral agent may be responsible for the proliferative process of leukemia. Moreover, it seems likely that a virus would constitute only one of a number of agents which must combine to cause leukemia in any given case, that is, to produce the self-perpetuating mechanism inherent in the leukemic disease. The abnormal cell proliferation in leukemia is, in fact, a "new way of life," or in the shorthand of language, "neoplastic." Thus, the self-perpetuating feature of the leukemic process, its relentless character terminating only with death of the individual - these features are synonymous with neoplastic disease. Leukemia may, therefore, be considered as generalized neoplasia of one of the white-cell-forming tissues. The "why" of this apparently purposeless proliferation is by no means clear, and must be considered as the central investigative problem of leukemia, if not of hematology. The all-encompassing problem is that of growth, more particularly that of abnormal and successful neoplastic growth. Should the various complex mechanisms involved in growth be solved some day, one would be a long way toward solving the problems of abnormal or neoplastic growth and thus of leukemia itself.

DISTRIBUTION OF BOVINE LEUKEMIA

The distribution of leukemia may be determined roughly from publications in the veterinary litsrature. Many are reports of clinical cases and others come from meat inspection findings. At present there are so few from certain regions that the impression is gained that the disease seldom occurs in these locations.

Extensive reports of leukemia originate from Europe where in some areas, it is the dominant tumor of cattle, for example, West and Northern provinces of Germany. Reports from Sweden and Estonia also indicate that the leukemia is common. In the United States, meat inspection figures provide an insight into the distribution of the disease and a guide to the incidence of the disease. There are five main centers in the U.S.A. actively involved in investigating various aspects of leukemia; Philadelphia (Pennysylvania), Ames (Iowa), California, Wisconsin, and Manhattan (Kansas). These centers do not reflect distribution of leukemia.

Germany

The first quite extensive description of bovine leukemia cases was published in Germany where Siedamgrotzky (1878) described his observations in black and white cattle of Saxony. Schottler and Schottler (1934) reported that bovine leukemia was first observed in Memel (Lithuania) and that it penetrated west. By 1920 it was widespread. Knuth and Volkmann (1916) investigated leukemia in cattle herds. Up to World War II bovine leukemia was confined and established in German provinces. Cases reported by veterinary practitioners ranged from 100 to 380 cases per 100,000 head of cattle in the East German provinces; reports from

other communities indicated 700 to 1,000 cases per 100,000. In southern provinces of Germany the incidence was low. The high incidence of leukemia in Germany was traced to importation of cattle after World War II, large movements of cattle during 1944-1945 from East to West, and changes in methods of cattle farming which have occurred in the later years, first to form a division of the large farm and later by the combination to form the new large collective farms, seemed to increase the spread of leukemia considerably (Weisner, 1961).

Since 1953 extensive investigations of bovine leukemia has been carried out. The work was started by Gotze (1953) and his coworkers and now being continued by Rosenberger (1961) and cŏworkers in Hannover. Cattle movement from infected areas to free areas was incriminated as the main reason for spreading leukemia.

Sweden

In Sweden the appearance of bovine leukemia caused an increased interest in the disease during the last 20 years. The disease has been known for many years in Kalmar. Since 1945 there has been more than 100 percent increase observed in the cases of leukemia. It was shown by evaluation of cattle slaughtered in 1944 that 0.25 percent were condemned because of leukemia. In 1949 this increased to 0.54 percent. It has been reported that in areas hard hit by the disease, 100 out of every 100,000 head of cattle are affected (died or condemned) annually. Further more, the disease has appeared in areas where it previously was seldomly found (Bendixen, 1967).

However, it still does not seem to have gained a foothold in Norrland. Hjarre (1958) stated that an increase in the incidence of leukemia has been demonstrated among cattle in herds where piroplasmosis vaccination

had been introduced, and also that the increased incidence occured the year after vaccination was adopted on a broad basis. The material for the production of vaccine originated from leukemia areas (Olson (1961). Cattle in the southern part of the country are more affected.

U.S.A. and Canada

As mentioned earlier, the occurrence and spread of bovine leukemia in the U.S.A. and Canada has not been clearly reported. A number of publications regarding cause; Lewis and Savage (1930); Boyd and Karlson (1934); Frank and Thompson (1940); Jasper et al. (1946); Boyd et al. (1947); Thompson and Roderick (1942); Sautter and Sellers (1948); Marshak and Dutcher (1965); Ferrer (1972); Ferrer et al. (1974); Miller et al. (1969); Graves and Ferrer (1976). There does not seem to be any doubt that bovine leukemia occurs enzoolically in many states especially the Midwest states and California. Feldman in 1929 and 1932 reported that leukemia had an especially high frequency in adult cattle and that it was responsible for condemnation of approximately 0.011 percent of adult slaughter cattle. Schlotthaner (1928) called the attention to the fact that leukemia in cattle occured frequently in Minnesota, which was varified by Karlson in 1942 and Sorensen and Anderson (1964) in their studies. Starr and Tyler Young (1941) described cases found in Alabama, and Theilen et al. (1961) reported that in Government-inspected slaughterhouses in the U.S. 0.017 percent of slaughtered cattle were condemned because of leukemia in 1958. The highest percentage of cattle were found in areas east of the Rocky mountains. The incidence presumably doubled from 1945 to 1958, though this may be due to more accurate reporting. Information about the incidence of leukemia relative to the cattle population in the individual states has not been published but there is a

strong indication that the incidence in Minnesota and Wisconsin is close to that of Germany.

According to statistics of the Federal Meat Inspection Service (FMIS), the number of cattle condemned for leukemia at the time of slaughter in the U.S.A. has been increasing. From 1917 to 1925, the leukemic rate can be calculated to be 0.4 to 0.6 per 10,000 cattle slaughtered. In 1938, the rate was 1.4 per 10,000. For unknown reasons, the rate decreased, ranging from 0.92 to 0.99 per 10,000 for the years 1950 to 1953, and then increased to 1.8 and 1.7 for the years 1958 and 1964, respectively.

Recently, using immunodiffusion, Baumgartener et al. (1975) investigated the incidence of leukemia in the six North-Central States of U.S.A. and found 10.2 percent reactors out of 4,394 dairy cattle in five states. These reactions came from 66 herds with a total cattle population of 3,281, the reactor rate varied from 2.1 to 44.4 percent. In beef cattle, the incidence was 1 percent. Thus, if serological methods are employed to survey the whole U.S.A. cattle population, a higher incidence of leukemia would be expected than that found by meat inspection (Appendix A).

From the statistical figures, an average of 7,000 carcasses are condemned annually; dollarwise, this amounts to about $3,920,000 besides associated losses due in production milk, meat and conception.

Russia (U.S.S.R.)

The yearly survey of the FAO/OIE (1960) indicated that bovine leukosis was spread all over the U.S.S.R. and was considered to be a serious problem. It's etiology was the subject of intensive investigation and the virus theory had not been verified.

Denmark

A systematic mapping of the occurrence and spread of bovine leukosis in Denmark had not previously been described. Earlier Danish workers had primarily directed their attention studying the etiological problems. This is valid for the work of Engelbreth-Holm and Plum (1941) who performed transmission experiments and for the work of Egehøj (1947), who performed experiments attempting to produce leukosis according to the theories of "protein poisoning," as had been set forth by workers such as Dobberstein and Seifried (1938). Egehøj (1945) performed systematic blood tests on cattle in a few leukotic herds, and his work showed stationary lymphocytosis in many cattle which was of considerable interest, partly because it verified earlier German observations such as those of du Toit, (1916), and partly because it showed that leukemia in Denmark was identical with bovine leukosis in Germany.

The articles mentioned here give the impression that 20 years ago Danish workers were aware of bovine leukosis in certain parts of the country. Additional information could be obtained by persuing medical records at for large domestic animals of the Royal Veterinary and Agricultural College in Copenhagen. Only a very few cases of bovine leukosis were registered before 1930. In the 1940's an increasing number of leukotic animals were admitted to the clinic.

Another source of information on occurrence of leukosis in earlier times are records of cases reported in questionnaires sent to all veterinarians in Denmark during 1953-54. The most accurate and detailed information concerned the years around 1950, but many valuable observations regarding cases detected earlier allowed a chronological survey of occurrence and spread of leukemia (Bendixen, 1965).

With the introduction of a systematic government-controlled eradication program in July, 1959, the opportunity was provided for continued mapping on a broad basis. There was an average of 4.1 cases of leukemia per 100,000 head of cattle per year for the whole country; 3.1 were the enzootically-occurring type of leukemia, 0.5 cases were young animal leukemia, and 0.1 cases were skin leukemia.

It is striking that enzootically-occurring leukemia had distinct localization. If an evaluation for each county is done, some counties in Sjaelland and Lolland-Falster (Sorø, Praestø, Holbak, and Maribo) were severely involved, while counties in other parts of the country, with the exception of a few in Jutland (Aarhus, Randers, and Aabenraa), must be considered as relatively leukosis-free. Up-to-date figures are not available.

Africa

The disease has attracted little attention in any part of Africa despite the widespread nature it apparently has in Nigeria.

India

In India the disease was identified in Buffaloes (Singh, 1968).

CLINICAL SIGNS AND PATHOLOGY

After a period of slow and insidious development, bovine leukemia presents variable clinical signs. Despite availability of ample feed and water, weight gain is slow and eventually becomes a loss. Body temperature, while usually normal, may slightly elevate temporarily and intermittently. However, clinical sign depend on what organs are affected. The following are the most commonly observed organic changes which have special relevence to pathological conditions important for diagnosing leukemia:

1. Lymph Nodes

The lymphoid tissue is the usual site of leukotic changes, and 98 percent of cases examined had one or more enlarged lymph nodes. However, involvement of all lymph nodes by the neoplasm is unusual in enzootically-occurring leukosis, and only a part of the lymph nodes are affected in about 75 percent of the cases. In this type of leukosis, the leukotic neoplasm changes often are predominantly in the internal organs such as heart, liver, kidneys, abomasal wall, uterus and the associated lymph nodes. The externally accessible lymph nodes in these cases are only slightly changed. It may be of interest for clinicians to note that 4 percent of leukotic cases had neoplastic changes localized in lymph nodes in the pelvic region and also in the most candal part of the abdominal cavity where they could be detected by rectal examination.

A thorough examination of the size, shape and consistency of lymph nodes is of value in the differential diagnosis of diseases causing lymph node enlargement, such as Corynebacterium pyogenese infections, actinobacillosis, or tuberculosis. The leukotic changes increase

affected lymph node to 5-10 times their normal size. The consistency is tensely elastic or turgid, and no fluctuation or softening. Usually the surface is even and smooth, and the lymph node is easily displaced in relation to it's surroundings, but in a number of cases infiltrative growth into the surrounding connective tissue and musculature occurs. The infiltration obscures the contours of the lymph node, which can be further distorted by edema occurring in the area. These changes occur most frequently around the superficial cervical lymph nodes and in the precordial mediastinum, from where they spread to the dewlap and along the sulcus jugularis.

The fast growing neoplasm fragments easily, and rupture of the vessels often occurs which are followed by formations of hematomas. The size and the form of affected lymph nodes can change considerably from day to day, and such changes frequently may be palpated by rectal examination.

Hyperplasia of the lymph nodes was often observed but could not be distinguished by palpation. This was especially true for the subcutaneous lymph nodes of the abdomen and thorax, and at the base of the tail. For this reason, Lubke (1939) tried to use biopsy and a histological examination of these lymph nodes for verifying the diagnosis in the early stage of the disease. He stated that the diagnosis could be made in this manner with an accuracy of about 85 percent.

2. Thoracic Cavity

During clinical examination, it is difficult to determine if the organs in the thoracic cavity are sites of leukotic infiltration. The suspicion that a case of morbus cordis, for example, is caused by leukosis will usually arise when neoplastic changes are found in other locations.

Among leukotic cases, tumor changes in the heart were found postmortem in 58 percent of the cases, but circulatory disturbances were found clinically in only 52 percent. The circulatory disturbances, as a rule, involved stasis phenomena of a relatively unspecific character, for example; tense jugular veins, reinforced venous pulse, edema of the dewlap, extended percussion sounds from the liver, and diarrhea. In some cases, abnormal cardiac sounds were detected, and increases in the heart rate were observed upon slight exertion.

The leukotic infiltrations can be diffuse or nodular and usually start in the right auricular wall and later, diffuse changes appear in the ventricular walls.

The precordial mediastinum is often the site of intense leukotic infiltrations which spread cranially through the apertura thoracis cranialis and up the neck along the sulcus jugularis.

The pulmonary lymph nodes and those lying parietally in the thorax are often the site of leukotic infiltration. However, morphological changes in the lungs themself can rarely be demonstrated.

3. Abdominal Cavity

Autopsies of leukotic animals showed macroscopically recognizable leukotic changes in the liver in 58 percent. The lymph nodes of the affected livers were usually enlarged. Cases also were found in which the hepatic lymph nodes were leukotic without macroscopically recognizable infiltrations in the liver. These leukotic changes do not, as a rule, cause distinctive clinical signs. Extended percussion sounds from the liver as well as a hepatic insufficiency with the accompanying general signs are found in other cattle diseases and can support a diagnosis of leukosis only if signs are present in other locations in

the animal.

Sometimes the spleen is the site of massive leukotic infiltrations which can result in splenic rupture and internal hemorrhage and sudden death. In other cases, splenomegaly is more moderate. Hiepage (1954) and Winquist (1959) considered that there was a connection between the leukemic blood changes and the degree of splenic change.

In the abomasal wall, leukotic changes are often found. In this location large lymphocytic masses frequently occur, and the abomasum walls and folds may be several centimeters thick. The pathological changes undoubtedly have mechanical effects on the function of the abomasum, for example, by reducing the ability of the abomasum to contract, by stenosis in the channels of the omasum, and by stenosis of the pylorus. Pressure from enlarged regional lymph nodes can also cause mechanical disturbances. In addition to this, damage to the mucosa of the abomasum can occur since the tumor tissue easily ruptures. Ulcerations in the surface sometimes cause severe hemorrhages, which are clinically manifested by melena.

Leukotic changes also occur in other parts of the digestive tract, especially in the small intestines and rectum and all the mesenteric lymph nodes may be involved. Rumen motility can be disturbed by leukotic infiltrations; thus, tympany, among other things, may be seen as chronic recurring bloat caused by disturbances in the motor function. Often the tympany seems to occur as a result of a compression of the esophagus by pressure from enlarged mediastinal lymph nodes (Jensen, 1974).

Usually leukotic changes in the gastrointestinal tract produce nonspecific signs such as loss of appetite, indigestion, ileus, and diarrhea. In suspected cases of leukosis, rectal palpation often

discloses additional tumor infiltrations of the pelvic cavity and the posterior part of the abdominal cavity.

Leukotic changes are often found in the kidneys that may be diffuse or nodular and result in enlargement and change in shape. Such changes are often detectable by palpation on rectal examination. In some cases, the leukotic infiltrations are localized in the renal pelvis and ureters. Involvement of the ureters produces urinary stasis, which results in distention of the calyces and hydronephrosis. These changes result in renal failure and if bilateral, uremia occurs. Leukotic changes may also occur in the wall of the bladder and urethra. Rectal examination in these cases can detect localization of tumor formations and thus explain any eventual difficulty to produce urine. Leukotic changes in the bladder wall may be diffuse or nodular and may fill the entire lumen. Bleeding and hematuria occur as a result of cellular destruction and rupture of vessels in the mucosa (Bendixen, 1965).

4. Reproductive Tract

All reproductive organs can be attacked but infiltrations in the uterus are the most common. The infiltrations can be either diffuse or nodular, depending on the condition of the uterus, since both the pregnant and the nonpregnant uterus can be the site of tumor formation. The horns, body of the uterus, the cervix and vagina may be infiltrated. This has diagnostic importance when rectally palpating the reproductive organs such as during insemination, treatment for infertility, or examinations during pregnancy.

In the nonpregnant uterus, the tumor masses may become large enough to obliterate the anterior uterine boundary. On palpation, the

neoplastic formations feel like relatively firm bulges with either a smooth or irregular surface, which in some cases resembles carbuncles in a pregnant uterus or a cacerated or mummified fetus. Neoplastic masses in the salpinx can produce changes in the thickness of the wall and may resemble tuberculous salpingitis without calcification. Leukotic masses of the diffuse and nodular leukotic masses are found frequently in the cervix. The nodules may have a large or a small stem and may be as large as a clenched fist. Deformation of vervical canal increases the possibility of bacterial contaminants ascending into the uterus. Metritis, therefore, is a frequent complication of uterine leukosis.

Leukotic infiltrations sometimes occur in a pregnant uterus. Depending on the stage of pregnancy at which these changes occur, the rate of growth, and on the size of the tumors, embryonic death, abortion, or dystocia may occur. Pregnancies have been successfully completed and without dystocia in spite of leukotic involvement of the uterus wall.

Leukotic infiltrations in the vaginal wall may be diffuse and nodular. The nodules may vary in size up to 5 to 6 centimeters in diameter. They are prominent in the lumen and are usually yellowish-white. Bloody or exudative mucosal defects are common on the surface of the nodules. Changes in the vaginal wall can be diagnosed by rectal examination of by a vaginoscope.

Macroscopically, recognizable leukotic changes are seldom observed in the mammary glands, and high milk production is found in many leukotic cows up to the premortal stage at which time the neoplasm affects the functions of the individual organs and leads to general deterioration

of the affected cow's condition. The supramammary lymph nodes are often extremely enlarged and frequently the site of leukotic infiltration (Jensen, 1974).

5. Other Organs

Leukotic infiltrations generally are not found in nerve tissues. Nervous disturbances often appear in leukosis because neoplastic masses in the vicinity of the nerves produce mechanical pressure which results in functional disturbances. The spinal canal is a common site for leukotic infiltrations. The tumors are located in the spinal cord membranes and often form a thick coat around the spinal cord. The size and location of the leukotic infiltration are the deciding factors in the degree and extent of the nervous disturbances. Since the infiltrations usually are located in the most posterior thoracic vertebrae and in the vertebrae of the lumbar and sacral regions, the paralysis develops most often in the posterior limbs and results in an ataxia which may gradually result in total paresis (Jensen, 1974; Bendixen, 1965).

The leukotic infiltrations in the spinal cord membranes often continue through the foramina intervertebralia along the nerve and out into the musculature of the loin. At the same time, the regional lymph nodes are usually affected. These pathological changes are diagnostically significant as they may be easily palpated by rectal examination and therefore be used to verify suspicion of leukosis in paretic cases.

The leukotic infiltrations have a tendency to localize in the orbital region. They appear to spread from the small amount of lymphoid tissue situated in the retrobulbar fatty tissue. Growth of the tumor mass pushes the bulbus oculi forward resulting in exophthalmus. Later,

the tumor mass penetrates beneath the conjunctiva and appears as a thickening under the eyelid. The eyelid function becomes hindered and the cornea dries, which results in its perforation. The tumor changes may then be complicated with panophthalmitis. The ocular changes may develop bilaterally, although in most cases a certain asymmetry is seen.

Leukotic changes are demonstrable in the bone marrow of a varying number of affected cattle. Kohler (1957) and Winquist (1958) examined the sternal marrow and found diffuse infiltrations. Kohler (1957) stated that the pathological changes must be characterized as a reticulosis and that bone marrow examinations ought to be used more widely as a diagnostic aid and as a supplement to the blood smear examinations. However, Kohler reported that examination of lymphocytes and lymphoblasts in the bone marrow did not demonstrate the same results as the blood smears. Among other findings, one cow with a tumor had normal bone marrow, just as some cows with lymphocytosis demonstrated nothing abnormal on the marrow examination.

In summary that the examinations performed did not indicate that bone marrow studies can be used instead of blood examinations to diagnose leukosis. Bone marrow as a tissue seems to be attacked to a varying degree and extent just as other tissues.

6. Blood

As already described, there are great similarities between the lymphocyte fraction in the subclinical and clinical stages. The difference is essentially one of degree. Even if aleukemic cases occur, the estimation of the white blood cell picture is most important.

CLASSIFICATION

The four forms of bovine lymphosarcoma or leukemia are clinically distinct: adult, thymic, calf and skin.

1. Adult form (three years or more). Scattered lymph nodes are enlarged and often the heart abomasum and uterus are affected. This is the most common form.

2. Thymic form. Relatively rare form with a large neoplastic tumor in the neck adjacent to the jugular vein and trachea in cattle about two years of age. A few lymph nodes are also affected.

3. Calf form. Nearly all lymph nodes and many internal organs are affected in this form, even at birth.

4. Skin form. This rare skin form is usually found in cattle, one and half to three years of age, and may be associated with lesions of the adult form.

According to Bendixen (1967) there are two major forms: enzootic and sporadic. Leukosis enzootica bovis is an enzootic neoplastic disease involving cells of the reticulo-histiocytic tissue of cattle and is known as lymphosarcoma, malignant lymphoma, leukemia, lymphadenosis, lymphoblastoma, lymphomatosis, and pseudoleukemia. The term leukosis was proposed by Ellermann and Bang (1908) as a general name for leukemia.

Dobberstein (1934) proposed that the term leukosis be used as designating for the corresponding disease of cattle. This term is preferred especially in East Europe as it is neutral in meaning and does not infer any clinical signs. Therefore it can be used as a general designation for tumor diseases involving leukocytes, even if etiological differences appear later.

There are two types of sporadic leukosis; leukosis sporadica juvenilis and leukosis sporadica cutanea. These two are the young animal leukosis and the so called skin leukosis as described above. Natural regression of lesions has been reported in some cases of calf and skin forms and has been suspected in some adult forms.

In previous surveys of the prevalence of leukosis, investigators relied on lymphosarcoma detected on necropsy inspection data from slaughter establishments, and repeated counts of circulating lymphocytes to detect persistent lymphocytosis. Studies based on these criteria have provided extrement information on the extent of leukosis in various countries and a concept of bovine leukemia as an infectious disease, as well as ideas for control.

As stated, leukemia may be considered as a generalized neoplastic proliferation of leukocytic tissues. Since three lines of white cells may be distinguished - granulocytes, lymphocytes, and monocytes - three main types of leukemia may be described: granulocytic, lymphocytic, and monocytic. To these three main types may be added others which occur occasionally as result of invasion of the blood by cells not normally circulating in it, such as lympho and reticulosarcoma or perhaps tissue mast cells. A special case may also be made for including myelomatosis among the leukemias since its constituent plasma cells (mature or immature) can nearly always be found in the blood if sought for, and may occasionally produce a leukemic picture.

Like other types of neoplastic proliferation, leukemia shows considerable variations in growth patterns from mild to severe. In such cases, examination of the tissue in question (whether bone marrow, lymph node, etc.) shows excessive numbers of the proliferating cell

types, the proliferation appearing to take place in an orderly fashion, with eventual maturation to mature cells. The course of such cases is ordinarily protracted; this is chronic leukemia. There are, varying degrees of chronicity.

