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BOVINE LEUKEMIA VIRUS: A MODEL VIRUS FOR THE HUMAN T LYMPHOCYTOTROPHIC RETROVIRUSES

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BOVINE LEUKEMIA VIRUS: A MODEL VIRUS FOR THE HUMAN T LYMPHOCYTOTROPHIC RETROVIRUSES

BY

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ABSTRACT

BOVINE LEUKEMIA VIRUS: A MODEL VIRUS FOR THE HUMAN T LYMPHOCYTOTROPHIC RETROVIRUSES

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The objective of these studies was to investigate the potential of BLV infection in sheep as a model system for studying the biology of leukemogenic retroviruses of animals and humans. A prospective study of the serologic, hematologic, and histologic changes of sheep infected with bovine leukemia virus (BLV) was done. Whole blood from a BLV seropositive cow was used to infect 8 sheep with BLV. Antibodies to BLV were detectable in the sheep 3 weeks after exposure and the antibodies have persisted for 120 weeks. All control sheep have remained seronegative. There were no differences between the hematologic counts and percentages of the infected and control sheep during the first 120 weeks of the study. However, one sheep did develop a leukopenia and lymphopenia 95 weeks after infection and died of histologically-confirmed lymphosarcoma 10 days later. A colony assay for culturing sheep peripheral blood lymphocytes in soft agar was standardized according to several important technical parameters. This assay was then used to study colony formation by lymphocytes from bovine leukemia virus-infected, aleukemic sheep as an alternative method for evaluating possible abnormalities in the interleukin 2 (IL 2)/interleukin 2 receptor system in these sheep. There was no difference in the number of lymphocyte colonies formed by cells from BLV infected and control sheep. Nor was there a difference in the number of colonies formed by lymphocytes from the BLV infected sheep when autologous serum was

replaced with either pooled serum from the infected sheep or from the control sheep. Thus, we were not able to demonstrate abnormalities in IL 2 activity in aleukemic sheep infected with BLV by using a lymphocyte colony assay. The biological similarities between BLV and human T lymphocytrophic retroviruses, especially HTLV I, suggests that BLV infected sheep may prove to be a useful animal model for studying mechanisms of leukemogenesis induced by both human and animal lymphocytotrophic oncogenic retroviruses.

This work is dedicated to my parents, Marvin and Alice Ruth Johnson, and to my sisters and brothers, Cynthia, Cheryl Janice, Idella, Kenneth Jerome, Michael, and Edgar Byron.

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LIST OF ABBREVIATIONS

AGID,	agar gel immunodiffusion
APC,	antigen presenting cell
ASS,	autologous sheep serum
ATL,	Adult T cell leukemia
BLV,	bovine leukemia virus
BstE II,	Bacillus stearothermophilus
BSV,	bovine syncytial virus
CDMEM,	complete Dulbecco's modified Eagle's medium
CEC,	Common European Community
CON A,	concanavalin A
CPSS,	control pooled sheep serum
EBL,	enzootic bovine leukosis
HBSS,	Hank's balanced salt solution
HLA,	human leucocyte antigen
HTLV I,	human T cell leukemia/lymphoma virus
IL 2 R,	interleukin 2 receptor
IL 2,	interleukin 2
IPSS,	infected pooled sheep serum
LNP,	lymphocyte nuclear pocket
MHC,	major histocompatability complex
NASS,	normal autologous sheep serum

p24,	protein 24
p51,	protein 51
PBL,	peripheral blood lymphocytes
PBMC,	peripheral blood mononuclear cells
PE,	plating efficiency
PHA,	phytohemagglutinin A.
PL,	persistent lymphocytosis
PSS,	pooled sheep serum
RE,	restriction endonuclease
RFLP,	restriction fragment length polymorphism
RIA,	radioimmunoassay
SIgG,	surface IgG
SIgM,	surface Igm
SN,	seronegative
TAA,	tumor associated antigen
SP,	seropositive
VE,	virus expression