It is important, however, to stress that with recognition of the acute and aleukemic forms of leukemia, the main phase of morphologic hematology as applied to leukemia was drawing to a close. Already the first "cytochemical" methods like peroxidase stains were being used and the first tissue cultures of leukemic blood had been made. Further research was focused progressively on the functional and dynamic aspects of leukemia, and chemical, biochemical, biophysical, biological, cytogenetic, and immunologic techniques were to supplement more and more the classic methods of histologists and cytologists. Although something of a renaissance of morphology has come about with introduction of new techniques such as phase-contrast and electron microscopy, only a few significant discoveries in leukemia have been made with these techniques. There can be no question that the simple act of looking at a cell still has considerable importance, but it is by the combined use of all available tools that progress is likely to come.

ETIOLOGY

Montemagno et al. (1957) and Papparilla (1958) reported isolating a virus-like agent from cell-free filtrate of tumor material from leukotic cattle. They made serial passages of the agent in embryonated hen's eggs but maintained no control. This made it difficult to say whether the agent was actually a leukotic virus.

Thorell in 1957 isolated virus-like particles from a cow with a leukotic tumor. He employed the same technique used in the isolating chicken erythroblastosis. The particle isolated had the same appearnce and the same chemical characteristic as erythroblastosis virus.

"Almost nothing is known of the causes, either immediate or remote, of leukemia. In a minority of cases some disease or incident can be found in the patient's life to which leukemia is sequential in time and to which, in some degree, may be related. In the majority of cases no such antecedent is demonstrable. Those etiologic conditions which can be traced or suspected in some cases stand in a position with relation to the disease which is too uncertain to allow them to be considered as exciting or predisposing causes." (Forkner, 1938). More than three decades after those lines were written, large parts of medicine have been revolutionized. Yet, progress can be recognized in the understanding of leukemia's nature and of some of the factors concerned in its production. What is more, confidence can be placed in the continuance and acceleration of such progress. Thus, it is now almost generally accepted that leukemia is essentially neoplastic in nature, and that a search for factors and mechanisms involved in carcinogenesis in general has relevance to neoplasia of the hematopoietic tissues.

It has become clear in recent years that the etiology of the various forms of leukemia is not uniform and that the same causative agents may not be involved. Probably what we designate as leukemia is actually a heterogeneous group of more or less closely related conditions whose origin is determined by the interaction of many different factors, some known, others as yet only surmised.

Among these factors are a galaxy of viruses of virus-like agents whose role in human and bovine leukemogenesis is by no means established. Underlying or associated with these factors is the genetic constitution which endows every individual with its own degree of susceptibility and response to extrinsic agents. It is also likely that immunologic factors play an important part in some of the leukemias.

To set the stage for a detailed discussion of the leukemogenic factors, a rather bried historic description will be given of the manner in which current views on etiology have developed. Basic to these is the conviction that the leukemias are, in fact, neoplasms of the hematopoietic tissues.

Although this section is concerned with bovine leukemia and its etiology, a good deal of the discussion must be based on the results of extensive experimental work carried out in other animals. Such studies are impossible in cattle especially in the field of virology where positive findings have largely come from fowl and rodent leukemias and related neoplasia. It is certainly pertinent to ask how far such findings can be extrapolated to bovine leukemia. To this question there is as yet no conclusive answers, although at least some bovine leukemias behave similarly to those in other animals, and may therefore have a related etiology (Dameshek, 1958).

Role of Viruses

As with lymphoid neoplasms of other animals, bovine leukemia is now established as a virus-induced neoplasm.

Evidence in support of an infectious nature:

1. Occurrence of multiple cases in herds.
2. Mode of transmission, both vertical and horizontal.
3. Frequency of isolation of c-type virus from tissues of animals infected with leukemia.
4. Epidemological data.
5. Transmission study.

Germans were the first to focus attention on the infections nature of bovine leukemia. Veterinary scientists in 1900 were more concerned with the hereditary aspect of leukemia and cited numerous examples of familial pattern to support this view. Schaper (1938) suggested that affected cattle of either sex as well as their parents and grandparents should not be used for breeding purposes. Weisner (1944) described occurrence of leukemia in 149 out of 150 daughters of a single sire and the bull himself also succumbed to the disease.

During the 1950s and 1960s studies of Gotze and associates (1953, 1954, 1956) in Germany and Bendixen (1960, 1963, 1965, 1967) in Denmark began to shift emphasis away from genetic factors to a concept of leukemia as a virus-precipitated disease with a long incubation period of even years (Gotze et al., 1954; Bendixen, 1960). Bendixen observed that leukemic cases were concentrated in certain parts of Denmark with annual incidence of 15 per 100,000 as compared to 1 per 100,000 in other parts of the country. Annual mortality rate in certain herds in enzootic areas was as high as 2 to 5 percent and leukemia followed in

some areas after several years following addition of an animal from a known leukemia herd, without any given breed or family hereditary disposition being apparent.

EVIDENCE IN SUPPORT OF INFECTIOUS AGENTS

Transmission Studies

Many transmission experiments were performed to verify the transmissible nature of bovine leukemia. These transmission studies were difficult because of the low morbidity when leukotic tumor cases were used for evaluation. In addition to this difficulty, the incubation period may require years. Therefore, each experiment requires extensive studies over a long period, which can perhaps be carried out in mice and poultry but involves many practical difficulties in cattle. The relationship between leukotic tumor cases and lymphocytosis was examined, because a causal relationship seems to exist between these two manifestations of leukemia and considerably more animals develop lymphocytosis than develop leukotic tumors. It is possible to use experimental conditions which are less extensive with shorter periods of observation when lymphocytosis is used to detect leukosis. It has been shown that about half of the cattle in a herd attacked by leukosis develop lymphocytosis. It is reasonable to assume that practically all cattles may have been exposed to the agent and that in most cases this exposure occurs much earlier than the tumoral changes (Bendixen, 1967).

Many transmission studies have been concluded as negatives simply because the investigators observed no tumoral changes. However, if appearance of lymphocytosis had been used for evaluation, some experiments may have been evaluated as positive. This would be true for the studies by Knuth and Volkmann (1916) who found lymphocytosis in young calves about 6 months after inoculation with material from leukotic

tumor cases. Schottler and Schottler (1934) found similar results. Dobberstein and Piening (1934) observed that fever and anemia developed in normal cattle inoculated with blood from leukotic cattle. They considered this sign as initial stage of leukosis.

In 1941, Engelbreth-Holm and Plum inoculated a number of calves with blood from leukotic tumor cases. Tumors did not develop but lymphocytosis was observed in more than half the calves inoculated.

The most extensive pioneering experiments were performed by Gotze in 1956 and Rosenberger in 1961. Gotze inoculated 17 calves with material from leukotic cattle. Lymphocytosis occurred in 10 of the calves, and also in an uninoculated control calf housed in barn where the experiment was carried out. Over a ten year period of observation, the control and five inoculated calves developed tumors.

To closely parallel the possible modes of natural transmission, such as placental transmission, transmission through milk, inoculation via contaminated hypodermic needles, and inoculation via tuberculin syringes used for testing a leukotic animal, similar experiments were performed. Lymphocytosis occurred in one or more animals and one animal exposed to transplacental inoculation died of leukosis (Bendixen, 1967).

Bodin et al. (1961) observed lymphocytosis in animals vaccinated against piroplasmosis by using blood from leukotic herds.

These findings verify the suspicion that an infectious agent is associated with leukemia and that the agent is transmissible via a number of routes. The findings, however, do not give information regarding the nature of this infectious leukemic agent.

More recently, Ferrer et al. (1975) found bovine leukemia to be a disease with low communicability both in utero and by contact (horizontal spread). They found approximately 10 percent of the fetuses from infected dams to be infected at birth. The majority of young calves separated from positive dams remained negative until reintroduced into a herd at 19 to 24 months of age when they became infected apparently by contact with positive milking stock. Olson et al. (1975) found very few animals less than two years of age positive except for transient colostral antibody.

Miller et al. (1969) used naturally-infected cattle as sources to first culture bovine leukemia virus from lymphocyte cultures. They inoculated 14 calves with lymphocyte cultures containing bovine leukemia virus (BLV) that was prepared from cows that died of leukemia and from two experimentally-inoculated calves (Miller et al., 1972). All the calves became infected with bovine leukemia virus within 13 months as evidenced by re-isolation. Five calves developed persistent lymphocytosis which appeared between 4 and 13 months post-inoculation.

Schmidt et al. (1975) inoculated nine calves from herds known to be free from bovine leukemia with cell-free tissue culture fluid from lymphocyte cultures of a cow naturally-infected with bovine leukemia virus. Nine control calves were inoculated with culture fluids from a cow not infected with leukemia virus. No control calves developed lymphocytosis or antibodies against bovine leukemia virus nor could the virus be isolated from them. Three test calves developed lymphocytosis and antibodies to BLV, and the virus was isolated from these three calves. However, none of the calves from these studies were maintained long enough to develop tumors but the development of lymphocytosis

(persistent) in eight out of the nine calves was significant.

Olson et al. (1972) inoculated 13 lambs (one to two weeks of age) with lymphocytes from bovine lymphocyte cultures producing BLV particles. Eleven lambs became infected as evidenced by reisolation of BLV from their lymphocytes. Within 27.5 months, five lambs died of leukemia.

Olson et al. (1969) in studying parameters they considered important in leukemia found that: (1) Lymphocytosis were constantly associated with leukemia and often followed experimental inoculation; (2) An increase in lymphocytic nuclear projections in cows from multiple case herds, as well as in experimentally-inoculated cattle; and (3) C-type particles were demonstrable in lymphocyte cultures stimulated by phytohemagglutinin. In their transmission studies they used tumor cell suspensions prepared by pressing a leukotic tumor through a tissue mincer and adding an equal volume of Hank's Balanced Salt solution containing 100 IU penicillin and 500 mg of streptomycin per ml. Buffy coat cells were also obtained for inoculation by centrifuging 300 to 500 ml of blood, Table 1-2.

Seventeen cows were inoculated by both subcutaneous and intraperitoneal routes with 1 to 2 ml of cell suspension from tumor or buffy coat while in utero at fetal ages ranging from 99 to 259 days. One calve inoculated in utero developed generalised lymphadenopathy at 9 months of age which persisted for 5 weeks. Attempts to transplant lymph node tissue during this period to other calves failed. This enlarged lymph node had hyperplasia of both lymphoid and reticular elements but there was no invasion of the capsule or alteration of the normal architecture.

TABLE 1

Reactions of calves inoculated during fetal or neonatal life with bovine lymphosarcoma material

Donor and type	Inoculum	Recipient	Age (days)	Nuclear Projections	Reaction of lymphocytosis in culture: Type C particles	Reaction of lymphocytosis in culture: Age when cultured (months)	Reaction of lymphocytosis in culture: Lymphocytosis
		Fetal inoculates					
3081 Calf	Tumor	213	210	+	+	37	none
3081 Calf	Tumor	214	202	-	+	37	slight
3081 Calf	Tumor	215	213	+	+	36	slight
3081 Calf	Tumor	216	215	+	+	36	moderate
3084 Thymic	Tumor	219	181	+	+	35	slight
3088 Adult	Tumor	222	259	+	+	32	transient
3088 Adult	Tumor	224	198	-	not done		transient
3089 Adult	Tumor	220	253	-	-	36	moderate
3089 Adult	Tumor	223	201	+	+	33	moderate
3089 Adult	Buffy Coat	226	184	-	-	33	transient
3091 Adult	Tumor	228	181	-	-	31	very sligh
3091 Adult	Tumor	229	177	+	+	31	none
3093 Calf	Tumor	227	184	-	+	28	transient
3093 Calf	Tumor	232	134	+	+	22	transient
3094 Adult	Tumor	233	102	-	-	23	transient
3098 Adult	Tumor	243	99	+	-	9	transient
3100 Adult	Buffy Coat	239[1]	242	-	-	21	slight
		Neonatal inoculates					
3081 Calf	Tumor	210[2]	6	-	+	38	none
3081 Calf	Tumor	212	1	+	+	38	none
3088 Adult	Tumor	221	1	-	+	33	slight
		Irradiated					
3100 Adult	Blood	244	14	-	+	19	very sligh
3113 Adult	Tumor	281[1]	5	-	+	16	very sligh
3115 Adult	Tumor	286	2	-	-	12	none
3117 Thymic	Tumor	304	15	-	-	12	none
3117 Thymic	Tumor	305	9	-	-	12	very sligh
3139 Adult	Tumor	311[1]	14	-	-	10	none
3123 Adult	Tumor	325[1]	7	-	-	4	none

[1]Inoculated with tissue from its dam.

[2]Died at 4½ years with lymphosarcoma.

Reference: Olson et al. (1969).

TABLE 2

Occurrence of nuclear projections in lymphocytes (2% or more) and C-type particles in PHA-stimulated lymphocyte cultures.

	Nuclear projections		
	Lymphoid tissue	Blood	C-type particles[2]
Lymphosarcome			
Adult	22/22[1]	16/16	9/12
Thymic	1/2	1/1	-
Calf	0/4	0/2	0/1
Multiple-case herd			
Lymphocytosis	-	14/14	10/10
Suspect	-	4/7	3/4
Non-lymphocytosis	-	2/18	5/21
Inoculated cattle			
Fetal	-	9/17	10/16
Neonatal	-	1/3	3/3
Irradiated	-	0/7	2/7
Controls	0/8	0/18	2/26

[1]Animals with projections or particles/number examined.

[2]Blood or tumor examined.

Reference: Olson et al. (1969).

Nine calves were inoculated with the tumor suspension during the first 15 days of post-natal life. Combinations of different inoculation routes were used including intravaneous, subcutaneous and intra- or peri- lymph nodes. Six inoculated calves received 200 to 350 Roentgens total body irradiation 24 to 96 hours prior to inoculation. Another calf was inoculated by cross circulation in which 6 litres of blood were exchanged (Olson, 1969). This quantity of blood was estimated to exceed the total blood volume of the recipient calf. One irradiated calf inoculated with tumor suspension around the superficial lymph nodes developed enlarged lymph nodes and intramuscular tumor masses at the sites of inoculation three weeks later. While many inoculated calves had elevated lymphocyte counts, none developed persistent lymphocytosis.

Olson et al. (1969) also fed 13 pigs with minced leukemic material and 27 other pigs were fed colostrum from infected cows. No test pigs or any of the 24 control pigs exhibited evidence of neoplastic disease when killed six to nine months later.

Schmidt et al. (1970) inoculated eight cows with primary leukocyte cultures and also from the fifth passage. Two cows developed a markedly persistent lymphocytosis. One other cow had a moderately raised lymphocytosis which lasted for one year before the lymphocyte count returned to normal. In a second group, seven cows were injected with culture material from the 10th to the 25th passage; one developed a persistent lymphocytosis. The affected animal received leukocyte cultures from the 15th to 17th passage. This experiment demonstrated that inoculation of primary leukocyte cultures derived from cattle with leukemia could result in development of persistent lymphocytosis

and thereby indicating the presence of an infections agents. The lymphocytosis was considered to be the preclinical stage of bovine leukemia. The results also indicated that the infections agent could be cultivated *in vitro* and that it gradually lost its activity.

In another similar experiment, Graves and Ferrer (1976) Tables 3-6 demonstrated that BLV could infect *in vitro* cells of human, simian, bovine, canine, caprine, ovine and bat origin. Cultures of these cells were cultivated with BLV-infected cells or inoculated with cell-free BLV preparations, and continuously showed the presence of cells with the internal BLV antigen, as well as BLV-induced syncitia. Virus replication was abundant and increased with passage in bat lung cells and was moderate but constant in fetal canine thymus cells. The amount of cells released by simian and caprine cultures was low. The bovine embryonic kidney culture was initially established to be free from foamy-like bovine syncytial virus, parainfluenza 3 (PI_3); infectious bovine rhinotracheitis (IBR), and bovine viral diarrhea (BVD), Tables 3-6.

Burroughs and Cardinet (1973) infected Madin-Darby bovine kidney cells (MDBK) with lymphatic tissue from a leukemic cow and used this as source of virus to infect a calf by different routes; nostrils, eye and mouth. After 106 days, they were able to recover the virus from the lymphatic tissue. One of six calves inoculated developed persistent lymphocytosis for two years.

Theilen *et al.* (1965) conducted transmission studies. Thirteen calves from inoculated cows bred by one bull over two years were maintained for five years for observation. Two of seven calves that nursed their inoculated dams developed persistent lymphocytosis, and five

TABLE 3

Cell cultures used

Cell culture	Origin	Refs.
Uninfected		
A204[a]	Human rhabdomyosarcoma	2, 15
DHS-FRhL-1[a]	Rhesus monkey lung	30, 35
FCf_2Th[a]	Canine thymus (NBRL)	2
S-743[a]	Caprine ovary	
TbILu[a]	Bat lung (ATCC)	2, 30
FLS[b]	Ovine spleen	34
BESP[c]	Bovine embryo spleen	6
WI-38[d]	Human lung	19
PT001 Sp[e]	Chimpanzee spleen (NBRL)	
Infected		
AJ-147[c]	Bovine BC lymphocytes from a cow (AJ-147 with persistent lymphocytosis	6
$BLV\text{-}bat_1$[c,f]	TbILu cells experimentally infected with BLV	
$BLV\text{-}bat_2$[c,g]	TbILu cells experimentally infected with BLV	
BS-2[c]	Bovine fibrosarcoma; cell culture releasing BSV	7
FLS.NI-1228[b]	FLS fells experimentally infected with BLV	34
NBC-13[c]	Thoracic duct lymphocytes from a leukemic cow; continuous lymphoid cell culture infected with BLV	10, 14, 18

[a]Supplied by Dr. M. Lieber (Meloy Laboratories, Springfield, Va.).

[b]Obtained from Dr. J. M. Van Der Maaten (National Animal Disease Center, Ames, Iowa).

[c]Initiated in our laboratory.

[d]Purchased from the American Type Culture Collection (ATCC), Rockville, Md.

[e]Obtained from the Naval Biomedical Research Laboratory (NBRL), Okland, Calif., through the courtesy of Dr. W. A. Nelson Rees.

[f]Initiated by cocultivation of TbILu cells with AJ-147 BC cells.

[g]Initiated by cocultivation of TbILu cells with FLS.NI-1228 cells.

Reference: Graves and Ferrer (1976).

TABLE 4

Propagationof BLV in monolayer cell cultures infected by cocultivation

Host cells (species)	BLV-infected cell inoculum	Assay	Results at following times after inoculation				
			1-4 wk	6-8 wk	10-12 wk	14-18 wk	20-22 wk
TbILu (bat)	FLS NI-1228	RDDP[a]	20,090	31,753	62,567	136,400	162,977
		IFA[b]	21	32	20	25	25
		Syncytia[c]	+++	++	++	NT[d]	++
DBS (simian)	FLS NI-1228	RDDP	20,038	NT	19,366	15,480	33,940
		IFA	45	40	33	30	30
		Syncytia	++	NT	++	+	+
FCf_2Th (canine)	FLS NI-1228	RDDP	29,810	33,115	27,518	31,722	33,940
		IFA	50	35	50	40	50
		Syncytia	NT	+	NT	+	+
FLS (ovine)	FLS NI-1228	RDDP	9,294	11,670	NT	2,436	NT
		IFA	35	20	NT	20	NT
		Syncytia	+	NT	NT	NT	NT

[a]Performed on 20 to 40 ml of supernatant culture fluids. Results are expressed as the cpm of (^{3}H) TMP incorporated into polydeoxythymidylate product during incubation for 60 min at 37°. Values were corrected by subtracting 3 times the cpm of the blank.

[b]The percentage of positive cells in IFA test with reference BLV serum was determined by examining at least 200 cells.

[c]Results are expressed as the approximate percentage of the monolayer sheet shwong syncytia (+, 1 to 10%; + +, 10 to 25%; + + +, 30%).

[d]NT, not tested.

TABLE 5

Transmission of BLV to monolayer cell cultures by inoculation cell-free virus preparations

Host cells[a]	Inoculum	Assay	Results at following times after inoculation			
			4-8 wk	10-12 wk	14-18 wk	20-22 wk
TbILu	CFP from BLV-bat$_1$ (passage 27)	RDDP[b]	400	0	5,970	50,206
		IFA[c]	0	25	30	35
		Syncytia[d]	+	++	+++	++
TbILu	CFF from BLV-bat$_2$ (passage 27)	RDDP	0	0	9,650	57,030
		IFA	1	12	25	35
		Syncytia	+	++	+++	+++
DBS	CFF from BLV-bat$_2$ (passage 41)	RDDP	3,760	3,369		
		IFA	10	2		
		Syncytia	+	+		
PT001Sp	CFF from BLV-bat$_2$ (passage 36)	RDDP	0			
		IFA	12			
		Syncytia	++			

[a] The monolayer cell cultures were pretreated with DEAE-dextran for 30 min prior to inoculation.

[b] Performed on 20 to 40 ml of supernatant culture fluids. Results are expressed as the cpm of (^{3}H)TMP incorporated into polydeoxythymidylate product during incubation for 60 min at 37°. Values were corrected by subtracting 3 times the cpm of the blank.

[c] The percentage of positive cells in IFA test with reference BLV serum was determined by examining at least 200 cells.

[d] Results are expressed as the approximate percentage of the monolayer sheet showing syncytia (+, 1 to 10%; + +, 10 to 25%; + + + = 30%).

Reference: Graves and Ferrer (1976)

TABLE 6

IFA analysis of cell line BLV-BAT_2

Cell culture	Reference sera for					
	BLV	BSV	PI_3	IBR	BVDV	R-29
BLV-bat_2 (passage 22)	+	-	-	-	-	-
NBC-13	+	-	-	-	-	-
BSV-infected BS-2 cells	-	+	-	-	-	-
PI_3-infected cells	-	-	+	-	-	-
IBR-infected cells	-	-	-	+	-	-
BVDV-infected cells	-	-	-	-	+	-
R-29-infected cells	-	-	NT[a]	NT	NT	+

[a] NT, not tested.

Reference: Grages and Ferrer (1976).

calves reared on artificial diet had no lymphocytosis. The two calves with persistent lymphocytosis eventually developed leukemia, Tables 7-13.

Onuma et al. (1977) studied the in utero infection of BLV in lambs. They thymectomized several sheep fetuses and removed their tails at 58 to 65 days of gestation for tissue culture. BLV antigens were detected in serial culture tissue from fetuses whose dams and sires were both BLV-positive.

All these findings support the infectious nature of bovine leukemia. The disease can be transmitted vertically and horizontally and BLV can be cultured in various tissue cultures and maintain infectivity for a considerably long periods.

Role of Genetic Factors and Vertical Transmission

The importance of genetic factors in bovine leukemia was first recognized by European scientists early in this century but was not given the attention until recently. Swedish scientists reported a positive correlation between the occurrence of leukemia in dams and their off-spring (Marshak and Dutcher, 1965). Bendixen (1963) reported that bovine leukemia had a familial pattern in Danish 'leukosis herd'.

Marshak and Dutcher (1965) observed remarkable familial aggregations in many of the multiple case herds in Eastern U.S.A. In another multiple-case herd of purebred Jersey cattle, 22 animals were lost; 17 were either daughters or granddaughters of one sire, Tables 14-17. On the basis of similarity to other tumor diseases in mice and chickens and also on the familial occurrence of leukemia in Danish cattle, Bendixen (1965) studied the possible role of genetic and environmental factors in bovine leukemia. He quoted Hartenstein (1897) as having noticed a herd

TABLE 7

Age, Breed, and Sex distribution of 9 cases of lymphosarcoma in calves

Calf No.	Age (months)	Breed	Sex
30	4	Hereford	M
47	2.5	Guernsey	F
55	6	Guernsey	F
66	5	Guernsey	F
84	6	Jersey	F
99	6	Holstein-Friesian	F
100	4	Holstein-Friesian	F
102	4	Holstein-Friesian	F
103	3.5	Holstein-Friesian	F

Reference: Theilen et al. (1965).

TABLE 8

Hemograms in 7 calves with lymphosarcoma

Calf No.	RBC/ccm x 10^6	PCV (%)	(Hb (GM./ 100 ml.)	WBC/ cmm.	WBC differential* (%)							
					B	N	L	AL	M	E	B	U.C.
47**	2.3	12	3.0	2,000	4	4	72	0	20	0	0	0
66*	8.9	30	8.5	17,5 0	1	13	81	0	3	0	0	2
84*	8.4	32	9.6	44,350	1	3	16	2	1	2	0	0
99*	5.4	23	7.0	6,550	11	26	54	0	7	0	0	2
100*	3.3	16	3.6	10,25	3	5	85	1	6	1	0	0
102*	5.1	25	6.0	5,200	4	27	34	0	28	0	1	0
103*	4.1	17	6.0	26,000	0	5	83	0	1	0	1	0

*WBC = White blood cell; B = band neutrophil; N = neutrophil; L = lymphocyte; AL = atypical lymphocyte; M = monocyte; E = eosinophil; B = basophil; and U.C. = unclassified.

**Massive bone marrow infiltration.

Reference: Theilen et al. (1965).

TABLE 9

Serum proteins in 6 calves with lymphosarcoma

Calf No.	Total protein (Gm./100 ml.)	Albumin (Gm./100 ml.)	Globulin (Gm./100 ml.)			
			Alpha 1 + 2	Beta	Gamma	A/G tatio
66	5.19	4.21	0.44	0.18	0.36	4.3
84	6.80	3.70	0.50	0.50	2.10	1.2
99	5.40	3.12	0.80	0.30	1.18	1.4
100	4.95	3.90	0.65	0.32	0.08	3.7
102	6.05	3.88	1.10	0.70	0.37	1.8
103	6.40	4.16	1.16	0.65	0.43	1.9

Reference: Theilen et al. (1965).

TABLE 10

Serum enzymes in 4 calves with lymphosarcoma

Calf. No.	Glumatic oxalacetic transaminase*	Glutamic pyruvic transaminase*
66	250	..
99	240	42
100	51	18
102	41	..

*Sigma-Frankel units.

Reference: Theilen et al. (1965).

TABLE 11

Serum electrolytes in three calves with lymphosarcoma

Calf No.	Sodium (mEq./L.)	Potassium (mEq./L.)	Magnesium (mEq./L.)	Phosphorus (mEq./L.)	Calcium (mg./ 100 ml.)	Chlorine (mg./ 100 ml.)	Blood urea nitrogen (mg./ 100 ml.)
99	146	4.8	2.6	7.6	9.5	107	30
100	141	5.6	3.3	9.8	8.0	99	28
102	165	7.0	1.4	7.7	9.1	103	17

Reference: Theilen et al. (1965).

TABLE 12

Age at time of inoculation, breed, date, route of inoculation, source of inoculum, and type of inoculation for experimental cattle

Animal No.	Age	Breed*	Date of inoculation:1958	Route of inoculations**	Source of inoculum: donor cow identification	Type of inoculation
1	8 yr.	J	June	e.d.	WMH 1	Lymph node cells
24	4 yr.	H	June	i.v.	WMH 1	Tissue culture cells
259	5 mo.	H	June	e.d. & s.c.	WMH 1	Lymph node cells
266	5 mo.	H	June	i.v.	WMH 1	Trypsinized lymph node cells
276	5 mo.	H	June	i.v. & i.m.	WMH 1	Trypsinized lymph node cells
249	2 yr.	H	Dec.	i.v.	UCV 112	Whole blood
342	3 yr.	J	Dec.	i.v. & s.c.	UCV 112	Trypsinised lymph node cells
343	3 yr.	J	Dec.	i.v. & s.c.	UCV 112	Trypsinized lymph node cells
927	4 yr.	H	Dec.	i.v. & s.c.	UCV 112	Buffy coat cells

*J = Jersey, H = Holsterin-Friesian.

**e.d. = Epidurally; i.v. = intravenously; i.m. = intramuscularly; s.c. = subcutaneously.

Reference: Theilen et al. (1965).

TABLE 13

Length of experiment and ratio of hemograms indicating lymphocytosis for inoculated cows and their progeny

Inoculated cows			Progeny							
							Colostrum-and Nursed			Whole milk-deprived
No.	Length of experiment (months)	L/T*	No.	Sex	length of experiment (months)	L/T*	No.	Sex	length of experiment (months)	L/T*
1	11	0/5	...	.	..	....	....	.	..	....
24	66	11/54	789	M	50	2/35	....	.	..	....
249	74	12/55	590	M	39	31/37	1073	F	37	0/34
259	76	4/56	697	M	53	16/39	1042**	F	40	15/36
266	80	41/64	591	F	53	6/39	831	M	41	1/35
276	77	49/58	678	M	53	3/40	829	F	11	0/11
342	12	2/12	370	M	43	15/27	....	F	..	.....
343	75	2/12	...	.	..	.....	372	F	40	1/29
927	75	52/60	592	F	59	41/46	834	M	46	14/43

*Lymphocytosis/total hemograms, at 1-month or greater intervals.

**1042 was foster-nursed on a cow from a multiple-case herd with persistent lymphocytosis.

Reference: Theilen et al. (1965).

where cases of leukemia were found among related cows. Czymoch (1937) reported that 16 of 20 cases of leukemia were traced to one bull. Fortner (1953) studied the off-spring from two bulls in the same herd and found that one of the bulls had sired 50 calves of which 11 died of leukemia where only one of 120 calves sired by the other bull developed leukemia.

Bendixen (1965) also quoted Bottger as saying leukemia is more than a hereditary disease as it's appearance is also determined by environmental factors.

Wiesner (1961) investigated certain bulls to see if they had favorable or unfavorable influence on the occurrence of leukemia in their off-spring. He concluded that such influence could not be traced, but he made an important finding when he compared the appearance of leukemia in a large number of cow families and found that approximately twice as many daughters of cows with leukemia also developed leukemia than did the daughters of normal cows.

Other investigators, have found that parents free from any evidence of leukemia produced off-spring which became affected. Olson (1961) found by examining conditions in 14 affected herds that a significant correlation existed between daughters and dams regarding the incidence of leukotic tumors. The daughters had a different incidence of leukemia and came from different sires which were normal. Hereditary factors can only partially explain this relationship. Bendixen (1965) considered that predisposition to leukemia may be genetic while the transition from subclinical to clinical stages may be determined by provoking environmental factors.

Jensen (1974), studying the pattern of transmission, removed 10

TABLE 14

Incidence of lymphosarcoma by sire groups
herd BF

Group	Total	Affected
H	9	6
C (son of H	14	7
Ma (son of H)	9	3
T	20	2
Others	63	4
Totals	115	22

P = 0.0016 for daughters of sire H.

P = 0.00002 for daughters and granddaughters of sire H.

Reference: Marshak and Dutcher (1965).

TABLE 15

Incidence of lymphosarcoma by cow families
herd BF

Family	Total	Affected
B	8	4
J	10	3
A_1	21	5
M	9	2
Others	67	8
Totals	115	22

P = 0.0425 for family B.

Reference: Marshak and Dutcher (1965).

TABLE 16

Lymphocytosis status in multiple-case herds-
first sampling

A. None - 10 herds	
B. Partial - 6 herds	
C. Generalized - 6 herds	
D. Incomplete data - 1 herd - appears not to have lymphocytosis	
Total: 23 herds	

Reference: Marshak and Dutcher (1965).

TABLE 17

Lymphocytosis status in multiple-case herds after
Repetitive Sampling

A. Lymphocytosis maintained	3 herds
B. Lymphocytosis lost	4 herds
C. Lymphocytosis increased	1 herd
D. Lymphocytosis increased and then decreased again	3 herds
E. Original normal levels maintained	5 herds
F. Repetitive data for evaluation:	
(1) Incomplete	3 herds
(2) Unavailable or unattainable	4 herds
	23 herds

Reference: Marshak and Dutcher (1965).

calves from infected cows at birth and raised them in isolation without neither colostrum nor milk from their dams. The calves were found to be infected; one died of leukemia at 479 days of age, and three others became hematologically-positive at 194, 207 and 619 days of age, respectively.

Jensen (1974) also studied fetuses from leukemic cows delivered by Cesarean section, denied their dam's colostrum and milk, and raised in isolation. Two calves developed leukemia. In one herd, Guernsey and Holstein cattle had been maintained together for 20 years but during a subsequent three-year period, five Holstein and no Guernsey cows developed leukemia (Jensen, 1974).

A herd of 350 cattle in which annual observations are made is providing valuable information on BLV infection.(Baumgartner et al., 1975). Tests for BLV antibody and cultures for virus indicate infection can be apparent, though rarely, at less than one year of age. An infected cow had a calf which was infected at less than one year of age but her next calf remained negative for two years. Cows up to eight years old have been negative on one or two annual tests, then had both BLV antibodies and BLV on culture. A pair of twins were both negative at five years of age then one became infected and remained so for three successive years while the other was negative. Evidence for recovery from BLV infection has, thus far, not been clearly evident. Usually cows once infected remained so, although there can be variations in antibody titer. These observations supported the earlier concept of transmission from dam-to-progeny as well as by contact between cattle.

The question of infection via semen from infected bulls has received preliminary examination. Two bull studs cooperated in this study involving 33 herds where there were 190 progeny from 11 bulls with BLV infection. Sera were obtained from about 1,000 cattle over one year old including the progeny, and where possible, their dams. Haphazardly selected other cows were also tested to provide an index of BLV infection in the 33 herds. In three herds there were no reactors to the complement fixation test and in the other 30 herds, the reactor rate varied from 2 to 60 percent. There were only 29 (15%) reactors among the 190 progeny from the 11 infected bulls. In the same herds, there were 386 progeny from 21 negative bulls and 73 (19%) of their progeny were reactors. There were 52 progeny from infected dams, 48 percent of which were reactors compared to 7.6 percent reactors in 224 progeny from negative dams. Thus the maternal infection status was far more important than that of the sire. In addition, there were many reactor progeny when both the sire and dam were negative which indicated infection from another source, probably contact (Baumgartner et al., 1975).

While some recorded information suggested genetic susceptibility, the majority supports the hypothesis of vertical transmission of an infectious agent. These studies also demonstrated that bovine leukemia is a virus-induced disease and that the virus:

a) can infect calves in which lymphocytosis and/or a neoplasm develops;

b) induces antibody production in inoculated calves;

c) can be re-isolated from inoculated calves; and

d) can also infect lambs.

This also supports the fact that BLV is leukemogenic. One of the difficulties in studying the oncogenic potential of BLV in cattle is the expense of maintaining cattle for long enough to develop neoplastic a period up to 5 to 8 years. This explains why the incidence of bovine leukemia in USA is lower than in Europe. The average life of cattle in the USA is 5 years or less while in Europe, Africa, Asia and many other parts of the world cattle live an average of 12 years or more (Baumgartner et al., 1975).

MODE OF TRANSMISSION

Studies involving experimental and natural cases strongly suggest that bovine leukemia is an infectious disease that can be transmitted vertically and horizontally. From the nature of leukemia, the virus must be a "moderate" type which produces an integrated course of cellular infection. The cell-virus combination must have a characteristic and behavior different from those of the cell alone with respect to genetic or nongenetic influences. It is conceivable that the virus penetrates into the cell at a time when the cell's ability to produce antibody is still not developed either in fetal period or during a short period after birth. The invasion may occur vertically to the fetus, or via milk factor through raw milk from mother to the neonate, or horizontally from affected to in-contact. From the present data, it is difficult to say whether transmission occurs preferentially in one or another or possibly less equally either way. The spread of leukemia within a herd may occur vertically, but since raw milk is used not only for the cow's own calf but also for other young animals in the herd, horizontal transmission is also possible via milk. Transmission from urine, feces, saliva, etc. from infected cattle to those in contact is a distinct possibility.

Transmission via parents has been emphasised. This is the method of transmission of chicken erythroblastosis or myeloblastosis.

In modern cattle practice, many diagnostic, therapeutic and prophylactic procedures are performed which increase the risk of transferring BLV from infected to susceptible cattle, for example, blood transfusion. Inoculation with piroplasmosis vaccines has been incriminated in active

spread of leukemia in North German provinces and some parts of Sweden. Bodin et al. (1961) stated that the infectious agent was transferred with the vaccine. They found that visceral lymphocytosis had been transmitted when vaccinating against Newcastle disease. Rosenberger (1961) reported that lymphocytosis occurred under controlled conditions in cows that were inoculated intracutaneously with a tuberculin needle which had just been used to inoculate a cow with leukemia. Lymphocytosis was observed in cows from which blood samples had been taken with unsterilized needles and syringes used on leukemic cattle. These procedures present a potential mode of transmission of leukemia both within an infected herd and between herds.

In Eastern Europe bovine leukemia has been associated with tick-borne diseases, as there was strong correlation between the appearance of the two diseases. On this basis, Ixodes ricinus was thought to be responsible for transmitting leukemia. However, this was later found to be incorrect (Bendixen, 1965). Ratcliffe and Stubbs (1935) in investigations to determine whether avian leukosis could be transferred via mosquitoes or mites found that transmission could not be achieved in this manner. The virus remained viable in the mosquito stomach for up to three hours but showed no activity after 24 hours and could not be followed in the life cycle of the mosquito.

To investigate the possibility that BLV might have a reservior in other animals, 2321 sera were tested and found negative for precipitins to BLV antigen. These included 792 pigs. 479 goats, 382 sheep, 7 ponies, 689 wild animals (17 species), and 72 wild birds (5 species). In addition 175 animals (mice, rats, hamsters, rabbits, guinea pigs, chipmunks, grey and ground squirrels, kittens, and fawns) and 23 birds

(chickens, pigeons, colurnix quail, and doves) were inoculated with BLV and after more than one year, none had developed tumors or antibodies against BLV (Baumgartner et al., 1975).

There had been an investigation to determine whether wild deer and roe deer living in the same locality as leukotic herds were susceptible by leukemia. They grazed the same areas as the cows and calves, and they were hosts for the same mites and ticks. No evidence was found that any of the deers developed leukemia. Also the possibility that leukemia can be transferred from roe deers to cows through mites or ticks as intermediate hosts was not substantiated. Thus all available evidence supported the fact that leukemia is an infectious disease caused by a C-type virus that is transmitted both vertically and horizontally.

PATHOGENESIS

Until recently various hypotheses have been advanced to explain the pathogenesis of bovine leukemia. This was due primarily to the fact that the actual etiological agent(s) and factors were merely speculative!

Jensen (1974) suggested that the causative agent possibly transmits the disease from infected dam to susceptible fetus in utero or to the newborn calf via infected colostrum and milk. During a prolonged pre-tumorous period, the agent acts directly on lymphoid tissue and stimulates neoplasia at one or more sites. With primary proliferation in lymphoid tissue, the sinusoids become occluded and the germinal centers are destroyed and replaced with neoplastic lymphocytes and lymphoblasts, and the affected lymph nodes become enlarged. During early stages of leukemia the neoplastic cells are restricted to the nodal parenchyma, but eventually the nodal capsule is invaded and surrounding tissues infiltrated. Commonly the neoplastic cells metastasize to other organs. Myocardium, bone marrow, abomasal wall, kidney, post orbital fat, pelvis and spinal epidural tissues are often affected. Muscles, skin, liver, nerves and other organs are susceptible but less commonly involved. The metastatic growths pressurise and destroy cells and the invaded organ loses some or all of its function and architecture. In the blood, lymphocytosis may be transistory or permanent, mild or severe. In advanced stages, some peripheral lymph nodes may be conspicuously enlarged.

Tumor Formation

It is the cells of the reticulo-histiocyte system (RHS) that are the site of virus-induced neoplastic changes. The cells of the RHS have special functions of phargocytosis and antibody production. These functions explain why leukemia has such a long latent period and so development from subclinical to clinical stage. The varied picture of the disease, in which neoplastic changes can occur in nearly all parts of the body indicate that all cells of the RHS are invaded by virus and that the time and place for triggering neoplastic changes are determined by various eliciting (etiologic) factors. The changes could easily be produced by alterations in the balance between the agent-host cell combination and antibodies. It is quite likely that the agent readily penetrates into the cell at a time when the ability of the host to produce antibody against the virus is still underdeveloped (immunological immaturity).

With the viral agent, the development of leukemai passes through several stages, which last for varying periods of time depending on the individual, before neoplastic changes are detected. The process of disease development can stop at any time at any stage.

After cellular penetration, the virus influences the characteristics and the function of the cell in a way which culminates in the acquisition of autonomy. The development of autonomy does not depend on the virus alone as other factors play a part e.g. genetic, physiological condition of the individual etc.

The presence of the virus in the cell, especially if invasion occurs intrauterinely or just after birth, plays a role during clonal selection which occurs among individual cell clones during the entire

life of the organism but with its greatest effects in the fetus and the young. RHS is one of the tissues in which cell replacement is most active and therefore clonal selection occurs most rapidly. This is of great importance throughout the an animal's life during which it must adjust in order to live in immunological balance with microbes of its environment. Leukemia virus is believed to induce more cell divisions in the RHS system. This means that the virus carrying RHS cells would eventually dominate the RHS cell clones. Eventually large portions of the total RHS contain only cells with a strongly proliferative tendency.

Under the influence of secondary factors (nutrition, homones, genetic, etc.), the proliferative activity of RHS increases and this is manifested by an increased production of lymphocytes or lymphoblasts, hence the observed lymphocytosis usually associated with the disease and which is considered a sign that the disease can progress from latent stage to subclinical stage. This has been considered by some workers as the pre-tumorous stage of leukemia (Bendixen, 1965).

If the proliferative tendency of RHS continues, hyperplasia of new cells leads to observed increase in lymphoid tissues and eventually the aggregates of lymphoid tissue become conspicuously enlarged. This is the tumor stage.

On the other hand, changes in proliferative characteristics of RHS cells to autonomous growth may be the condition that produces tumoral changes. This may be caused by a change in the hereditary characteristics of the cells as a result of somatic mutation. Mutational frequency increases with mitotic activity. The ability of cells with autonomous growth characteristics is much greater than that of

the other RHS cells so that their clones gradually overgrow and replace other slower growing and nonautonomous clones. Hence, autonomously growing clones gradually dominate at first a small part of RHS and finally the whole RHS. This is the manner in which leukemia progresses from a subclinical to tumorous (clinical) stage as result of somatic mutation.

To account for the variable nature of lymphocytosis, it is reasonable to attribute this to immunological tolerance of infected animals so that RHS cells do not exhibit a proliferative reaction to leukemic virus. It is also likely that antibodies are produced against the virus or the virus-carrying cells and the antigen-antibody reaction which follows either forces out virus carrying cells or neutralises or in some way eliminates the virus. If neutralization occurs, a state of balance is produced which could be broken at any time. Burmester (1957) cited chicken leukosis as an example in which antibodies against the leukemia virus play a role in this manner.

Current view on the mechanism of leukemogenesis is based on five main theories:

1) Somatic mutation.

2) Deletion.

3) Virus.

4) Metabolic.

5) Endocrine.

Each theory attempts to explain the neoplastic nature of leukemia and how the precursors of the neoplastic cells become transformed.

Schultz (1958) believed that the change from normal to neoplastic involved a change in cellular hereditary! What is basically altered

is the cellular genotype?

Somatic mutation

This arose from early observation of Boveri (1929) concerning the appearance of chromosomes in malignant tumors. Grossly visible abnormalities such as nondisjunction and multiple mitoses were common and could lead to an asymmetrical distribution of the chromatin material. This resulted is an abnormal and unbalanced cellular metabolism controlled by the damaged chromosomes. Mitotic abnormalities would generally lead to cell death but in a few cases to establishment of cell lines endowed with the capacity for excessive proliferation, the altered cells surviving and transmitting their progeny; tumor thus arises!

Recently developments in cytogenetics have helped to bolster the mutation theory particularly in leukemia where consistent chromosomal abnormality has been observed in mouse and clonal progression, with development of new karyotypes, has been observed to run parallel with acquisition of obviously malignant characteristics. It is quite likely that when gross changes are found in leukemia they do not themselves represent required leukemogenic mutation rather they are submicroscopic changes in the genetic apparatus. Once genetic imbalance occurs it predisposes the surviving affected cells to a sequence of further and progressively more detrimental changes. Also the initial damage makes them more vulnerable to the activity of extrinsic leukemogenic factors - this initial damage or imbalance, however, need not to be mutagenic but may be congenital. There are arguments against the somatic mutation theory. One of these is that leukemia can be induced indirectly.

Kaplan (1954) induced lymphosarcoma in the thymus by irradiating thymectomised mice with X-rays and then transplanted the thymus graft from other unirradiated animals. Tumors arose in the grafted mice and was shown to be derived from the nonirradiated donor cells. More recently, it was shown by Gross (1958) that irradiation could activate latent leukemogenic virus in mice. Therefore the indirect method most probably is virus-mediated. On this basis such provocative factors such as environment, nutrition and hormones in cattle act in similar indirect ways to precipitate leukemia in an infected animal in the latent or subclinical stage (Burch, 1960, 1962). In cattle eventhough a certain homogenization of hereditary characteristics has occurred in attempts towards improving productivity, the degree of inbreeding is far from that achieved in strains of mice with a hereditarily-conditioned high incidence of leukemia. Changes in cattle breeding practices during recent years by artificial insemination has led to a considerable change in the genetic characteristics has caused no changes in the occurrence of leukemia either within the general cattle population or within an infected herd.

The deletion theory postulates that leukemia is caused by loss or deletion of specific enzyme systems concerned with cellular differentiation and normal function with a consequent overactivity of those systems associated with cellular proliferation. Such deletion are thought to be produced by either the virus itself or a combination of carcinogens with cellular proteins and especially nucleoprotein (Miller and Miller, 1952).