CHAPTER I

ENZOOTIC BOVINE LEUKOSIS AND BOVINE LEUKEMIA VIRUS: A LYMPHOID CANCER OF RETROVIRAL ORIGIN

INTRODUCTION

Enzootic bovine leukosis (EBL) is a naturally-occurring, lymphoid cancer of cattle caused by the retrovirus bovine leukemia virus. Though BLV infection is pandemic, the prevalence of the infection in cattle herds is between 0 and 90 percent. Only about 30 percent of the cattle infected with BLV develop persistent lymphocytosis (PL), a benign lymphoproliferative phase of infection with this virus. Only 5 percent of cattle with PL develop malignant lymphoma. Thus, EBL and BLV have been significant in the past primarily because of the international trade restrictions placed on seropositive animals and not because of the associated malignant lymphoma. Because of the high infection to disease ratio, the cost prohibitiveness of any form of therapy of lymphoma in cattle, and the poor prospects for a vaccine, the emphasis of veterinary epidemiologic studies has been on the investigation of the modes of transmission of BLV and on the development of control and eradication programs to prevent the transmission of the agent and its associated detrimental economic consequences. During the early 1980's, the results of these studies were being placed into effect for the cattle population, and interest in EBL and BLV began to wane. However, the recent discovery of the human T lymphocytotrophic retroviruses (HTLV I, HTLV II, HIV) and their associated diseases (adult T cell leukemia, hairy cell leukemia, AIDS), as well as new animal lymphocytrophic retroviruses (bovine immunodeficiency-like virus or bovine lentivirus, feline immunodeficiency virus) has caused resurgence of interest in EBL and BLV. This interest is prompted by the potential usefulness of this natural disease of animals as a model system to study newly recognized human and animal

diseases, as well as the fear of BLV and other animal retroviruses as zoonotic agents. The epidemiologic, zoonotic and other pertinent features of EBL and BLV arising from numerous studies have been reviewed here in order to enable veterinary epidemiologists to understand the most recent developments about BLV in the bovine population and to enable human epidemiologists to utilize those developments as a potential basis for understanding and further investigating various aspects of retrovirus infections of humans at the epidemiologic, clinical, cellular and subcellular levels.

BRIEF HISTORY OF EBL

The initial descriptions of leukosis in cattle appeared in German medical literature in 1871 (1). Thus, Europe is considered to be the homeland of enzootic bovine leukosis. EBL is thought to have originated specifically in the Memel area, and the disease then moved westward (2). Since the number of leukotic tumor cases in West Germany increased above previous levels after World War II, extensive research on bovine leukosis was initiated in that country (3).

Numerous reports of EBL arising in virtually every other eastern European country also appeared during the post-war period. European cattle probably infected with BLV were imported from the shores of the Baltic sea to the U.S. at the end of the nineteenth century. Though the initial report of EBL in the U.S. appeared prior to WWII, several reports followed the post-war era (4,5). These reports suggest that EBL was a common disease in the U.S. during that time. The infection has spread in American and Canadian cattle and is now highly prevalent in many dairy

herds in these countries. The direction of importation of cattle has reversed, and highly sought U.S. and Canadian cattle infected with BLV have contributed to the dissemination of the virus to cattle in numerous other countries in Europe and South America as these farmers attempt to upgrade their herds.

BLV: THE ETIOLOGIC AGENT OF EBL

Early investigators of EBL suspected an infectious etiology of the disease. Their suspicions were based upon the occurrence of tumors in some herds but not in others. A high prevalence of lymphosarcoma was observed in Swedish cattle in which the practice of using fresh bovine blood infected with Babesia bovis as an immunization procedure against piroplasmosis had been carried out for a seven year duration, thus raising suspicion of the transfer of an infectious agent in the blood (6). The development of "naturally occurring" lymphatic leukosis of unknown origin in a single flock of sheep was reported simultaneously (7), and the piroplasmosis studies of cattle stimulated Enke to recall that the sheep with leukosis had received injections of Babesia-infected blood from a cow in a herd with EBL several years prior to the leukosis epidemic in the sheep. The use of sheep in BLV research intensified these suspicions of an infectious agent. An experimental study resulted in the successful induction of lymphosarcoma in sheep with bovine lymphosarcoma material (8), thus leading Enke to suspect that the "naturally occurring" leukosis originally reported in sheep was not at all natural for the ovine species, but was induced by an infectious agent in the blood of the Babesia-infected cows.