What undergoes deletion are components of enzymes systems involved in cell maturation. The actual deletion may be immunologic in origin.

By this theory combination between eliciting agent (carcinogen, etc.) and cellular protein is required. This leads to loss of normal self-markers and thus formation of potentially autoantigenic protein. This, in turn, provokes formation of autoantibodies and the resulting antigen-antibody reaction is the basis for eventual emergence of malignant cell clones, which have lost the faculty of differentiation and are thereby proliferate freely.

The deletion may affect the inhibitors of cell divisions (clones) which must check the proliferation of the stem blast (alpha) cells. Such inhibitors are postulated to be produced by the more mature members of the cell series and thus reduction in number of more mature cells would automatically diminish the quantity of inhibitors available thereby ensuring increased stem cell proliferation.

DIAGNOSIS OF BOVINE LEUKEMIA

Until recently, the causative agent of bovine leukemia was unknown and accurate diagnosis was only achieved by histopathology. For several years investigators tried to establish relationship between leukemia and the C-type virus constantly identified in relation to leukemia. A break through occurred in 1977 when Onuma et al. and Miller et al. published results of their investigation which finally established bovine leukemia virus (C-type virus) as the etiological agent.

For a long time, European scientists, in Denmark (Bendixen, 1965) and Germany (Gotze, 1956) employed hematological examination to determine bovine leukemia. Not only did they rely on this for diagnostic purposes but they also based their eradication programme on persistent lymphocytosis.

Three methods are now used separately or in combination to diagnose bovine leukemia.

1. Hematology
2. Immunological including virological studies
3. Histopathology

Hematological Examination

The demonstration that a lymphocytosis appears in many apparently normal animals in cattle herds in which leukotic tumor cases occur was first made by Knuth and Volkmann (1916) and du'Toit (1920). Later, similar observations were made by others, such as Schottler and Schottler (1934), Thromahlen (1935), and Egehøj (1945). This finding was further elaborated when Gotze and his associated (1953, 1954, 1956)

published studies which provided a diagnostic method for demonstrating leukosis in cattle in the subclinical or clinical stages. This method, the so-called leukosis key, rests on a quantitative estimation of the leukocytes.

Winquist (1958) proposed that the number of lymphocutes be used as the sole basis for determining leukosis, because the number of heterophilic granulocytes varied widely in cattle and introduced an element of uncertainty when the total leukocyte count was used as a basis for determining leukosis. Referring to the work by Moberg (1955), Winquist proposed that animals more than 3 years old with up to 6,000 lymphocytes per mm^3 be considered normal; 6,000 to 12,000 as suspicious and more than 12,000 as highly suspicious leukosis. Animals younger that 3 years old were not included in the evaluation.

Hematological examinations carried out since 1956 by Bendixen in a number of Danish cattle herds in which leukotic tumors were found at regular intervals over several years verified that lymphocytosis is found in a considerable number of apparently normal cows. By performing blood examinations on all cattle at regular intervals, experience was gained regarding the following characteristics lymphocytosis: the type of shifts that occurred in the composition of leukocytes and duration of these shifts; the number of cattle with lymphocytosis in the individual herds; and the relation of lymphocytosis to the leukotic tumor stage. Both quantitative and qualitative changes occur in the leukocytic picture. In order to evaluate the pathological shift in the composition of leukocytes as carefully as possible, the leukocytic picture or composition in normal cattle was

determined.

Classical methods for leukocyte examinations were used in the hemotological examinations, namely: total leukocyte count by placing a dilution of the whole blood in a counting chamber; and differential counts and also morphological examinations of stained smear preparations. The methods were standardized and thoroughly worked out so that they can be undertaken with greatest possible accuracy in routine diagnostic work.

In order to improve evaluation of hematological results, a method of classification into groups was devised, making it possible to consider both quantitative and qualitative changes occurring in the lymphocytes.

Hematological Examination Technique

1. Collection of Blood Samples.

Careful precautions are required to be taken during collection and transportation of blood samples to avoid damaging leukocytes which can result from shaking, changes in temperature, and other conditions of storage. The blood is taken from the jugular vein using as gentle compression of the vessel as possible, preferably without a tourniquet. Stabilization is accomplished with 10 i.u. of heparin in 10 ml blood.

a) Preparation of blood sample vial:

One drop of a sterile physiological saline solution containing 250 international units of heparin per ml is pipetted into the bottom of each vial. The fluid is removed by holding the vial at 37°, and the vial is closed with a tight fitting cork stopper. The vial must be completely dry inside, as moisture will increase the possibility of microbial growth, especially fungal, during storage.

Immediately after the blood has been taken and as soon as the stopper has been fitted into place, the blood sample is inverted five or six times, and the formation of foam is avoided. The samples are transported to a laboratory by car whenever possible, and as quickly as possible. Dilutions and smear preparations are undertaken suitable places at room temperature and in dry air, as these preparations are always completed within the first five- to eight hours after collection of the blood samples. The leukocyte counts are made immediately or at least within 10-12 hours following

dilution.

2. Determination of Leukocyte Count per mm^3 of Whole Blood.

Classical methods were used for this determination. The procedure uses a Bürker-Türk counting chamber and the Ellermann method of dilution, following the instructions given by Christensen and Moustgaard (1944). A known quantity of whole blood is pipetted into a known amount of diluting fluid, causing hemolysis of the erythrocytes and staining of the leukocytes. The number of leukocytes in a small volume of the dilution is counted, and by knowing the dilution the number of leukocytes per mm^3 can be calculated.

a) Apparatus:

Counting chamber (Bürker-Türk), Ellermann diluting pipettes (475 mm^3 and 25 mm^3), small test tubes with rubber stoppers, glass beads, and hand counting apparatus. For larger numbers of samples, an automatic burette (BB glass vial filler, model III), having an accuracy of delivery of approximately $\pm.5$ l, is used to pipette the diluting fluid.

b) Reagent:

Methyl violet-acetic acid solution, 50 mg. methyl violet, 500 mg. glacial acetic acid, and distilled water to 100 ml.

c) Procedure:

Ellermann's dilution method - 475 mm^3 of methyl violet-acetic acid solution are added with a standard pipette or automatic burette into a small test tube. Twenty-five mm^3 of whole blood is blown into the diluting fluid. A glass bead is added to the test tube, the tube is closed with a rubber stopper, and the contents mixed thoroughly for approximately 30 seconds by rotation in a mixing

apparatus. An aliquot is transferred to a Bürker-Türk counting chamber.

d) Counting:

A binocular microscope having a magnification of about 75X is used for counting. Total counts are made of five 1-mm^2 fields, and the number of leukocytes calculated using the following equation:

$$\text{Number of leukocytes per } mm^3 = \frac{(x)\ (10)\ (20)}{5}$$

where x is the number of leukocytes counted in 5 fields. The height of the counting chamber is 0.10 mm, each field is 1 mm^2 and the dilution is 25 + 475 mm^3, or 1:20.

3. Determination of Leukocyte Composition in the Smear.

In addition to the total leukocyte count, a differential count was also made for each sample. The differential count was made to determine the percentage of lymphocytes and lymphocyte-like cells and also to determine whether special pathological cells, so-called leukotic cells, could be demonstrated. Smears are made as quickly as possible after a sample is taken, while the staining and the differential count could be postponed until later. The method used was the rapid May-Grünwald-Giemsa method, as described by Christensen and Moustgaard (1944).

a) Preparation of the Smear:

The slide method was used. It is better to use a relatively thick cover slip for studying the morphology of the lymphocytes. To avoid damaging the cells it is necessary to be extremely careful

in making the smears, and they should be made in a room with dry air in order that the drying will occur relatively rapidly.

b) Staining:

To obtain uniform color shades it is necessary that the pH value of the water used in staining and for rinsing be constant. This constant pH is obtained by using a phosphate buffer solution adjusted to pH 6.4.

c) Reagents:

May-Grünwald's stain is prepared by dissolving 250 mg. methylene-blue in acetone-free methyl alcohol made up to 100 ml. Giemsa's stain is made by dissolving 0.5 g of a mixture of equal parts of methylene-azur, eosin, and methylene-blue in 33 g glycerin and adding acetone-free methyl alcohol. Phosphate buffer solution pH 6.4 is made from a stock solution consisting of $Na_2HPO_4 12\ H_2O$ 6.16 g, KH_2PO_4 6.63 g, and distilled water in a volume of 1,000 ml. The stock buffer solution is diluted with distilled water 1:20 before use.

d) Procedure:

The staining is done directly on the slide as follows:

1. May-Grünwald's solution 18 drops, time 1 minute, no pouring off.
2. Giemsa's solution 6 drops, time 1 minute, no pouring off but careful mixing.
3. Distilled water 18 drops, time 1 minute, no pouring off but careful mixing.
4. Rinse in running distilled water.
5. Let stand in beaker with distilled water, time 1 minute.
6. Blot with filter paper and dry in the air.

e) Differential count:

At least 200 leukocytes are counted per smear, and they are divided by morphology into lymphocytes, monocytes, and heterophilic and basophilic granulocytes. At the same time any abnormal appearing cells, especially the so-called leukotic cells are noted (Bendixen, 1965).

4. Calculation of the Number of Lymphocytes per mm^3 of Whole Blood.

This value was calculated on the basis of leukocyte count and the percentage of lymphocytes determined by differential counts. In this regard all mononuclear cell forms except monocytes were included in the lymphocyte fraction. Under normal conditions, this means that lymphocytes, prolymphocytes, and lymphoblasts. Prolymphocytes and lymphoblasts are primitive types of cells frequently seen in the circulating blood of normal cattle. In many cases abnormal appearing mononuclear forms of leukocytes occur in the blood stream in leukotic conditions.

Sources of Error

The accuracy of the leukocyte determination depends on many factors, such as the technique of blood sampling, procedure of preparation, transport, storage, and also the precision of the cell counting method used. The working conditions for a study which must be accomplished under the practical conditions of veterinary practice set narrow limits on how sensitive the counting technique may be.

Proper treatment of the blood samples during and after their collection has great importance. Leukocytes are very easily damaged, destroyed, or agglutinated as a result of mechanical factors or

changes in temperature during transport and storage. Fröba (1958) stated that the cell types are destroyed in the following order: heterophilic granulocytes, lymphocytes, acidophilic granulocytes, and basophilic granulocytes. Improper treatment can easily result in a leukocyte count that is too low and a distribution of cell types which is inaccurate. Cells in early stages of disintegration are difficult to identify (Bendixen, 1965).

Therefore, different methods of preparation, of transport, and storage were tested in order to find those which were best suited for diagnostic work.

Samples were taken from 11 animals, and two blood samples from each animal were stabilized with heparin and two with sodium citrate. One heparinized and one citrated blood sample were sent to the laboratory by rail. Dilution and counting were performed the following day. The other samples were transported directly to the laboratory by car. Dilutions were performed the same day, counting the next day. Ten cell counts were performed on each sample.

The variance analysis shows that the determinations of leukocyte counts as well as the lymphocyte percentages and the lymphocyte counts differed for individual animals ($P < 0.01$). The average value for the total leukocyte count of samples transported by car (7,468) is significantly larger than the average value (6,858) for the samples transported by rail ($P < 0.01$). The average value for the total leukocyte counts of blood samples stabilized with heparin (7,132) was not significantly different from the value obtained for samples treated with sodium citrate (7,195).

The variance analysis shows that the relationship of the method of stabilization to the method of transport was insignificant. However, the value found for the variance quotient was relatively high ($0.10 > P > 0.05$).

	Method of stabilization	
Method of transport:	Heparin	Sodium Citrate
Car.....	7,333	7,604
Rail.....	6,931	6,785

There was a significant relationship between the white cell picture in individual cows and the method of stabilization used. This relationship possibly results from individual differences existing in the concentration of the different cell types among the leukocytes. Some cattle examined had a relatively high concentration of heterophilic granulocytes. Since these granulocytes autolyze more rapidly than other cell types, the total leukocyte number falls faster in cattle with heterophilia. A significant relationship exists between the leukocyte counts in individual cows and both the stabilizing method and the method of transportation. This relationship was presumably related to the concentration of granulocytes, as indicated by the fact that a similar relationship involving the percentages of lymphocytes was not found. Because the number of lymphocytes is calculated on the basis of the total leukocyte count and the percentage of lymphocytes a significant relationship occurs here also.

Concerning the percentage of lymphocytes, the average value was 54.9 percent for blood stabilized with heparin, which was significantly higher than the average value 51.7 percent for citrated blood ($P < 0.05$).

Conversely, the average value 53.6 percent for samples transported by car was not significantly different from the average value 53 percent for samples transported by rail. There was a significant relationship between the method of stabilization and the method of transport.

	Method of stabilization	
Method of transport:	Heparin (Percent)	Sodium Citrate (Percent)
Car.....	53.4	53.9
Rail.....	56.5	49.6

Heparin was advantageous over sodium citrate for blood samples transported by rail.

For lymphocyte counts the average value (4,046) for blood samples transported by car was significantly higher than the average value (3,645) for the corresponding samples transported by rail ($P < 0.01$). The average value (3,925) for samples stabilized with heparin was not significantly different from that for samples stabilized with sodium citrate (3,766), but still the variance quotient was relatively high. In this case, a significant relationship also existed ($P < 0.01$) between the method of stabilization and the method of transportation, as may be seen from the following:

	Method of stabilization	
Method of transport:	Heparin	Sodium Citrate
Car.....	3,955	4,137
Rail.....	3,895	3,395

A significant reduction in the lymphocyte counts occurred when the citrated blood was sent by rail.

In addition to cell decomposition, cell agglutination was also an important cause of error. This source can be reduced by avoiding storage of the blood samples or by reducing the storage periods as much as possible. The tendency to agglutinate was greater in samples exposed to prolonged period of transportation and storage when sodium citrate was used for stabilization than heparin.

Heparin-stabilized and sodium citrate-stabilized samples were taken from the same animal. One sample of each type was counted immediately after collection, another sample was counted after storage at 4° for 24 hours, and a third sample after storage at 20° for 24 hours. Ten cell counts were performed on each sample. The t-tests showed no significant differences between results obtained for leukocyte counts of heparinized blood which had not been stored and that stored at 4° and 20° for 24 hours.

On the other hand, it was shown that when sodium citrate was used significant differences resulted in the counts of unstored blood and blood stored at 4° for 24 hours. The differences were observed for total leukocytes and for lymphocytes. Storage at 20° for 24 hours resulted in a moderate but non-significant reduction of the cell count. For the total leukocyte and total lymphocyte counts results with stored blood were below these obtained for unstored blood (Bendixen, 1967).

No significant differences in the lymphocyte percentages were found between stored and unstored blood.

It can be concluded that when the chief object is to preserve as many leukocytes as possible for up to 24 hours storage, heparinization is preferable to sodium citrate. When heparin was used to stabilize

the sample 4° or 20°, storage had no damaging effects. This finding is important for blood samples collected under practical field conditions.

Leukocyte counts were made for a number of cows having varying numbers of white blood cells. The counts were performed to determine the spread of values as it would occur in routine diagnostic work. The blood sample from each cow was counted 10 times, and the spread (s) calculated for each sample. The size of s showed that numerically it was dependent on the size of the average value. This value as a rule fell between ca. 500 and ca. 900 for a total leukocyte count under 10,000, with a percent deviation of ±8-15. In a few cases the deviation increased to 20. The s value was somewhat smaller for the lymphocyte percentages. Numerically, as a rule, the s value ranges from 2-4, giving a percent deviation of seldom more than ±2-10. Thus, the spread in numerical value of experimental results for lymphocyte counts can almost always be kept to s = 300-900, at which the percent deviation becomes ±10-15.

A certain inaccuracy must be taken into consideration for determinating lymphocytes. The accuracy can be considerably increased by a more extensive counting procedure for each single sample and by reducing the transport distance and the period of storage, etc. An increase in the accuracy is possible only to a limited extent under practical conditions in which a very large number of samples have to be examined (Bendixen, 1967).

Finally, the agreement between the first and the second leukocyte determination in two tests carried out on the same animal was investigated. From this investigation some idea of the dependability of

hematological diagnosis for the individual cows was gained. In this examination blood samples were taken from 100 animals. Statistical analysis showed that the mean differences between the first and the second samples from these cows were 200 leukocytes per mm^3 for the total white cell count, 0.5 percent lymphocytes for the differential count, and 143 lymphocytes per mm^3 for the total lymphocyte count. A t-test showed that these differences were not significantly different from 0; or, in order words, the working assumption that no difference should exist between duplicate samples collected from the same animal cannot be denied.

The spread in the average differences between the first and the second samples was large. The spread in the total leukocytes was 1,907, in lymphocyte percentage 7.2, and in lymphocyte number 1,402. These differences could not be considered as resulting from the previously discussed influences of preparation, transportation, storage conditions, or the accuracy of the counting technique. The differences must have resulted from variations in the leukocyte concentrations in the blood of the cows which occured during the period between the two samplings. For example, the changes may have occurred as a result of handling the animal which was necessary for drawing the blood sample.

In developing methods for making divisions into groups, these uncertain factors had to be taken into consideration. The order of magnitude of the spread also was taken into consideration when deciding on the limits between group I, group II, and group III (Table 18 and 19). Setting up a group for doubtful results (group II) which required repeating the examination before a final decision was

made was of special importance. Finally, it must be remembered that morphological evaluation of lymphocytes also was taken into consideration when the division into groups was decided.

The value of using such groups as a basis for evaluating results is shown in Table 20 where a comparison between the first and second samples is made. These results showed that complete agreement existed between the determinations carried out in duplicate in 86 percent of the cases. The other 14 percent were displaced into the nearest neighboring group.

Using the evaluations described in the previous section, the importance which the following factors have for securing reliable results in routine diagnostic work was shown: preparation, conditions of transport, period of storage, and accuracy of the counting technique. The methodology described represents an attempt to achieve optimal conditions for preserving leukocytes in the blood samples. In routine work where samples are collected from all over the country, it is especially important to make an effort to see that the period of time mentioned for the duration of examination are closely observed. To avoid damage during transportation it is advisable for samples to be taken directly to the laboratory by car.

Determination of the White Blood Cell Picture for Normal Cattle

The normal values for leukocytes in normal cattle are given in Table 18. The values reported by Coffin (1953), Holman (1956), Schalm (1961), and Wirth (1950), were taken from a monograph by Bendixen (1965). Original observations were published by Greatorex (1954), Winquist (1954), and Straub (1956) for young animals, by Moberg (1955)

TABLE 18

Statistical calculation of normal leukocyte values in cattle of different age

Age in years	Leukocytes per mm^3			Lymphocytes percent			Lymphocytes per mm^3		
	Number of samples	Average	2.s	Number of samples	Average	2.s	Number of samples	Average	2.s
0-1	31	9,200	±4,100	31	78.8	±15.4	31	7,300	±3,500
1-2	35	9,100	±4,300	36	70.0	±19.6	35	6,300	±3,300
2-3	36	8,600	±4,100	39	59.6	±16.3	36	5,000	±2,500
3-4	20	8,200	±3,800	20	55.8	±16.3	20	4,600	±2,400
4	49	7,500	±2,700	51	51.6	±18.0	49	3,800	±1,600
	171			177			171		

Reference: Bendixen (1967).

TABLE 19

Groups in which animals are divided according to age and the number of lymphocytes per mm^3

Age in years	Normal	Dubious	Leukemic
0-1.....	10,000	10,000 - 12,000	12,000
1-2.....	9,000	9,000 - 11,000	11,000
2-3.....	7,500	7,500 - 9,500	9,500
3-4.....	6,500	6,500 - 8,500	8,500
4.....	5,000	5,000 - 7,000	7,000

Reference: Bendixen (1967).

TABLE 20

Comparison of the first and the second blood-examination, when 100 animals were evaluated according to the grouping procedure

	2nd examination			Total
	I	II	III	
1st examination:				
I.....	78	11	0	89
II.....	3	6	0	9
III.....	0	0	2	2
Total.....	81	17	2	100

for heifers and cows (Moberg, 1955), and for cows Ferguson (1945). Gotze et al. (1954) reported (Table 21) normal values for adults as well as young animals with the development of the "leukosis key", but further information about normal values was not given. Lack of information about obtaining the values may also be made of some of the veterinary manuals. Each individual investigation was limited regarding the number and age of the animals examined. This drawback makes it difficult to use the results as a basis for evaluating compiled examinations of herds to determine existing conditions. In addition, these studies did give significant differences in the normal values. The fact cannot be overlooked that breed and environment can determine differences from country to country.

1. Quantitative Evaluation of Lymphocytes and Lymphocyte-Like Cells in Normal Cattle

A relatively large herd in which cases of leukosis had never been detected was selected for this study. All the cattle were included in the examination, so that all classes in terms of age and physiological condition (lactation and pregnancy) were represented. The general health was satisfactory. The normal values were obtained from a total of 171 cattle. Age had a considerable influence on the lymphocyte count. The greatest limiting value for lymphocytes in young animals under one year of age was around 10,000 per mm^3, but for adult cattle more than four years old, the value was about 4,000-5,000. This result is obtained both because the average values were higher for young animals and because the spread was larger for young animals than for adults (Bendixen, 1967).

TABLE 21

Survey of the normal values of cattle, reported by different authors

Author	Number of animals	Age	Calculated average number of lymphocytes	Number of leukocytes	Ly. Percent	Mo. Percent	Neu. Percent	Acid. Percent	Bas. percent
Coffin (1953)	...	Adults	8,750	4,500-13,00	40-70 (52)	3-15 (9)	15-55 (30)	1-15 (8)	0-1 (0.5) ....
Holman (1956)	...	-do-	5,740	7,000- 2,000	52.0-11.8	7.0-2.7	33.0-9.8	11.0-11.9	0-2
Schalm (1961)	...	-do-	9,000	4,000-12,000 7-9,500	45-75 (58)	2-7 (4)	15-45 (28)	2-20 (9)	(0.5) 0-0.5
Wirth (1950)	...	-do-	6,300	5,000-10,000 (8,000)	37-63 (53)	3-10 (5)	25-50 (35.9)	3-8 (6)	(0.1) ....
		Young animals	.....	12,000-15,000 (10,000)	.....	.....	.....	.....	0.5
Gotze et al.(1954)	...	Adults	6,000	5,000-10,000 (8,000)	40-60 (53)	3-10 (5.5)	25-50 (34)	3-8 (7)	0.5
	...	Young animals	9,750	10,000-15,000	45-65 (55)	3-12 (7.5)	25-50 (32)	0-8 (5)	....
Gotze et al.(1954) (leukosenogle)	...	Adults	6,000	10,000	60	.....	.....	.....	....
	...	Young animals	7,800	12,000	65	.....	.....	.....	0.2
Moberg (1955)	48	Heifers not pregnant	7,770	11,116-406	69.9	1.7	22.1	6.1	0.1
	51	Heifers pregnant	6,200	9,286-330	66.7	1.9	21.5	9.7	0.3
	33	Cows 1st calf	5,060	8,225-331	61.5	1.5	25.0	10.7	0.2
	35	Cows 2d-3d calf	4,800	7,795-407	61.6	1.5	24.4	11.6	0.1

Contd..

Table 21 continued.

	36	Cows 4th calf or more	4,250	7,106-322	59.8	1.7	25.9	13.4	....
Greatorex (1954)	233	Calves at time of birth	4,400	8,700	50.8	2.8	45.0	1.1	....
	233	1-week-old calves	.....	.....	62.5	3.7	32.0	1.5	....
	233	2-4-month-old calves	6,700	9,700	68.6	3.4	27.0	1.9	....
	233	4-6-month-old calves	6,300	9,300	68.2	5.7	21.7	4.9	....
	233	12-month-old calves	.....	.....	67.0	2.2	27.7	3.0	0.3
Winquist (1954)	7	1-week-old calves	4,700	8,800	53.7	3.8	42.0	0.2	0.1
	19	1-9-week-old calves	6,100	8,500	71.9	2.5	24.8	0.7	0.1
Straub (1956)	20	Calves 1st day	5,830	12,540	46.5	3.4	49.6	0.4	0
	26	Calves 2d-7th day	4,870	10,952	44.5	3.6	47.7	0.7	0.1
	48	Calves 2d-4th week	6,080	10,992	55.3	6.9	35.7	0.9	0.2
	72	Calves 2d-3d month	6,940	11,030	62.9	5.7	28.4	1.6	0.4
	29	Calves 4th-7th month	6,4000	11,693	54.7	9.9	32.4	1.4	
	14	Calves 11th-15 month adults.	7,990	12,392	64.5	4.0	25.9	5.0	0.4
Ferguson et al. (1945)	25		3,670	8,912	41.2	7.9	34.7	14.9	0.6

Reference: Bendixen (1965).

A division into groups (Table 22) was introduced for evaluating routine diagnostic work, thus making the survey easier. The purpose of the division into groups was to select those cattle with lymphocytosis. For quantitative data, the estimation was made by determining the lymphocyte count and a morphological evaluation of lymphocytes in a smear preparation was made at the same time

Group I. Cattle with normal blood cell picture.

II. Cattle with moderately increased lymphocyte counts.

III. Cattle with lymphocytosis.

Boundaries between the groups were determined from quantitative results in normal animals. The upper limits for the individual age classes fell in group II with a margin of approximately 1,000 on either side. By this method, some normal blood pictures fell into group II, but all cattle completely normal in a quantitative evaluation were placed in group I. The more doubtful cases were reexamined if a decision needed to be made. All cattle in normal herds in which the blood examination was repeated after they had fallen into group II on the first examination, fell into group I on the second examination (Table 22). The reason for such a moderate and temporary increase in lymphocytes must be assigned to particular conditions in the animal and possibly to special conditions in its physiology.

Lymphocytosis and Leukemia

The association of lymphocytosis with bovine leukemia originated from Eastern Europe. Danish and German officials considered persistent lymphocytosis diagnostic and based their eradication and control program on this finding.

TABLE 22

Comparison of the first and the second blood-examination when 100 animals were evaluated according to the grouping procedure

	2nd examination			Total
	I	II	III	
1st examination:				
I.....	78	11	0	89
II.....	3	6	0	9
III.....	0	0	2	2
Total.....	81	17	2	100

Reference: Bendixen (1965).

TABLE 23

Statistical calculation of normal leukocyte values in cattle of different age

Age in years	Leukocytes per mma^3			Lymphocytes percent			Lymphocytes per mm^3		
	Number of samples	Average	2.s	Number of samples	Average	2.s	Number of samples	Average	2.s
0-1	31	9,200	±4,100	31	78.8	±15.4	31	7,300	±3,500
1-2	35	9,100	±4,300	36	70.0	±19.6	35	6,300	±3,300
2-3	36	8,600	±4,100	39	59.6	±16.3	36	5,000	±2,500
3-4	20	8,200	±3,800	20	55.8	±16.3	20	4,600	±2,400
4	49	7,500	±2,700	51	51.6	±18.0	49	3,800	±1,600
	171			177			171		

Reference: Bendixen (1967).

TABLE 24

Groups in which animals are divided according to age and the number of lymphocytes per mm^3

Age in years	Normal	Dubious	Leukemia
0-1.....	10,000	10,000 - 12,000	12,000
1-2.....	9,000	9,000 - 11,000	11,000
2-3.....	7,500	7,500 - 9,500	9,500
3-4.....	6,500	6,500 - 8,500	8,500
4.....	5,000	5,000 - 7,000	7,000

Reference: Bendixen (1967).

On the other hand researchers in North America were skeptical especially about the East European idea of eradicating bovine leukemia based on hematological findings alone.

According to Marshak et al. (1970) several observations suggested that lymphocytosis was not a reliable indicator of bovine leukemia:

(1) the frequently documented occurrence of familial aggregations of keukosis within affected herds;

(2) the inconstant presence of persistent lymphocytosis among cattle comprising multiple-case herds in the Eastern United States;

(3) the presence of numerous cattle with persistent lymphocytosis in herds which failed to yield any known cases of leukosis, and

(4) the observation that 36 percent of the leukosis cases presented to the University of Pennsylvania Veterinary Hospital failed to exhibit lymphocytosis at the time of admission.

Tables 26-30 show some of the results of longitudinal studies as they relate to persistent lymphocytosis, development of clinical leukosis, and familial aggregation of cattle with persistent lymphocytosis within several study herd categories.

TABLE 25

Survey of the results of hematologic examination of animals in different herds: leukosis-herds, observation-herds, herds with one case of juvenile leukosis and skin-leukosis, and leukosis-free herds

Type and number of herds	Number of animals in the herds			Number of examined animals			Group I		Group II		Group III	
	Adult	Young	Total	Adult	Young	Total	Adult	Young	Adult	Young	Adult	Young
Leukosis-herds:												
85 with 1 tumor case.....	1,537	1,817	3,354	1,446	463	1,909	924	365	128	37	394	61
127 with 2 or more tumor cases.....	6,399	7,045	13,444	4,950	1,101	6,051	2,669	922	570	81	1,711	98
8 without tumor case.....	162	214	376	162	48	210	114	40	15	5	33	3
Observation-herds:												
160 with 1 tumor case.....	2,344	3,066	5,410	2,227	610	2,837	2,055	590	130	12	42	8
5 with more than 1 tumor case.....	76	99	175	46	22	68	38	20	6	1	2	1
17 without tumor cases.....	305	285	590	294	131	425	273	124	16	4	5	3
104 with 1 juvenile tumor case.....	1,624	2,098	3,722	1,562	521	2,083	1,514	514	45	6	3	1
32 with 1 skin-tumor case.....	345	447	792	324	129	453	307	123	16	6	1	..
188 leukosis-free herds.....	...	...	...	3,405	1,331	4,736	3,364	1,296	137	29	4	6

Reference: Bendixen (1967).

TABLE 26

Persistent lymphocytosis (PL) and leukosis case data for 25 leukosis herds in the Eastern United States

Herd code	Number months studied	Number cattle studied	Number with PL	% with PL	Total cases of leukosis	Number of new cases	Number new cases with PL	Number of months[1] since last leukosis case
AB	80	689	17	2.5	13	4	1	7
AE	92	352	22	6.2	9	6	2	1
AH	123	202	38	18.8	7	2	1	89
AI	96	161	13	8.1	9	1	0	55
BA	93	142	37	26.1	14	7	7	6
BB	115	77	26	33.8	3	3	2	75
BC	90	165	1	0.6	4	0	0	75
BD	67	200	14	7.0	3	2	1	34
BE	88	133	5	3.8	5	0	0	88
BF	87	262	77	29.4	35	23	19	0
CD	82	145	0	-	2	1	0	56
CG	31	111	5	4.5	4	0	0	46
DC	16	66	20	33.3	3	0	0	6
DE	67	88	7	8.0	6	2	2	22
DG	77	390	6	1.5	2	0	0	87
DJ	77	359	6	1.7	2	2	1	27
EC	77	644	27	4.2	1	1	1	23
EG	65	81	2	2.5	3	0	0	66
EJ	26	103	5	4.9	1	1	1	8
FD	54	254	1	0.4	1	0	0	56
GA	33	182	9	4.9	3	3	1	7
GC	33	98	5	5.1	4[2]	0	0	35
GD	18	88	10	11.4	7[2]	4	2	12
GF	26	121	3	2.5	3[2]	0	0	30
GG	12	260	20	7.7	3[2]	2	1	3
Totals		5,373	376	7.0	147	64	42(65.6%)	

[1]Herds CG, DC, DE and EJ have been dispersed preventing any furhter observations. The intervals listed for these herds represent the time between the last leukosis case and the time of dispersal.

[2]These herds are believed to have had numerous additional cases which cannot be confirmed retrospectively. The totals given in the table reflect the minimal number of cases in these herds.

Reference: Abt et al. (1970).

TABLE 27

Persistent lymphocytosis (PL) data for 24 herds without leukosis in the Eastern United States

Herd code	Number months studied	Number cattle studied	Number with PL	Percent with PL	Classification of heard during the period of observation
AA	20	42	4	9.5	Control
AC	34	124	0	-	Control
AD	82	81	0	-	Control to contact
BG	86	446	8	1.8	Control
BH	86	82	0	-	Control
BI	86	291	0	-	Control
BJ	86	478	85	17.8	Contact
CA	85	152	11	7.2	Control
CB	84	125	1	0.8	Control to contact
CC	84	183	11	6.0	Control to contact
CE	82	97	19	19.6	Contact
CI	82	148	1	0.7	Control to contact
DH	77	409	2	.0.5	Contact
DI	77	288	0	-	Contact
EA	76	152	3	2.0	Contact
EB	22	113	2	1.8	Contact
EE	12	44	2	4.5	Control
EH	65	174	0	-	Contact
EI	4	87	9	10.3	Probable contact
FA	4	136	15	11.0	Probable contact
FE	54	114	0	-	Contact
FF	54	95	1	1.1	Contact
FJ	36	72	0	-	Control
GB	34	212	0	-	Control
Totals		4,145	174	4.2	

Reference: Abt et al. (1970).

TABLE 28

Association of familial aggregations and absolute lymphocyte count levels in sire group

Sire code	Herd code	Number of offspring	Type of trend	Computed chi square	Probability
0752	BB	8	Lymphocytosis	150.000	0.001
	BH	6	Normal	10.349	0.05
	BI	10	Normal	13.621	0.10
0454	AB	7	Lymphopenia	17.318	0.02
	CG	5	Normal	8.833	0.05
	DH	6	Normal	5.229	0.30
	DJ	4	Normal	2.622	0.30
	EC	3	Normal	3.315	0.10
0465	AB	18	Lymphocytosis	40.533	0.01
	CG	4	Normal	7.397	0.05
	DG	13	Normal	9.118	0.70
	EA	8	Normal	0.080	0.80
	EC	18	Lymphocytosis	50.399	0.001
1529	GA	20	Lymphocytosis	50,399	0.001
0014	AH	9	Lymphocytosis	55,200	0.001
	BA	8	Normal	9.330	0.20
	BJ	3	Lymphocytosis	22.441	0.001
	CI	23	Normal	13.510	0.90

Reference: Abt et al. (1970).

TABLE 29

Changes in the absolute lymphocyte configurations in herd BF (June 1962 to June 1965)

Date	Under 60 months			Over 60 months			Number of cases	
	N[1]	b[2]	F[3]	N[1]	$\bar{Y}$[4]	F[3]	New Cases	Cumulat. Cast Total
June 1962	19	+0.0139	18.43	13*	2.29	22.52	1	12
Nov. 1962	61	+0.0127	80.38	11	1.86	6.84	2	14
Apr. 1963	54	+0.0131	66.55	14	2.06	8.79	1	15
July 1963	57	+0.0071	45.07	15	2.08	8.51	-	15
Sept.1963	60	+0.0074	57.05	15	2.07	6.94	3	18
Dec. 1963	64	+0.0056	47.00	16	2.18	6.46	-	18
Mar. 1964	68	+0.0017	28.42	18	2.10	5.95	-	18
June 1964	57	-0.0010	12.21	19	2.19	8.02	1	19
Sept.1964	80	+0.0010	26.50	18	2.18	8.63	-	19
Dec. 1964	81	-0.0025	15.72	22	2.15	10.06	-	19
Mar. 1965	89	-0.0038	11.14	22	1.99	6.95	1	20
June 1965	92	-0.0046	7.50	23	2.01	8.23	2	22
			D.F.=1 and			D.F.= N-1/69		

[1]Denotes the number of animals sampled.

[2]Denotes the regression coefficient from least squares regression analysis.

[3]Denotes the variance ratio value. $P<0.001$ for all F values from June 1962 to March 1965 in the under 60 months of age group. $P>0.01$ for all F values in the over 60 months of age group and for the June 1965 value.

[4]Denotes the mean value of the logarithmic representations of the absolute lymphocyte counts.

*This sampling includes animal BFO 13 who was in the milking line and appeared healthy to the owner. He log value was 5.1 (329,000/mm^3 abs. lymphocytes count) and she died 6 months later. The values obtained if she is excluded are: N = 12, $\bar{Y}$ = 2.06, F = 9.46.

The herd was maintained as a commercial dairy at a level of approximately 70 total animals until March 1964, at which time it was purchased by the University of Pennsylvania and moved from Virginia to New Bolton Center, Kennett Square, Pa. Since then, herd size has approximately doubled due to the retention of a high percentage of new calves.

Eleven cases of lymphosarcoma have developed since June 1962, doubling the number of cases recorded between 1956 and June 1962. Since each time depicted represents a 3 month span, it is evident that the last 11 lymphosarcoma deaths have tended to occur in time-related aggregations. A marked aggregation (4 cases) occurred in May 1962).

Reference: Marshak et al. (1970).

TABLE 30

Investigations on the influence of certain chronic diseases and diagnostic inoculations on the lymphocyte fraction.

	Number of animals	Group		
		I	II	III
A. Tb. testing.....	122	114	7	1
B. Avian tb pos.....	207	201	6	0
C. Para-tb pos.....	64	62	2	0
D. F & M-vaccination:				
1 month before.....	101	100	1	0
1 month after	97	97	0	0

A. Blood sampling and reading of skin reaction both made 72 hours after injections (i.e.) of bovine and avian tuberculin.

B. Blood cell picture evaluated for animals showing positive reaction for avian tuberculosis.

C. Blood cell picture evaluated for animals showing positive reaction for paratuberculosis in the complement fixation test.

D. Blood samples taken from herd before and after foot-and-mouth disease vaccination.

Reference: Hugson (1970).

SEROLOGICAL AND VIROLOGICAL DIAGNOSIS

Introduction

Several laboratory attempts have been made to determine a specific serological diagnostic method. Crunder (1956) tested a leuko-agglutination technique be dependable results were not achieved. Hug (1957) was unable to develop a reliable complement fixation test . Biochemical tests for demonstrating new components or alterations in the normally occurring components of serum or plasma, and which would be characteristic for leukemia have been investigated. Hartung (1954) and Greve (1957) found that no typical changes in the serum protein fractions during bovine leukemia could be demonstrated, and Aberg (1957) found a small reduction in the protein-bound carbohydrates but it was not considered to be characteristic for bovine leukemia.

The agar gel test has been found useful for differential diagnosis. There is an inverse proportionality between the amount of gamma globulin in the blood serum and the time period required for gelation (Bendixen, 1965), which is the reason why many bacterial infections give a positive agar gel reaction. Such alterations in the serum protein are not observed in bovine leukemia. Because many infectious diseases cause enlargement of lymph nodes the possibility of leukemia frequently arises and the agar gel diffusion reaction provides a simple and rapid differential diagnostic method.

The situation has now changed. Many refined serological and virological methods are available for diagnosing leukemia or at least diagnosing bovine leukemia virus infection. BLV has been finally established as the primary causative agent of bovine leukemia

(Onuma et al., 1976; Van Der Maaten et al., 1977). Therefore, all the serological and virological techniques for identifying the virus, its antigens or antibodies are considered diagnostic procedures for leukemia. There are three main serological techniques:

1. Immunodiffusion (ID)
2. Immunofluorescent (IF)
3. Complement Fixation (CF)

Each method demonstrates certain antigen-antibody reactions and selection depends on what is desired. Sera from 175 cattle were sent to three different laboratories (New Bolton Center, School of Veterinary Medicine, University of Pennsylvania; Department of Veterinary Science, University of Wisconsin; and National Animal Disease Laboratory, Ames, Iowa) by a fourth agency for testing by the above three methods. Each laboratory was proficient in one of the respective methods and received the sera as unknowns. No antibodies to bovine leukemia virus were found in 146 sera by the three methods. Of 29 sera with antibodies, 16 were positive by all the three tests, five were positive by CF only, one by IF only, one by ID only, five by both CF and IF, and one by CF and ID. There was good agreement in the results of the three methods. The variations were considered to be due to sensitivity of the methods and quantitative differences in antibodies to different antigens as recognised by the methods of testing. Recently two BLV antigens, and ether sensitive (es) and ether resistent (er) have been demonstrated. The CF test can measure antibodies to both when using culture fluid as the antigen. ID with specific antigens can demonstrate either es or gs (group specific) antigens, and IF appears to demonstrate antibody to gs antigen (Olson, 1975).

Application of Serologic Tests

The gel diffusion test for gs precipitin as specifically designed for BLV antibodies has been used in a preliminary survey. In one, about 1,000 cows in 11 selected herds were tested and 222 reacted. Five herds with no lymphosarcoma for 13 to 33 years had a lower percentage of reactors (2% to 16%) than six herds with 24 cases of lymphosarcoma in the last seven years (24% to 42% reactors). BLV was demonstrated by cultures in 100 of 117 reactors including cattle in five herds with no lymphosarcoma. The survey was expanded to over 7,000 cattle in the north central states of USA. Reactors were found in two thirds of 100 herds involving a total of 4,400 dairy cattle and only one seventh of 50 herds with a total of 2,800 beef cattle. It is significant that few cattle less than two years old reacted to the gel diffusion test, which suggested a slow development of BLV infection.

In 22 herds with BLV infection, 13.5 percent of 1,354 cows were reactors to BLV gs antibody test and 10.4 percent of 96 bulls. The prevalence of leukemia appeared to be similar in bulls and cows.

No reactors to gs antigen were found in 100 dairy cows in ten nerds on the Island of Jersey and 16 cattle on Bornholm Island of Denmark, or in 126 dairy cattle in an isolated herd in the United States. Lymphosarcoma or persistent lymphocytosis has not been diagnosed in the islands or in the isolated U.S. herd for more than 25 years (House, 1975).

Sera from 97 cattle were received from Denmark, and tested as unknowns for precipitins to BLV gs antigen and the results were correlated with the history of the cattle. Antibodies were found in 19 cattle sera from herds with multiple cases of lymphosarcoma or

cases of persistent lymphocytosis. Sera from two cattle with lymphosarcoma and two cattle with persistent lymphocytosis were negative. Sera from 32 cattle in herds with no history of lymphosarcoma or persistent lymphocytosis were negative. Sera were also negative from 42 cattle in the clinic of the Royal Veterinary College Copenhagen, each affected with a variety of diseases other than lymphosarcoma (House, 1975).

Sera from 416 cattle were received from Sweden and tested as unknowns for BLV gs antigens and the results correlated with the history of the cattle. There were 197 sera from four multiple case herds and 24 to 39 percent of the cattle in each herd were reactors. Cattle in the vicinity of Kalmar, Sweden, had a high incidence of lymphosarcoma. Fifteen of 130 sera obtained at the Kalmar abbatoir had precipitins. Thirty-six sera were submitted from cattle with "leukosis" (lymphosarcoma and/or lymphocytosis) and 20 had antibody to BLV. Sera from 53 cattle on the island of Aland, now apparently free of bovine "leukosis", were all negative (House, 1975).

The practical application of serology in bovine lymphosarcoma is still being explored. A positive test for antibodies involving precipitins, complement fixation, or immunofluorence merely indicates the animal has or has had infection with the virus. A few cows from which the virus can be isolated and a few cows with lymphosarcoma do not have BLV antibodies.

All circumstances must be considered when interpret test results in a herd of cattle. Herds with previous cases of lymphosarcoma and several reactor cows should be watched for signs of leukemia since some reactors will develop lymphosarcoma.

Rationale for Serological Diagnostic Procedures

Detection of virus infection by serological means is based upon the fact that most viruses elicit production of specific antibodies in an infected host. These antibodies are produced and liberated into the blood over a period of time, where they circulate for a variable period depending upon the particular virus inducing their formation. Blood may be withdrawn at any time and the serum tested for the presence of antibodies by determining its ability to combine with known viral antigens. Demonstrating a positive reaction between serum and a particular virus is regarded as evidence of previous exposure of the individual to the virus.

Although any serum specimen may be examined for antibodies, the mere detection of antibody against a particular virus does not indicate that the individual from whom the serum comes has had previous experience of the virus. Detection of antibodies per se gives no information concerning the time at which the individual may have come into contact with the virus. Even if the antibody is quantitatively determined its titer can, with few exceptions, give little useful information. The exceptions are those cases where the particular antibodies are short-lived when high titers may be cautiously interpreted as evidence of recent infection (Hoskin, 1967).

Diagnosis, as opposed to detection, of virus infection by serological means depends upon demonstrating the appearance, or a rise in titer, of circulating antibody during the course of the disease. Demonstrating a change in level of antibody against a particular virus with time means that two serum specimens must be obtained and

examined. One is taken early in infection (acute), the other later on when antibodies have reached their maximum level (convalescent phase).

As a result examining the paired sera, four possible serological findings may be encountered.

1. No present infection - both have no antibody.
2. Present infection - no antibody in acute phase but present in convalescent serum.
3. No present infection, but previous infection and or immunization - antibodies are present in both sera in equal quantity.
4. Present infection plus previous infection and/or immunization - antibodies are present in both sera but larger quantity in convalescent than in acute phase (Hoskin, 1967).

The three main serological methods employed for diagnozing BLV infection are not considered as barely detecting the presence of infection but as conclusively diagnostic even though none are employed for comparing rising antibody titers.

Complement fixation test gives less emphasizes to group specificity and therefore tends not to detect rises of antibody against individual virus serotypes. The other two serological tests: immunofluorescence and immunodiffusion emphasize type or strain specifity.

Complement Fixation Test

The basis of this technique lies in the property of antigen-antibody complex to combine with complement. By mixing in test tubes complement and two distinct antigenic systems, the first one being

variable under investigation and the second a constant consisting of sheep erythrocytes (rbc) and an anti-sheep antibody (hemolysin) The second system acts as an indicator through which the reaction, positive or negative, given by the first, is visualised. As one of the effects of complement on the sheep rbc - anti-sheep antibody complex is hemolysis, absence of hemolysis in the tube indicates that there was no free complement, hence that it had been adsorbed by the first system. When a known positive antigen is used, this result indicates that the serum had antibodies against it and when a known positive serum is used, that the antigen is effective (House, 1967).

Circulating antibodies detected by CFT appear to be the result of a recent or relatively recent infection. Their rate of development is relatively slower than that of neutralising or hemagglutination inhibition antibodies consequently the test is useful as an aid to diagnosis. It is relatively simple to perform, technically dependable and well adjusted to large numbers of specimens. With infection due to virus that belongs to a group of antigenically related agents, specificity cannot always be anticipated in the diagnosis, but at least the group in which the virus falls can be ascertained. As a tool for conducting serological surveys in a population, the CFT is of little help because, in general, CF antibodies are short lived in the host after clinical infection and of low titers in asymptomatic ones, so that only those with current or recent infection are detected. This is particularly important in BLV since infected cattle seem to maintain high precipitating and CF antibodies (House, 1967).

Anticomplementary effect, a property that some sera and antigens have to combine with or destroy complement, and nonspecific reactions such as given by some tissue extracts, exudates and bacterial filtrates, which in the presence of normal or antigenically unrelated sera make complexes capable of fixing complement - although neither sera nor extract is anticomplimentary - constitute the two main causes of error and sources of difficulty in interpreting CF test results (Tables 31-32).

The most reliable method for eliminating anticomplementary and nonspecific reactions of antigens and sera is to purify them by eliminating the interfering substances. Extraction of antigens with organic solvents, centrifugation, filtration and adsorption are good methods. Removing anticomplementary action from sera is almost impossible, in which case the sera can be used only at higher dilution. Moderate heating usually clears low degree of anticomplementary effects. The temperature required is slightly higher than for inactivation of complement. Centrifugating heated sera at 18,000 rpm for 30 minutes further improves the sera. When using sera from animals experimentally immunized, care should be taken, to reduce nonspecific reactions by not inoculating an animal with infected tissue from the species used as a source of material for preparing the antigen, unless the same species supplies the serum, infected tissue or antigen.

1. <u>The Test</u>:

While the essentials of the test are constant, the detail of how to carry it out varies. Volumes of reagents, vessels, containers, incubation of first phase, amount of complement, criterion for end-point

TABLE 31

Titers of complement fixing antibodies to bovine C-type virus in the leukosis herd animals

Animal Number	Date of Serum Collection: 1973 May	June	July	Aug	Oct	Nov	Dec	1974 Jan	Feb	Apr	May	June	July	Sept	Oct	Nov
42	4	4	8	4	4	4	8	8	8	4	4	8	8	16	8	8
186	16	8	4	4	AC[a]	16	32	16	-[b]	8	16	16	-	-	-	64
267[c]	16	16	xx[d]	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
323	-	-	-	8	8	-	8	-	8	-	8	-	-	-	-	-
328	16	64	16	-	32	64	32	32	32	32	32	32	32	xx	4	8
342	8	8	16	32	16	64	32	32	8	8	8	8	16	8	8	8
362	16	8	16	8	16	8	16	32	32	32	32	16	8	16	-	4
46274	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46270	-	-	-	AC	AC	-	AC	8	8	8	xx	4	-	8	-	-
46271	-	-	-	-	8	8	8	8	8	8	8	4	4	-	4	-
94637	-	-	-	-	-	-	-	-	-	16	16	8	16	4	xx	16
94638	-	-	-	-	-	-	-	-	AC	AC	16	4	4	-	-	-
94642	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-
94976	-	8	16	16	8	8	8	8	4	AC	AC	4	-	-	8	8
94977	-	-	-	AC	-	-	-	4	-	-	4	-	-	-	-	-
94435	xx	xx	xx	AC	-	-	-	-	-	-	-	32	AC	AC	-	-
94876	xx	xx	xx	xx	xx	xx	AC	AC	-	-	-	-	-	-	-	-
94694	xx	xx	xx	xx	xx	xx	xx	xx	xx	AC	4	-	-	-	-	-
94695	xx	xx	xx	xx	xx	xx	xx	xx	xx	-	-	-	-	-	-	-
94696	xx	xx	xx	xx	xx	xx	xx	xx	xx	AC	4	-	-	-	-	-

[a] Anticomplementary

[b] No detectable antibody at serum dilution = 1:4

[c] Died of lymphosarcoma

[d] Not done

Reference: Table et al. (1976).

TABLE 32

Comparison of hematological classification and complement fixation tests on bovine leukosis herd animals.

Animal number	Hematology[a]	Complement fixation[b]
42	0/16	16/16
186	16/16	11/16
267	2/2	2/2
323	12/16	5/16
328	16/16	14/15
342	16/16	16/16
362	15/16	15/16
46274	0/15	0/16
46270	15/16	5/15
46271	2/16	10/16
94637	1/16	6/15
94638	0/16	3/1
94642	0/16	1/16
94976	2/16	11/16
94977	3/16	2/16
94435	0/13	1/13
94876	0/10	0/10
94694	0/7	1/7
94695	0/7	0/7
94696	1/7	1/7
Total tests	101/269 37.6%	120/267 44.9%

[a]Lymphocytosis (Suspect and Positive classification)/Total Tests.

C.F. Titer = 1.4/Total Tests.

Reference: Tabel et al. (1976).

hemolysis, concentration of rbcs, compositionof diluent, etc. vary. Some of the details are important e.g., amount of complement, criterion for end-point hemolysis, and incubation phase. In general, the end point for hemolysis is taken as 100 percent hemolysis. This is less sensitive than the 50 percent hemolysis end-point. For the 100 percent end-point two units of complement are used and for the 50 percent five units. There are six units to consider in the entire list one unit each taken by serum and antigen, two units by hemolytic system, (one volume of hemolysin and one of erythrocytes suspension), and two units by complement. These are volume units (Hoskin, 1967).

2. Technique:

1) Diluent used is veronal buffer which consists of diethyl barbiturate (barbitone), sodium barbiturate, magnesium chloride (hydrated), calcium chloride and distilled water pH 7.3-7.4.
2) Antigen - Prepared or obtained commercially.
3) Serum - Inactivated to destroy complement that may be present and also eliminates anticomplementary or nonspecific effects. Temperature 56-60°C depending on species.
4) Complement - Commercially lyophilized complement available. Unit of complement is the smallest amount that gives complete hemolysis (or 50% hemolysis) complement must be titrated before every test.
5) Hemolysis - Commercial anti-sheep hemolysin in 50 percent glycerin available. It is necessary to re-titrate the hemolysin in the system. Titration determines the minimal hemolytic dose (MHD) i.e. amount that completely hemolyses the sheep rbc in presence of 3-4 units of

complement. An excess of hemolysis must be avoided to prevent agglutination of cells before hemolysis occurs.

6) Titration of hemolysin -

Amboceptor is diluted to 1:100, then in 2-fold steps to 1:12:800 or higher. To a series of test tubes is introduced in descending order, 0.1 ml of each dilution. To all tubes are then added 0.1 ml of the cell suspension and 0.4 ml of complement so that 3-4 units are used. All tubes are incubated at 37°C for 30 minutes. The highest dilution giving complete hemolysis is the MHD, and in the test 3 MHDs are used.

7) Test

A preliminary titration of complement is performed to determine the smallest amount that completely hemolyse the rbc's. In the test 1.8-2 unit are used.

A 2.5% suspension of sheep rbc and a dilution of hemolysin that contains 3 MHD per 0.1 ml are prepared. Equal volumes of these are mixed, held at room temperature for 15 minutes and 0.2 ml of the mixture added to the tubes of the titration, shaken an incubated in water bath at 37°C for 30 minutes. A tube with the hemolytic system alone is included as a control of cell stability. The titration is read at the end of the incubation and the smallest amount causing complete hemolysis is the unit of complement.

The test proper is then conducted using disposable plastic trays. In micro test, drops rather than ml are used. Using an 18 gauge hypodermic need as dropper, each drop is approximately 0.025 ml.

Dilution of sera and antigen depends on the object of the test.

i) If the test is to determine the titer of an antigen and of relationship between viruses. Simultaneous dilution of sera and antigen are used - checker board titration, the antigen and sera are diluted in increasing 2 fold dilutions beginning at 1:4.

ii) If the test is for identification of virus, test the antigen at two or three dilutions representing 2, 8 or higher 32 units, respectively, against typing sera in 2 fold dilutions beginning at 1:4 or 1:8 and follow with six or eight dilution depdnding on titer.

iii) Determination of antibodies in serum against reference antigen. Dilution of serum of 1:4-1:8 up are tested against a constant amount of one or various antigens representing 4-16 units. Control - normal antigen + normal serum. Distribute serum dilutions in 1 drop amounts. Titrated complement 1.8-2 units added next one drop of antigen is added. The mixture placed in cold at 4°C for 18 hrs. Also place the titrations of the complement at 4°C overnight. At end of the 18 hr. the mixtures are held at room temperature for 15 minutes. 2 drops of 2.5% suspension of sheep rbc added and 3 MHDs of hemolysin and the whole set placed at 37°C for 30-40 minutes to minimise agglutination before hemolysis. At end of this period the test is read.

Complete hemolysis = 0

No hemolysis = 4+

Intermediate values = 1+, 2+, 3+

The titer of serum or antigen is determined by the highest dilution that gives a 3 or better fixation.

The exact techniques used in diagnosis of or detection of antibodies in BLV infection bears little modification. Tabel, Chander (1976) used tissue culture fluids of fetal lamb spleen cell cultures chronically infected with bovine C-type virus. Fluids of non-infected fetal lamb spleen cell cultures were used as control.

Complement titrations and the "plateau method" of optimal hemolysis determination were carried out.

The test itself was conducted in the microtiter system with 5, 50% hemolytic units of guinea pig complement and 2.8% suspension of sheep rbc. The guinea pig complement was supplemented by 1% fresh bovine serum. The lowest serum dilution tested was 1:4.

Results:

Antibodies to bovine type-C-virus were demonstrated in 17 out of 20 animals and 120 out of 267 serum samples (44.9%) were CF antibodies positive.

In another study Ressang et al. (1976), used the same technique of CFT as Tabel et al. (1976), but before reading the tests the microplates were spun at 650 g for 30 seconds. Their studies compared the sensitivity of gel-diffusion, hematology, indirect fluorescence antibody technique, and the immunoperoxidase test. They found that complement fixation test was more sensitive than hematological examination 3.9 percent, gel-diffusion 8.5 percent but less so than fluorescent antibody technique 13 percent and immunoperoxidase 11.2 percent. Complement fixation is nearly equally as sensitive, 9 percent as gel-diffusion 8.5 percent (Tables 33-34).

TABLE 33

Comparison of the results of hematological, serological, virogical and electron microscopical (EM) examinations for the diagnosis of bovine leukemia in 75 selected animals

Code number of animals	Origin	Hemato-logy	Serology (Fluorescing antibodies)	BLV isolation	EM
1	Exp. Station I	+	+	+	+
2		-	+	+	0
3		-	+	+	+
4		-	+	+	0
5		-	+	+	+
6		-	+	+	0
E 357		-	+	-	-*
8		-	+	+	-*
9		-	+	-	-
10		-	-	-	-
11		-	+	+	+*
1	Exp. Station II	+	+	+	+
2		-	+	-	-*
3		-	+	+	0
4		-	+	+	0
5		-	+	-	-
6		-	+	+	+
7		-	+	-	-
8		-	+	+	-*
9		-	+	-	-
10		-	+	-	-
11		-	+	+	+
12		-	+	-	-*
13		-	+	+	+*
14		-	+	-	-
15		-	+	+	+
16		-	+	+	0
51953	Farm J. G.	+	+	+	+
51850		-	+	-	-
15803		-	+	-	-
51849		-	+	-	-
42673		-	+	-	-
E 355		-	+	-	-

0 EM evaluation not possible because of bad condition of leucoyte culture.

*) screened 50-100 cells instead of the usual 150.

contd..

Table 33 continued.

Code number of animals	Origin	Hemato-logy	Serology (Fluorescing antibodies)	BLV isolation	EM
E 242	Farm G. J. B.	-	+	+	+
48568		+	+	+	+
48566		+	+	+	+
48564		+	+	+	+
48565		-	+	-	-
48567		-	+	+	+
50067		-	+	-	-
7896		-	+	-	-
1		-	+	-	0
2		+	+	+	0
3		-	+	+	+*
4		-	+	+	0
5		-	+	-	-
6		-	+	+	+
7		-	+	+	-*
8		-	+	-	-
9		-	+	-	-
10		+	+	+	+
345	Selected cattle	+	+	+	+
400	kept at the	-	-	-	-
486	central	+	+	+	+
523	Veterinary	+	+	+	+
561	Institute	+	+	+	+
632		-	-	-	-
859		+	+	+	+
2305		+	+	+	+
2396		+	+	+	+
2842		-	-	-	-
51186		+	+	+	+
Goat 2491	Experimental	-	+	-	-
2492	sheep and	-	+	-	-
2493	goats	-	+	-	-
2494		-	+	+	+
2495		+	+	+	+
Sheep 546		+	+	+	+
547		-	+	+	+
198		+	+	+	+
2857		+	+	+	+
385		-	-	-	-
386		-	-	-	-
387		-	-	-	-
388		-	-	-	-
75		20	67	43/67(64.2%)	32/58 (55.2%)

Reference: Ressang et al. (1976).

TABLE 34

The results of the ID FAT, the ID IPT, the M CFT and the GDT used to detect antibodies to BLV in foreign sera

Number of sera	Origin of sera	Number of animals with			Antibodies to BLV detected with the			
		Lympho-cytosis	Suspect lympho-cytosis	Normal blood counts	ID FAT	ID IPT	M CFT	GDT
97 (group II[a])	Copenhagen	19 / 97 (19.6%)	-	78	18 / 97 (18.6%)	17 / 97 (17.5%)	n.t.	11 / 97 (11.3%)
100 (group II[b])	Weybridge	1*	-	99	-	-	-	-
100 (group II[c])	Belfast	1	7	92	-	-	-	-
297		21	7	269	18	17	-	11

* = calf; n.t. = not tested

Reference: Ressang et al. (1976).

Immunofluorescence Technique

Although immunofluorescence techniques are applicable to all major virus groups, their adoption for routine use has been restricted by their complexity and dependence upon expensive materials and equipment. No one staining method is necessarily applicable to all viruses, and the exact procedure used in any instance depends upon the particular virus-cell-antibody system being studied. The information that can be made available is often obtained more readily by conventional serological procedures. It should be noted, however, that immunofluorescence techniques possess the important advantage of speed and specificity, so that under ideal circumstances positive identification of clinical material may be made on the same day that the patient is seen or material submitted. The procedure has been employed by many researchers for bovine leukemia.

Ferrer (1972) Tables 35-38 in comparing antigenic relationship between bovine, murine and feline leukemia viruses found that bovine leukemia antibodies did not react in an immunofluorescence test with rat cells transformed by an releasing abundant quantities of murine leukemia virus. Thus the BLV is antigenically unrelated to these viruses. In another study, the same author found that 16 out of 17 sera from leukemic cows reacted in immunofluorescence test with virus-containing cultures. In contrast no reaction were seen with sera from 12 normal cows in leukemia-free herd.

Studies by different workers such as Gillette et al. (1972) demonstrated abnormal antigen in bovine lymphosarcoma by IF (Tables 39-40).

TABLE 35

Indirect immunofluorescence tests with bovine serum 27-125[a] on cells releasing murine leukemia-sarcoma viruses

Cells tested	No. of fluorescent cells/ no. of cells examined[b]	
NBC-6 (grown in E20FSI)	102/410	(25)[c]
NBC-13 (grown in E20FSI)	34/217	(15)
LW-27 (rat Rad LV lymphoma)	0/514	(0)
78 A1 (MSV-MLV-infected embryo rat cells)	0/456	(0)
Normal rat lymphoid cells	0/580	(0)

[a]Serum absorbed *in vivo* (see text).

[b]Control smears incubated with a normal bovine serum (BI-038) previously absorbed *in vivo* (see text) and the conjugate or incubated with the conjugate alone showed less than 1% fluorescent cells.

[c]Numbers in parentheses, % of fluorescent cells.

Reference: Ferrer (1972).

TABLE 36

Absorption immunofluorescence tests with bovine serum 27-125

Cells used for absorption[a]			
Origin	No. ($\times 10^9$)	Immuno fluorescence reactivity of absorbed serum[b]	
NBC-13 (grown in E20FSI)	1.6	18/363[c]	(4.9)[d]
LW-27 (rat Rad LV lymphoma)	1.9	95/421	(23)
Normal bovine lymphoids cells[e]	1.7	91/354	(25)
None		75/360	(20)

[a]Serum diluted 1:10 and incubated with the indicated number of packed, frozen, and thawed (3 times) cells (1 volume of diluted serum per 2 volumes of packed cells).

[b]Tested against NBC-6 cells grown in E20FSI.

[c]No. of fluorescent cells/no. of cells examined.

[d]Numbers in parentheses, % of fluorescent cells.

[e]Obtained from the right prefemoral lymph node removed surgically from a cow (BI-371) in a leukemia-free herd.

Reference: Ferrer (1972).

TABLE 37

Indirect immunofluorescence tests on NBC cell lines with antisera against murine leukemia-sarcoma viruses

Cells tested	Anti-Rad LV serum[a]	Anti-GLV serum[a]	Anti-MSV-M serum[a]
NBC-6 (grown in E20FSI)[b]	0/650[c] (0)[d]	0/589 (0)	0/515 (0)
NBC-13 (grown in E20FSI)[b]	Not done	0/615 (0)	0/503 (0)
LW-27 (rat Rad LV lymphoma)	215/265 (82)	220/245 (91)	276/345 (80)
78 AI (MSV-MLV-infected embryo rat cells)	197/212 (92)	215/226 (95)	263/294 (89)

[a]Sera diluted 1:2 control smears incubated with the conjugate only or with a normal rat serum and the conjugate showed less than 1% fluorescent cells.

[b]Same samples of NBC cultures tested in experiments of Table 1. Between 15 and 20% of the cells in these cultures showed type C virus particles.

[c]No. of fluorescent cells/no. of cells examined.

[d]Numbers in parentheses, % of cluorescent cells.

Reference: Ferrer (1972).

TABLE 38

Absorption of the immunofluorescence activity of a goat anti-FeLV serum[a] for a rat Rad LV lymphoma

Cells used for absorption[b]		Immunofluorescence reactivity of absorbed serum[c]	
Origin	No. (x 10^9)		
NBC-13 (grown in E20FSI)[d]	2.4	409/434[e]	(94)[f]
78 AI (MSV-MLV-infected rat embryo cells	0.7	2/371	(0.5)
Normal rat lymphoid cells	1.3	237/262	(91)
None		223/231	(96)

[a]Preabsorbed with few calf serum and normal rat lymphoid cells.

[b]Serum diluted 1:5. Absorption with NBC-13 cells was carried out in 2 steps. The diluted antiserum was incubated first with approximately 1.8×10^9 cells (1 volume of diluted antiserum per 2 volumes of packed, disrupted cells) and then recovered and incubated again with approximately 0.6×10^9 cells (1 volume of absorbed antiserum per volume of packed, disrupted cells).

[c]Tested on LW-27.

[d]Cultures showing between 18 and 31% of cells with the virus immunocluorescence antigen detected by serum 27-125 were used.

[e]No. of fluorescent cells/no. of cells examined.

[f]Numbers in parentheses, % of fluorescent cells.

Reference: Ferrer (1972).

TABLE 39

Protocol for production of antiserums in calves with bovine lymphosarcom preparations

Anti serum No.	Calf No.	Injection schedule				
		Donor No.	Tumor prepara-tion	No. of injec-tions	Total tissue injected (Gm.)	Route of injection
1	204	3081	S,SS	23	445	IM, SC, IP
2	205	3081	S,SS	15	95	IM, SC, IP
3	201	3082	S,SS	23	445	IM, SC, IP
4	203	3082	S,SS	15	95	IM, SC, IP
5	203	3082	CCS	3	20	IM
6	179	3084	S,SS	17	195	IM, SC, IP
7	179	3088	S	6	250	IM
8	185	3084	S,SS	15	95	IM, SC, IP
9	185	3088	CCS	3	20	IM

S = Tumor suspension; SS = sonicated tumor suspension; CCS - coupled cell suspension; IM = intramuscular; SC = subcutaneous; and IP = intraperitoneal.

Reference: Gillette et al. (1969).

TABLE 40

Results of immunofluorescent staining tests with bovine antitumor serums

Cattle No.	Lymphoid tissue site	Labeled gamma globulin from antiserums* 1 (donor 3081)	3 (donor 3082)	6 (donor 3084)	7 (donors 3084 and 3088)	9 (donors 3084 and 3088)
AFFECTED WITH TUMORS						
3081	Prescapular lymph node	+ +	-	-	-	-
	Kidney	+ +	-	ND	ND	ND
3082	Tumor	-	-	-	+	+
	Heart	-	+	-	+	ND
3084	Prefemoral lymph node	-	-	+	+ +	+
	Heart	-	-	-	+	ND
3088	Iliac lymph node	-	-	+	++	ND
	Kidney	-	-	+	+	+ + +
	Tumor	ND	ND	ND	+	ND
3092	Iliac lymph node	-	+	-	-	-
	Heart	-	-	+	+	ND
3093	Mandibular lymph node	-	-	-	+	-
	Liver	-	+	-	-	ND
3096	Prefemoral lymph node	+	-	+	+	-
3100	Tumor	+ +	+	+	+	ND
	Prescapular lymph node**	-	-	ND	-	ND
	Prefemoral lymph node	-	ND	ND	-	ND
3106	Tumor	+	-	-	-	ND
	Kidney	-	-	+	-	ND

Contd..

Table 40 continued.

Cattle No.	Lymphoid tissue site	Labeled gamma globulin from antiserums*				
		1 (donor 3081)	3 (donor 3082)	6 (donor 3084)	7 (donors 3084 and 3088)	9 (donors 3084 and 3088)
3107	Tumor	-	-	-	+ +	ND
	Kidney	+	-	-	+	ND
3108	Prescapular lymph node	+	-	-	-	ND
	Heart	+	+	-	-	ND
NONAFFECTED CONTROLS						
1	Prescapular lymph node	-	-	-	-	-
2	Prefemoral lymph node	-	-	-	-	-
3	Iliac lymph node	-	-	-	-	-
4	Mammary lymph node	-	-	-	-	-
5	Prefemoral lymph node	-	-	-	-	-

*Antiserum numbers (1, 3, 6, 7, and 9) are those from Table 1.

+ + + = Bright green fluorescence

+ + = Less bright green fluorescence

+ = Weak green fluorescence

- = No specific fluorescence

ND = not done

**Tumor cells were not found in tissue.

Reference: Gillette et al. (1969).

The main application is, however, as a procedure for determining the location of viral antigens in an infected cell and the site in the cell at which the elaboration of antigens is completed. It is also possible to follow infection of individual cells in sections or cell cultures when no abnormality is as yet otherwise seen.

The basis of the entire methodology (Coons et al., 1941) is that antibodies in sera when conjugated with fluorescent substances retain combining site free to react with antigens and the resulting antigen - antibody complex is visualized by fluorescence.

Both direct and indirect techniques are used in studies of animal viruses. The former is mainly applied to particular diagnostic problems, for instance, detection of rabies virus in brain tissue shere a single labelled antiserum suffices. The indirect method is preferred in other cases where the problem is to determine presence of antibodies in the sera against one or several viruses. It is the method of choice for BLV. To label each serum might be a difficult and expensive task but with the indirect method only one labelled antiserum for each animal species involved is required.

1. Procedure:

Prepared antigen is incubated with antibody labelled with a fluorescent dye, usually fluorescein isothiocyanate (FITC). Labelled serum proteins that have not reacted serologically with the specimen are removed by washing and the preparation is mounted and examined by fluorescence microsocpy. Labelled antibody that has combined with viral antigen is excited by ultra-violet light and fluoreces. An apple green color characteristic of the antibody label, while unstained tissues usually emits a low level of blue-white autofluorescence.

2. Preparation of Antibodies:

Unknown or undesirable antibodies present in the serum to be conjugated can contribute to nonspecific staining. To minimise this:

a) Reduce the possibility of immunization with mixed cultures by purification of the viral antigen, preferentially by a plaque procedure or by three successive terminal dilution passages if the virus does not readily form plaque.

b) Purify the virus to remove antigenic components of the host cells and growth medium.

c) Take rigorous precautions to prevent introducting undesirable virus from subclinical or latent viral infections in the animal to be immunised, in the host system used for the preparation of viral antigen, and in the tissues stained with the labelled antibody. Sera should be screened for antibodies against adventitious viruses and if such antibodies are detected they should be removed by absorption with the homologous antigen.

d) Prepare virus in cells of the species of animal to be immunised, since antibodies are less likely to be induced by contaminating tissue from the same species.

e) Obtain pre-immunization serum for control purposes.

f) Immunize susceptible animals or use convalescent sera from animals with natural disease.

g) When feasible cells or tissues to be stained should be of a different species of animal from those used for preparing the immunizing antigen.

h) The immunizing schedule should be sufficient to allow for good antibody response without sacrificing specificity. Prolonged immunization can enhance minor antigenic components, which may give rise to undesirable cross-reactions.

i) Absorb conjugate with suspension of tissue or cells used in the preparation of the immunizing virus and or tissue or cells to be stained.

j) Use only the fraction of the serum globulin that contains the specific antibody.

3. Serum Fractionation:

To reduce the amount of nonspecific staining protein from the globulin fraction, diethyl aminoethyl cellulose (DEAE), ammonium sulphate $(NH_4)_2SO_4$ or DEAE-Sephadex is used.

When concentrated ammonium sulphate is added to serum, protein precipitate from the solution. Serum proteins precipitated by $(NH_4)_2SO_4$ are unaltered ans usually redissolve in fresh portions of the original solvents. Globulins are precipitated from the solution upon half saturation with the salt, and albumins are precipitated by complete saturation. If less salt is used, less globulin precipitates and more of the albumin remains in solution. Most fractionation is done with half-saturated sulfate (50% saturated) an added advantage is to use neutralised $(NH_4)_2SO_4$ by adding NaOH. The ammonium sulfate - serum mixture is allowed to stand at 4^oC overnight. The precipitated globulin sediments by centrifugation at 1000g for 30 minutes. Dissolve the precipitated globulin in distilled water to approximate the original volume of serum. Reprecipitate the globulins at 50 percent saturation with salt as before and centrifuge again. Three precipitations will

be sufficient to remove most of the hemoglobulin. Last precipitate should be dissolved in distilled water. Residual sulfate is removed by dialysis against saline or by passage through a sephadex column, or dialyze in cold against 0.85 percent NaCl until the sulfate is removed. Separation of gamma (Y-) globulin is by use of DEAE - sephadex columns on whole serum (Cherry et al., 1961).

Maniquist, Van Der Maaten and Boothe used sera from cattle with leukemia, normal cattle, or experimentally infected calves for the preparation of fluorescent antibody conjugates. Separation of Y-globulin fraction and conjugation with fluorescein isothiocyanate was carried out as described by Cherry et al. (1961) but the serum was not held overnight after the first addition of ammonium sulfate. Instead it was stirred for 10 minutes and the precipitate collected by centrifugation. Unconjugated fluorescein was removed by passing the conjugate through sephadix G-25 column. The conjugate was adsorbed with bovine liver powder before use.

Coverslips were rinsed in PBS, pH 7.2, fixed for 15 minutes in cold acetone, dried and stained with undiluted conjugate for 30 minutes at 37°C in a moist chamber. The dry coverslips were then mounted on slides using 10 percent glycerin as mounting medium and observed with a microscope equipped for fluorescence microscopy.

Direct Method

Antigen (A) + Fluorescent Antibody (FAb)

A - FAb (fluorescence)

Indirect

a) Antigen (A) + Rabbit antibody (RAb) A - RAb

b) A - RAb + Fluorescent antirabbit Y-globulin antibody

(FARY - GAb) A - RAb - FARY - GAb

Ferrer et al. (1974) used the indirect method and FITC-conjugated goat anti-bovine 7S globulin diluted 1:10 with solution of bovine serum albumin used for the staining. The specific (gs) antigen was obtained from BLV preparation; sephadex G-100 was used.

Immunodiffusion (Agar Gel-diffusion)

This technique depends upon the ability of antigen-antibody complex to form a visible precipitate in a semisolid medium. Various gelling agents can be used but as a rule agar is the most commonly employed agent, particularly with animal viruses.

The test pattern is arranged in such a way that the precipitate is a linear form in a thin agar layer. The technique can be used to identity an unknown antigen when using known reference serum. Conversely, the presence of specific antibody can be detected by means of known reference antigens.

The modern use of immunodiffusion finds application in many viral studies. It has the greatest advantage of enabling even very minute quantity of reagents to detect large quantity of antibody/antigen. Application of the test to diagnosis of several clinical infection had been attempted. Dumbell and Nizamudin (1959) described a simple and rapid diagnostic procedure for smallpox virus using, as antigen, suspended scrapings and fluids from the lesion as well as materials from infected embryonated hen egg. With known positive, serum precipitates occurred within two hrs. With entero-viruses, Schmidt et al. (1964) the test can be used successfully for early detection of coxsackie virus infection in man, while the test requires

concentrated antigen, the use of microtechnique and adequate screening permits routine use. The same workers found the test to give fewer heterotypic reactions than CFT and that it gave a higher proportion of antibody rises than did the neutralisation test. The test has also been used for investigating the distinct components in complex viral antigens and for studying their nature (Morisset et al., 1974). Illustrative instances of these applications are in influenza (Jensen, 1953), smallpox (Gispen, 1955), myxomatosis and fibromatosis (Fayet, 1959), adenoviruses (Pierra and Pierra, 1960) and arboviruses (Clarke, 1964). The main applications are for studying the antigenic variation of viruses and antigenic relationship among viruses.

Of the different types of immunodiffusion, the one commonly used in virological studies is the two dimensional, radial, double diffusion test. A combination of this method with electrophoresis results in a sensitive analytical method called immunoelectrophoresis (Grabar, 1960). An additional development consisting of use of radioactive-tagged reagent - radioimmunoelectrophoresis increases further the sensitivity of the method, Van Der Maaten et al., 1970 (Tables 41-43).

Recently Van Der Maaten and Miller (1976), after Miller et al. (1969) cultured BLV in short term lymphocyte cultures from infected cattle, adopted the BLV to grow in fetal lamb spleen cultures. This later development facilitated the production of viral antigens in large quantity for serological studies. The first antigen discovered was an ether stable antigen called p 24 or internal antigen. Numerous serological studies were performed with this antigen and it was shown that infection with the virus correlated with the presence of antibodies to this antigen.

TABLE 41

Results of immunodiffusion tests of sera from several species against bovine syncytial virus antigen

Species	Number tested	Number positive
Mouse	47	0
Hamster	29	0
Guinea pig	30	0
Rabbit	16	0
Sheep	85	0
Swine	101	0
Bison	120	0
Horse	11	0
Man	48	0

Reference: Van Der Maaten et al. (1970).

TABLE 42

Results of experimental inoculations of bovine syncytial virus into several animal species

Species	Route of inoculation	Number inoculated	Number infected[1]
Mouse	subcutaneous	5	0
	intravenous	6	0
Hamster	subcutaneous	5	0
	intracardial	6	0
Guinea pig	subcutaneous	5	0
	intracardial	5	0
Rabbit	intracerebral	2	2
	intranasal	2	2
	intraperitoneal	2	2
	subcutaneous	2	2
	intravenous	2	2
Sheep	intravenous	4	2
Swine	intravenous	4	0

[1]As determined by reisolation of the virus.

Reference: Van Der Maaten et al. (1970).

TABLE 43

Results of immunodiffusion tests of bovine sera against bovine syncytial virus antigen

Origin of serum	Number tested	Number positive	Percent positive
Lymphosarcoma cases	37	8	22
Multiple incidence herds	160	44	28
Apparently normal herds	530	110	21

Reference: Van Der Maaten et al. (1970).

Recently a second antigen of the virus was discovered by National Animal Disease Laboratory (NADL), the antigen is ether labile and of higher molecular weight and is a glycoprotein antigen (Miller et al., 1975). Pitmann Moore Laboratory have been working with the antigen and a serological procedure called bovine leukemia glycoprotein immunodiffusion test (BL-GID) has been developed. This is the first United State Department of Agriculture (USDA) licenced test for the BLV. The test is specifically designed for BLV infection but it is similar in principles to the equine infections anaemia (E.I.A.) test. Miller et al. (1970) compared the sensitivity of this test with those of CFT, hemotological, and the test based on the p 24 antigen. They found BL-GID to be as sensitive as CFT but more sensitive than both hematology and p 24 tests.

1. The BL-GID test:

The kit consists of:

- Glycoprotein antigen (powder)
- Antigen diluent 3 ml
- One vial of sterile serum 9 ml
- One vial of reagent serum
- One vial of negative reference serum 1 ml
- One vial of positive reference serum 1 ml
- One vial of weak positive reference serum

0.7 percent agar is used and is prepared thus : 7g of Nobles special agar carefully added to 1 liter of buffer solution and boiled to dissolve completely. The buffer is prepared by adding 2g of sodium hydroxide (NaOH), 70g of sodium chloride (NaCl) and 9g boric acid H_3BO_3 to a liter of distilled water pH 8-6.

In 100 mm petri-dishes, 15 ml of the 0.7 percent agar at 58°C is distributed to each dish and set aside for 1 hr to harden at room temperature (27°C) circular wells are cut around a central well so that each well is 3 mm away from its immediate neighbour and all the 6 wells run along the circumference of a circle radius 7 mm so that each of the 6 wells is 7 mm from the central well.

A plastic template and a fitted hollow stainless steel cutter (puncher) is used to punch the wells. The agar plug from the punched well is removed by aspiration using a pipette connected to a vacuum.

2. Filling the wells:

The test sera are placed in alternate wells around the central well using individual sterile pipettes dropper or tuberculin syringe fitted with a ½" 27g needle. The remaining peripheral wells receive the positive serum and the central well the antigen.

3. Essential procedures and precautions:

a. The petri-dish must be clearly labelled - names and dates indelibly written.
b. Each well must be named or numbered.
c. A work-sheet containing diagrammatic representation of the dish and well pattern and numbers is essential.
d. The sheet should also record time of test began and ended (read).
e. The wells should be filled just enough to a top meniscus.
f. Over filling and careless spilling of reagents can cause false results.
g. The dish should be (preferably) incubated in a humidified chamber at 27-30°C.
h. Results can be obtained between 48 to 72 hrs.

4. Results and observations:

Acurate interpretation of the reaction is essential.

(a) Positive - indicated by appearance of fine line between antigen and test serum which joins and continues into or with the control line.

(b) Negative - control line continues into the test serum well without bending or with slight bending toward the reagent serum.

(c) Weak positive - control line bends slightly towards the antigen well but does not continue on to form a complete line between antigen and test serum.

(d) Non-specific - lines which either cross the control line or tail to join smoothly.

5. Interpretation of results:

Positive - due to antigen - antibody diffusion towards each other and occurs only with positive test serum or with positive (reference) serum mistakingly lodged into the test well.

Negative - due to lack of antibody in the test serum (test animal not infected) or lack of antigen in the central well or due to lack of serum (failing to fill the test well with test serum).

Weak positive - due to very low antibody-serum or lack of enough werum in the test well or seepage of any positive serum under agar.

Non-specific - reaction of antigen-antibody from a source other than BLV.

Many other lines or forms can be seen. Haze around the well may be due to lipids or other materials in serum which obscure the line - a repeat of the test may clarify this condition.

Positive serum with a second line may indicate possible reaction to a second antigen of BLV. Very strong positive may be due to very high concentration of the samples, if diluted 1:4 or 1:8 and test repeated, a more distinct line may be obtained.

It is important to keep all reagents in good conditions - frozen once the antigen has been reconstituted. A good record sheet or workbook containing all details and fully labelled procedure is essential in order to correctly interprete the tests.

To investigate the relationship between infection with BLV (C-type virus), leukemia and lymphocytosis, Ferrer et al. (1974) examined leukemic cows and adult cattle from several herds by Fluorescent Antibody technique, Immunodiffusion and Electron microscopy (Tables 44-48). They found fluorescent antibodies reacting specifically with a virion antigen in the cytoplasm of BLV infected cells in 90 percent of leukemic cattle and in 80 to 100 percent of clinically normal animals with PL in multiple-case herds. The antibody titer of cattle with PL were lower than those of leukemic cattle. Clinically normal cattle with normal blood counts in multiple-case herd also showed fluorescent antibodies to BLV, but in each of these herds the incidence is 25 to 76 percent and in most cases, the titers of antibodies found in the nonlymphocytotic animal group were lower than those in the corresponding PL group. The antibody was also present in one third of the cattle in the single-case herd in which PL rate was not significant. On the other hand antibodies were also found in 4 out of 214 cattle from leukemia-free herds. Eighteen out of 20 cows were found positive for the BLV by electron microscopy, and none of 12 antibody negative cows.

The distribution of precipiting antibodies reacting in immunodiffusion tests with intraspecies group specific (gs-1) antigen correlated closely with the distribution of the IF antibodies. Thus, fluorescent and precipitating antibodies react with the same antigen because semipurified BLV gs-1 antigen completely removes the specific fluorescent activity of standard BLV reference serum (Tables 44-48).

A comparison of fluorescent and precipitating activities in the same bovine sera showed that the fluorescent antibody test is substantially more sensitive than the immunodiffusion test for demonstrating BLV infection.

TABLE 44

Distribution of fluorescent antibodies to BLV among cattle

Cattle category	No. antibody positive/total no. tested	Titer[b] distribution of positive sera		
		2-4	8-32	64-256
Leukemic[a]	62/69 (90)[c]	18 (29)[d]	24 (39)[d]	20 (32)[d]
Multiple-case BA herd				
PL[e]	15/16 (94)	1 (7)	14 (93)	0 (0)
Non L	24/48 (50)	14 (58)	10 (42)	0 (0)
Multiple-case BB herd				
PL	8/10 (80)	6 (75)	2 (25)	0 (0)
Non L	2/8 (25)	2 (100)	0 (0)	0 (0)
Multiple-case BF herd				
PL	32/35 (91)	17 (53)	15 (47)	0 (0)
Non L	16/21 (76)	14 (88)	2 (12)	0 (0)
Multiple-case BJ herd				
PL	24/24 (100)	8 (33)	14 (59)	2 (8)
Non L	16/34 (47)	13 (81)	3 (19)	0 (0)
Single-case BG herd	18/54 (33)	12 (67)	6 (33)	0 (0)
Leukemia-free BH herd	0/32 (0)	0 (0)	0 (0)	0 (0)
Leukemia-free BI herd	2/40 (5)	2 (100)	0 (0)	0 (0)
Leukemia-free GB herd	2/142 (1)	2 (100)	0 (0)	0 (0)

[a]Histopathologically confirmed cases referred to our clinic or occurring in the multiple-case study herds.

[b]Reciprocal of the highest serum dilution showing fluorescent activity.

[c]Numbers in parentheses, % of total.

[d]Numbers in parentheses, % of positive sera.

PL, clinically normal animals with PL; Non L, clinically normal, nonlymphocytotic animals.

Reference: J. F. Ferrer et al. (1974).

TABLE 45

Correlation between the presence of fluorescent antibodies to BLV and the presence of C-type virus particles in short-term BC cultures

Titer of fluorescent antibodies[a]	Presence of BLV in short-term BC cultures[b]
2	0/12
2-4	10/11
8-32	9/9

[a]Determined by the indirect immunofluorescence technique on acetone-fixed NBC-13 cells. Titers expressed as the reciproval of the highest antiserum dilution showing distinct fluorescent activity.

[b]Determined by electron microscopic examination.

Reference: J. F. Ferrer et al. (1974).

TABLE 46

Correlation between the incidence of fluorescent and precipitating antibodies to BLV

Cattle category	Fluorescent antibodies[a] (positive/total)	Precipitating antibodies (positive/total)
Leukemic[c]	55/60 (92)[d]	44/60 (73)
Multiple and single-case herds		
PL	49/51 (96)	38/51 (74)
Non L	67/148 (45)	23/148 (15)
Leukemia-free herd	4/120 (3)	2/120 (2)

[a]Determined by the indirect immunofluorescence technique on acetone-fixed NBC-13 cells.

[b]Determined in Ouchterlony immunodiffusion tests with a standard reference BLV gs-1 antigen preparation.

[c]Leukemic, histopathologically confirmed cases referred to our clinic or occurring in 3 multiple-case study herds; PL, clinically normal animals with PL; Non L, clinically normal, nonlymphocytotic animals.

[d]Numbers in parentheses, % of total.

Reference: J. F. Ferrer et al. (1974).

TABLE 47

Correlation between the titer of fluorescent antibodies and precipitating activity of bovine sera

Fluorescent antibody titer[a]	Precipitating antibodies[b] (positive/total)
2	0/85 (0)[c]
2-4	15/65 (24)
8-16	47/53 (89)
32	37/37 (100)

[a]Determinde by the indirect immunofluorescence technique on acetone-fixed NBC-13 cells. Titers expressed as reciprocal of the highest antiserum dilution showing distinct fluorescent activity.

[b]Determined in Ouchterlony immunodiffusion tests with a standard reference BLV gs-1 antigen preparation.

[c]Numbers in parentheses, % of total.

Reference: J. F. Ferrer et al. (1974).

TABLE 48

BLV gs-1 antigenic activity and immunofluorescence absorption activity of various antigen preparations

Antigen preparation	BLV gs-1 antigen activity[a]	Immuno-fluorescence absorption activity[b]
Ether-treated BLV from culture fluids of cell line NBC-13	+	+
Ether-treated homogenate of BLV-infected NBC-13 cells.	+	+
Ether-treated cell homogenate from a BLV-infected short-term BC culture[c]	+	+
BLV gs-1 antigen partially purified by column chromatography	+	+
Ether-treated cell homogenate from an uninfected short-term BC culture[d]	-	-

[a]Determined in Ouchterlony immunodiffusion tests with Reference Serum 27-125. All the precipitin lines of the positive antigen preparations showed reactions of identity with the line formed by a standard reference BLV gs-1 antigen preparation.

[b]The immunofluorescence absorption tests were carried out with Reference Serum 27-125 which, after incubation with the antigen preparation, was tested for residual fluorescent activity against acetone-fixed NBC-13 cells. +, complete absorption.

[c]Derived from a cow with PL in a multiple-case herd.

[d]Derived from a normal cow in a leukemia-free herd.

Reference: J. F. Ferrer et al. (1974).

CONCLUSIONS

In the first 10 years of this century, many investigators attributed the apparent rise in the frequency of human cancer to an increase in the consumption of flesh and supported the view by asserting that malignant disease had never been observed in vegetarians. Trotter (1911) published a series of 300 bovine tumors and challenged this view by pointing out that the etiologic significance of a meat diet could hardly apply to herbivorous species. This publication plus Peyton Rous' cell-free transmission of solid chicken tumor provided an early but crude example of the potential contribution which lies in a comparative approach to cancer epidemiology (Marshak and Dutcher, 1965). Fieldman (1932) complained about the meager and fragmentary nature of statistical data on tumors of lower animals. Since then veterinary epidemiology has begun moving toward areas other than mere collection of tumor cases and beyond the collection of statistics without a common denominator or adequate appreciation for the complexity of frequency rates and pitfalls of interpretation.

In considering leukemia of mammals, except man, several generalities are obvious.

1) Leukemias comprise the most commonly occurring group of neoplasms affecting animals besides the common laboratory and domestic species. Leukemia has been reported in monkeys, lions, rats, skunks, buffaloes, squirrels, kangaroos, deer, elephants, and african clawed toads (Marsha, 1965).

2) Lymphatic leukemia predominates overwhelmingly even though most of the clinico-pathologic features observed in human leukemia have been described also in laboratory and domestic animals.

3) It is not yet demonstrated conclusively that ionizing radiation or chemical carcinogens are involved in the etiology of leukemia. Recently, however, viruses have been incriminated as the etiological agents in mouse, avian and bovine leukemia.

4) Only in cattle is well-established epidemologic data available. In the rest of the domestic animals such data is still lacking. It is agreed that the incidence of leukemia in most domestic species is rising.

5) In both laboratory and domestic animals leukemia occurs in both old and young but the incidence is higher in the older animals.

6) Genetic factors have been extensively investigated in mouse but less so in domestic animals. This has been related to the high incidence of inbred murine strains.

Murine leukemia is utilized as reference and model because of the large volume of documentation available on this disease. It is important to emphasize that a mouse is not a cow and therefore inter-species generalisations, no matter how alluring, could be seriously misleading.

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Table 2. Number of Animals Condemned for Various Diseases and Conditions on Ante-Mortem Inspection, Fiscal Year 1973

Cause of Condemnation	Cattle	Calves	Sheep and Lambs	Goats	Swine	Equine
Degenerative and Dropsical Conditions:						
Anasarca	41					
Emaciation	155	10	16		46	
Miscellaneous	83		2		9	
Infectious Diseases:						
Actinomycosis, Actinobacil	84					
Listerellosis	5					
Mucosal Disease	2					
Swine Erysipelas					9	
Miscellaneous	5				5	
Inflammatory Diseases:						
Enteritis, Gastritis, Peritonitis	44	10			116	
Mastitis, Mammitis	42					
Metritis	32				8	
Nephritis, Pyelitis	13					
Pericarditis	100				9	
Pneumonia	340	50	34		55	
Miscellaneous	35	18	10		48	
Neoplasms:						
Carcinoma	4					
Epithelioma	1,638					
Malignant Lymphoma	18					
Miscellaneous	4				2	
Parasitic Conditions:						
Miscellaneous		1				
Pigmentary Conditions:						
Miscellaneous					1	
Septic Conditions:						
Abscess, Pyemia	99	2	4		617	
Septicemia	75	11			46	
Toxemia	60	1	6		48	
Miscellaneous	10	2			2	
Other:						
Arthritis, Polyarthritis	238	43	4		798	
Deads	4,885	7,762	8,177	373	72,681	33
Icterus		1		24	4	
Immaturity		11			3	
Injuries, Bruises	106	12	6		19	1
Moribund	1,715	1,498	220		832	9
Pyrexia	243	32	40		179	
Rabies	6		1			
Suspect Died in Pen	499	338	43	4	1,547	6
Tetanus	11	3			7	32
Uremia	17	810	6		1	
Miscellaneous General	22	12	4		18	
Total	10,631	10,627	8,573	401	77,110	81

Table 3. Number of Carcasses Condemned for Various Diseases and Conditions on Post-Mortem Inspection, Fiscal Year 1973

Cause of Condemnation	Number of Carcasses Condemned					
	Cattle	Calves	Sheep & Lambs	Goats	Swine	Equine
Degenerative and Dropsical Conditions:						
Anasarca	1,169	19	73	102	123	2
Emaciation	5,599	1,209	10,162	315	938	118
Miscellaneous	1,272	23	124		499	3
Infectious Diseases:						
Actinomycosis, Actinobacil	688	2			23	
Anaplasmosis	194					
Caseous Lumphadenitis			8,107	282		
Coccidioidal Granuloma	9					
Hog Cholera					2	
Leptospirosis					2	
Listerellosis		2				
Mucosal Disease	1	1				
Necrobacillosis and Necrosis	114	4			21	
Swine Erysipelas					3,514	
Tuberculosis Nonreactor	126				5,721	
Tuberculosis Reactor with Lesions	60					
Miscellaneous	49	3			35	
Inflammatory Diseases:						
Enteritis, Gastritis, Peritonitis	4,368	1,275	218	4	9,338	13
Eosinophilic Myositic	3,877	14			8	
Mastitis, Mammitis	964				44	
Metritis	1,931	8	106		1,063	
Nephritis, Pyelitis	3,185	145	564	1	3,176	7
Pericarditis	4,307	54	137		1,143	
Pneumonia	13,503	3,930	6,577	145	17,183	223
Miscellaneous	377	156	138		355	2
Neoplasms:						
Carcinoma	2,724		8		263	13
Epithelioma	16,615	1				
Malignant Lymphoma	5,676	47	10		1,699	4
Sarcoma	166	2	6		243	200
Miscellaneous	530	8	12		342	43
Parasitic Conditions:						
Cysticercosis	461	2	138		7	
Sarcosporidiosis	171	1	3,416		8	
Stephanuriasis					3,063	
Miscellaneous	151	23	95		186	
Pigmentary Conditions:						
Melanosis, Non-malignent	61	10	12	2	377	
Miscellaneous	151	10	4	33	66	43
Septic Conditions:						
Abscess, Pyemia	10,665	479	837	122	35,474	22
Septicemia	7,002	1,465	529	16	9,425	49
Toxemia	2,915	94	267	1	2,407	4
Miscellaneous	254	14	29		3,544	
Other:						
Arthritis, Polyarthritis	1,849	2,135	1,201	11	23,225	3
Asphyxia	47	125	130		3,239	
Bone Conditions	70	28			158	
Contamination	1,476	169	274		6,759	5
Icterus	487	2,423	855	25	13,749	
Immaturity		380			16	
Injuries, Bruises	3,141	933	640	20	3,713	53
Sexual Odor					800	
Uremia	1,261	32	812	1	1,139	2
Miscellaneous General	165	5	125		267	
Total	97,831	15,231	35,606	1,080	153,357	809

Table 2. Number of Animals Condemned for Various Diseases and Conditions on Ante-Mortem Inspection, Fiscal Year 1974

Cause of Condemnation	Cattle	Calves	Sheep and Lambs	Goats	Swine	Equine
Degenerative and Dropsical Conditions:						
Anasarca	38					
Emaciation	61	2	5	1	50	
Miscellaneous	51	1	1		23	
Infectious Diseases:						
Actinomycosis, Actinobacil	107	1				
Anaplasmosis	1					
Caseous Lymphadenitis			2			
Listerellosis	2		3		1	
Swine Erysipelas					14	
Tuberculosis Nonreactor					98	
Miscellaneous	3		2		3	
Inflammatory Diseases:						
Enteritis, Gastritis, Peritonitis	20	8	3		79	
Mastitis, Mammitis	20		1			
Metritis	29				10	
Nephritis, Pyelitis	1				1	
Pericarditis	26				5	
Pneumonia	152	62	2		49	
Miscellaneous	20	7	1		45	
Neoplasms:						
Carcinoma	9		1		1	
Epithelioma	1,114					
Malignant Lymphoma	5				1	
Sarcoma	36					
Miscellaneous	1					
Residues:						
Drug	3					
Pesticide	1					
Septic Conditions:						
Abscess, Pyemia	60	2			498	
Septicemia	64	26	3		126	
Toxemia	24	5	3		13	
Miscellaneous	10				3	
Other:						
Arthritis, Polyarthritis	60	17	2		867	
Deads	5,967	7,596	4,391		88,978	180
Icterus					3	
Immaturity		25			1	
Injuries, Bruises	62	3			11	
Moribund	1,936	1,552	284		16,051	15
Pyrexia	196	37	5		106	
Rabies	7	7	6			
Skin Conditions					1	
Suspect Died in Pen	587	292	66	1	444	6
Tetanus	13	4	22		26	
Uremia	19	1	19		22	
Miscellaneous General	27		3			
Total	10,732	9,648	4,825	2	107,530	201

Table 3. Number of Carcasses Condemned for Various Diseases and Conditions on Post-Mortem Inspection, Fiscal Year 1974

Cause of Condemnation	Number of Carcasses Condemned					
	Cattle	Calves	Sheep & Lambs	Goats	Swine	Equine
Degenerative and Dropsical Conditions:						
Anasarca	1,695	8	25	2	64	7
Emaciation	4,897	773	2,758	104	797	110
Miscellaneous	1,589	35	95		477	40
Infectious Diseases:						
Actinomycosis, Actinobacil	839	1			31	
Anaplasmosis	306	2				
Anthrax					18	
Brucellosis	8				1	
Caseous Lymphadenitis			4,481	138		
Coccidioidal Granuloma	15					
Listerellosis	6				13	
Mucosal Disease	3					
Necrobacillosis and Necrosis	111	6			13	
Swine Erysipelas					3,769	
Tuberculosis Nonreactor	74	2			6,071	
Tuberculosis Reactor with Lesions	67	6				
Miscellaneous	49	1	13	2	33	
Inflammatory Diseases:						
Enteritis, Gastritis, Peritonitis	4,439	1,107	188		8,797	31
Eosinophilic Myositic	2,865	7	15			
Mastitis, Mammitis	791	3	8		46	1
Metritis	1,727	4	203		1,047	2
Nephritis, Pyelitis	2,946	139	520		2,998	
Pericarditis	2,921	43	134		1,209	1
Pneumonia	12,744	3,609	2,725	1	12,741	282
Miscellaneous	372	211	106		354	10
Neoplasms:						
Carcinoma	2,892	7	22		321	14
Epithelioma	18,310	1			2	
Malignant Lymphoma	4,669	52	10		1,075	4
Sarcoma	328		5		221	224
Miscellaneous	520	4	13		462	6
Parasitic Conditions:						
Cysticercosis	288		256		8	
Sarcosporidiosis	73	1	950	3	7	
Stephanuriasis					1,808	
Miscellaneous	25	14	100		114	
Pigmentary Conditions:						
Melanosis, Non-malignant	65	9	7		322	56
Miscellaneous	201	4	3		59	
Residues:						
Drugs	130	35	8		1	
Pesticide	24	3			4	1
Miscellaneous	8	1				
Septic Conditions:						
Abscess, Pyemia	10,400	463	924	3	33,197	41
Septicemia	8,099	2,609	498		9,241	66
Toxemia	3,061	255	266		2,285	7
Miscellaneous	164	3	24		5,715	
Other:						
Arthritis, Polyarthritis	1,572	1,698	945	3	21,662	
Asphyxia	55	141	30		2,824	2
Bone Conditions	29	1	3		294	
Contamination	748	265	111	4	5,895	4
Icterus	400	999	1,299	3	11,229	
Immaturity		316	1		11	2
Injuries, Bruises	3,236	505	198	1	4,368	71
Sexual Odor					507	
Uremia	1,264	18	111		741	6
Miscellaneous General	141	9	27		158	
Total	95,166	13,370	17,082	264	141,010	988

Table 2. Number of Animals Condemned for Various Diseases and Conditions on Ante-Mortem Inspection, Fiscal Year 1975

Cause of Condemnation	Cattle	Calves	Sheep and Lambs	Goats	Swine	Equine
Degenerative and Dropsical Conditions:						
Anasarca	18		1			
Emaciation	76				44	
Miscellaneous	63	1			15	
Infectious Diseases:						
Actinomycosis, Actinobacillosis	108					
Johne's Disease	1					
Necrobacillosis and Necrosis	4				1	
Swine Erysipelas					4	
Tuberculosis Nonreactor		1				
Tuberculosis Reactor with Lesions	10					
Miscellaneous	6		3		2	
Inflammatory Diseases:						
Enteritis, Gastritis, Peritonitis	44	4			49	
Mastitis, Mammitis	33		1			
Metritis	32	1			13	
Nephritis, Pyelitis	5					
Pericarditis	33					
Pneumonia	218	52	14		37	1
Miscellaneous	25	4	2		27	
Neoplasms:						
Carcinoma	4					
Epithelioma	1,006					
Malignant Lymphoma	10					
Sarcoma	1					
Miscellaneous	1	1				
Residues:						
Drug	1				21	
Septic Conditions:						
Abscess, Pyemia	32	3	1		189	
Septicemia	40		6		19	
Toxemia	55	1	5		7	
Miscellaneous	9	2	4			
Other:						
Arthritis, Polyarthritis	20	6	3		662	
Deads	8,486	12,359	11,688	129	71,923	303
Icterus	3				2	
Immaturity		29				
Injuries, Bruises	66	1			29	2
Moribund	3,666	2,083	366	2	747	18
Pyrexia	136	11	18		80	
Suspect Died in Pen	755	407	67	7	567	11
Tetanus	22	48			42	1
Uremia	20		16			
Miscellaneous General	30	2	1		12	1
Total	15,039	15,016	12,196	138	74,492	337

Table 3. Number of Carcasses Condemned for Various Diseases and Conditions on Post-Mortem Inspection, Fiscal Year 1975

Cause of Condemnation	Number of Carcasses Condemned					
	Cattle	Calves	Sheep & Lambs	Goats	Swine	Equine
Degenerative and Dropsical Conditions:						
Anasarca	1,938	6	44	1	26	25
Emaciation	10,055	1,832	6,276	17	859	354
Miscellaneous	1,600	34	113		370	15
Infectious Diseases:						
Actinomycosis, Actinobacillosis	1,038				19	
Anaplasmosis	281	1				
Brucellosis					1	
Caseous Lymphadenitis			11,201	255		
Coccidioidal Granuloma	21	6				
Johne's Disease	2					
Listerellosis		3			5	
Mucosal Disease	1	2				
Necrobacillosis and Necrosis	121				12	
Swine Erysipelas					3,149	
Tuberculosis Nonreactor	80	1			5,192	
Tuberculosis Reactor with Lesions	154					
Miscellaneous	81		2		51	1
Inflammatory Diseases:						
Enteritis, Gastritis, Peritonitis	4,713	1,377	265		9,880	47
Eosinophilic Myositis	3,225	4	42		21	
Mastitis, Mammitis	1,015		4		51	2
Metritis	1,885		151		867	
Nephritis, Pyelitis	3,499	227	757		2,776	12
Pericarditis	4,328	61	203	8	1,297	4
Pneumonia	12,134	3,816	6,972		13,362	456
Miscellaneous	389	442	267		417	8
Neoplasms:						
Carcinoma	2,824				204	32
Epithelioma	20,485	5				
Malignant Lymphoma	4,160	68	31		831	13
Sarcoma	231	1			171	
Embryonal Nephroma					7	
Miscellaneous	512	10	8		284	259
Parasitic Conditions:						
Cysticercosis	276		594		5	
Sarcosporidiosis			3,466			
Stephanuriasis					2,796	
Miscellaneous	80	56	169	25	72	1
Pigmentary Conditions:						
Melanosis, Nonmalignant	91	12	6		364	154
Miscellaneous	465	1	1		93	5
Residues:						
Drug	89	76	3		9	
Pesticide	25	1	6		3	
Miscellaneous	24				2	
Septic Conditions:						
Abscess, Pyemia	11,413	554	1,391	6	32,223	47
Septicemia	7,329	1,730	953	1	3,507	153
Toxemia	3,398	287	798	1	2,815	4
Miscellaneous	186	11	47		1,036	1
Other:						
Arthritis, Polyarthritis	1,360	1,848	1,451	1	19,797	
Asphyxia		418	55		2,447	
Bone Conditions	88	13	6		68	
Contamination	881	56	328	5	7,229	3
Icterus	392	2,238	1,632		10,169	1
Immaturity		545		2	26	
Injuries, Bruises	5,290	838	572	2	4,902	152
Sexual Odor					512	
Uremia	1,142	47	1,105		929	2
Miscellaneous General	170		16		382	6
Total	107,471	16,627	38,935	324	129,238	1,757

Table 2. Number of Animals Condemned for Various Diseases and Conditions on Ante-Mortem Inspection, Fiscal Year 1976

Cause of Condemnation	Cattle	Calves	Sheep and Lambs	Goats	Swine	Equine
Degenerative and Dropsical Conditions:						
Anasarca	27		1		1	
Emaciation	56	1			34	5
Miscellaneous	83	1	1		15	
Infectious Diseases:						
Actinomycosis, Actinobacillosis	150					
Coccidioidal Granuloma	1					
Listerellosis	5					
Swine Erysipelas					4	
Miscellaneous	6		3		8	1
Inflammatory Diseases:						
Enteritis, Gastritis, Peritonitis	39	22	18		78	
Mastitis, Mammitis	29					
Metritis	26				10	
Nephritis, Pyelitis	6				2	
Pericarditis	40					
Pneumonia	424	38	9		42	2
Miscellaneous	29	5	4		29	
Neoplasms:						
Carcinoma	6					1
Epithelioma	1,023					
Malignant Lymphoma	20				2	1
Sarcoma	1					
Miscellaneous	17	1				18
Pigmentary Conditions:						
Miscellaneous					1	
Residues:						
Drug	1				20	
Septic Conditions:						
Abscess, Pyemia	58	3			534	
Septicemia	48	3	6		36	1
Toxemia	58	2	4		16	
Miscellaneous	9	3	3		4	
Other:						
Arthritis, Polyarthritis	54	14	6		636	
Deads	7.952	16,625	8,513	90	70,270	274
Icterus	4				2	
Immaturity		14				
Injuries, Bruises	61	2	1		31	
Moribund	3,164	1,874	381	2	817	31
Pyrexia	131	19	20		80	1
Suspect Died in Pen	919	386	41	3	638	7
Tetanus	21				22	1
Uremia	33		13			
Miscellaneous General	49	2	1		26	
Total	14,550	19,015	9,025	95	73,358	343

Table 3. Number of Carcasses Condemned for Various Diseases and Conditions on Post-Mortem Inspection, Fiscal Year 1976

Cause of Condemnation	Number of Carcasses Condemned					
	Cattle	Calves	Sheep & Lambs	Goats	Swine	Equine
Degenerative and Dropsical Conditions:						
Anasarca	1,776	8	62	1	27	29
Emaciation	8,861	1,644	5,787	22	556	176
Miscellaneous	1,774	46	91		304	28
Infectious Diseases:						
Actinomycosis, Actinobacillosis	833	2			10	
Anaplasmosis	308					
Caseous Lymphadenitis			11,637	126		
Coccidioidal Granuloma	23		1		1	
Johne's Disease	1					
Necrobacillosis and Necrosis	121	77			10	
Swine Erysipelas					3,165	
Tuberculosis Nonreactor	86	1			4,996	
Tuberculosis Reactor with Lesions	60					
Miscellaneous	62	2	8		44	3
Inflamatory Diseases:						
Enteritis, Gastritis, Peritonitis	5,087	1,617	289		9,129	43
Eosinophilic Myositis	3,878	9	28		28	
Mastitis, Mammitis	1,012	2	4		43	1
Metritis	1,932	5	161	1	904	5
Nephritis, Pyelitis	3,825	257	639		2,661	3
Pericarditis	4,585	76	223		1,350	14
Pneumonia	13,398	4,368	6,912	8	12,553	232
Miscellaneous	462	249	404		424	2
Neoplasms:						
Carcinoma	3,328	6	23		233	52
Epithelioma	19,131	1				
Malignant Lymphoma	5,814	80	29		1,047	13
Sarcoma	267	4	7		291	105
Miscellaneous	545	13	5		373	65
Parasitic Conditions:						
Cysticercosis	135		189		4	
Sarcosporidiosis			3,695			
Stephanuriasis					2,322	
Miscellaneous	88	3	133		74	1
Pigmentary Conditions:						
Melanosis, Nonmalignant	87	18	21		460	130
Miscellaneous	513	4	3		100	1
Residues:						
Drug	75	69	3		11	
Pesticide	29		19		9	
Miscellaneous	25	2			2	
Septic Conditions:						
Abscess, Pyemia	11,582	534	2,116	6	28,136	37
Septicemia	7,749	2,318	828	1	3,878	77
Toxemia	3,535	384	627		2,738	16
Miscellaneous	169	154	32	1	53	2
Other:						
Arthritis, Polyarthritis	1,468	2,786	2,102		19,332	2
Asphyxia	56	272	41		2,727	4
Bone Conditions	21	1	2		38	1
Contamination	1,135	36	316	1	5,111	
Icterus	477	2,133	1,688	1	9,884	1
Immaturity		517	1		27	
Injuries, Bruises	4,199	1,034	355	19	4,430	60
Sexual Odor					444	
Skin Conditions					78	
Uremia	1,364	45	1,265	1	874	2
Miscellaneous General	244	13	18		356	5
Total	110,120	18,790	39,764	188	119,237	1,110

BOVINE LEUKEMIA: ETIOLOGIC, PATHOGENETIC AND DIAGNOSTIC STUDIES

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AN ABSTRACT OF A REPORT

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Bovine leukemia (lymphosarcoma, leukosis, malignant lymphoma) is a neoplastic disease of cattle believed to be caused by an oncogenic virus (C-type) with genetic and environmental influencing agents. It is characterized by tumorous lesions which can occur in any part of the body principally the lymphoid tissue, abomasum, heart, uterus, and other internal organs. It is world-wide in distribution with highest recorded incidence in Germany. There are enzootic and the sporadic forms which are further classified into juvenile and skin forms.

This report covers bovine leukemia (BLV) in general with emphasis on etiology, pathogenesis, and diagnosis. Evidence in support of the infectious nature of leukemia includes: familial occurrence; transmission studies; reactivity of cell cultures originating from leukemic tissue; and other epidemologic observations, and the presence of virus-like particles in both milk and sections of lymph node and tissue from infected cattle examined by electron microscopy.

Transmission studies using leukemic tissues as inoculum have supported the infectious nature of bovine leukemia. Epidemiologic studies in both Europe and U.S.A. favor the hypothesis of vertical transmission thus proving genetically determined infection in utero or transmission through infected dam's milk. Some diagnostic procedures involving blood transfusion and other technical practice have been found to be a means of transmitting the disease. Cell cultures prepared from affected lymph nodes or other cell-free extracts of infected tissue used as inoculum for cell lines produced syncytia. Presence of virus-like particles in milk from

from leukemic as well as some apparently normal cattle supports the viral etiology as well as the fact that apparently normal cattle shed active virus in their milk. The disease is also horizontally transmitted as seen by feeding clinically healthy calves with milk from infected cows and by the appearance of the disease after introducing an infected cow in a previously leukemia-free herd. The disease spreads both vertically and horizontally.

The virus has been isolated, characterized and purified. Buffy coat (BC) cells from lymphoid cultures (short-term cultures) and New Bolton Center (NBC) cell lines had been sources of virus for the study. Two antigens - ether-sensitive and resistant internal antigens (p.24) and ether-sensitive glycoprotein or external antigen have been isolated. These antigens have been extensively employed in various serological studies and sensitivity of each technique was compared. Most leukemic cattle and cattle in multiple-case herds possess antibodies to BLV. These antibodies are detectable by immunofluorescent antibody technique (IFA), immunodiffusion - the so called bovine leukemia glycoprotein immunodiffusion test (BL-GID), and by complement fixation (CFT). The BL-GID test detects antibodies against the ether-sensitive antigen and by comparison, is more sensitive than the test against the internal, ether-resistant antigen and is as sensitive as the CFT.

Immunodiffusion tests have made it possible to detect a number of cattle that are virus carriers or have passively acquired precipitating antibodies. What is not certain is whether these antibodies are an indication of immunity or a reaction against certain viral precursors or components without influence on the

carrier state. Also not all virus carriers produced precipitating antibodies and some frank leukemic cattle were found to be negative. On the other hand, BLV virus could not be demonstrated by cultures from some cattle with antibodies. Another peculiar feature of bovine leukemia is that cattle with juvenile and thymic forms of the disease have neither demonstrable leukemia virus nor antibodies yet their tumor tissue is capable of inducing the virus infection in test calves and sheep.

Electron microscopy studies have shown that BLV virus is released by budding into cytoplasmic vacuoles and retained within the cell to be released only when the cell disintegrates, thus explaining the low infectivity of the virus.

One important manifestation of bovine leukemia is lymphocytosis. This has been and still remains controversial. Lymphocytosis is considered to be a pretumorous stage of leukemia and a persistent lymphocytosis is diagnostic. Leukemia is associated with significant blood alterations either qualitatively or quantitatively, thus hematological techniques are valuable diagnostic aids for bovine leukemia. Various factors, however, affect blood cell counts: collection, storage, method of stabilization, age of the animal, and physiological status at the time of collection.

The pathogenesis of the disease is based on the BLV virus having affinity for lymphoid tissue where it acts directly or indirectly to stimulate neoplastic changes. The sinusoids become occluded and the germinal centers destroyed and replaced by neoplastic lymphocytes and lymphoblasts. The affected tissue consequently enlarges. These neoplastic cells may metastasise to various organs within the body principally abomasum, myocardium,

and uterus.