Viruses had been shown to cause tumors in the chicken, mouse, cat, and monkey (9), and valiant searches for a similar agent in cattle were made during the sixties. A significant breakthrough came in 1969 (10) when it was reported that the leukocytes of some cattle, after culture for 2 to 3 days, produced virus particles (Fig. 1.1, Fig 1.2) similar to the particles from cells of other species with leukemia. The virus was isolated only in cows with adult lymphosarcoma, but not in cattle with sporadic bovine leukosis, the calf form of leukosis, or the skin form of leukosis. In herds with multiple cases of EBL, the virus was isolated from all cows with PL, a benign lymphoproliferative state of BLV infection. The virus was not isolated from cattle in EBL negative herds. Several other reports of the observation of these viral particles soon followed the initial report (11-14). Calves inoculated with cultures of the virus became infected themselves and some developed PL, thus confirming its transmissibility and its role in EBL (15). Antibodies to the virus were found in infected cattle (16,17), and the development of subsequent serologic tests enabled large-scale epidemiologic investigations of the bovine population (13,14). The results of all of these early studies provided sufficient evidence to fulfill most of the virologic and seroepidemiologic criteria necessary for the establishment of a cause-effect relationship between cancer and viruses (20).

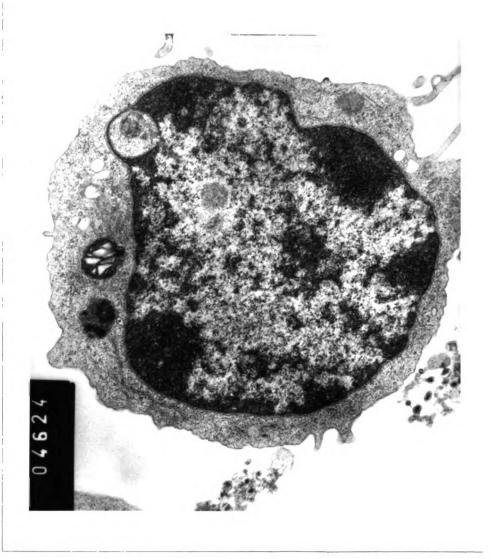

Fig. 1.1. Bovine leukemia virus particle budding (VB) from the surface of a lymphocyte, cell-free (CF) viral particles, and a lymphocyte nuclear pocket (LNP). Courtesy of Dr. A. F. Weber, Dept. of Veterinary Biology, Univ. of Minnesota.

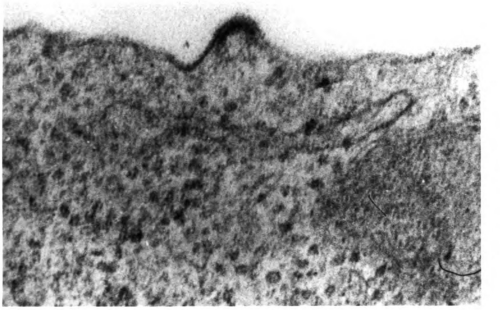

Fig. 1.2. Bovine leukemia virus particle budding (VB) from the surface of a lymphocyte.

DESCRIPTIVE EPIDEMIOLOGY OF EBL AND BLV

A review of the descriptive epidemiology of EBL and BLV has yielded information of variable quality. There are two reasons for this: 1) appropriately designed descriptive epidemiologic studies of the agent and the disease are rare, and 2) neither the disease or the agent is notifiable except in a few countries. Most of the studies were sample surveys or prospective cohort studies of a small number of cattle herds (21). The sampling techniques in many of the studies were nonrandom or were not described and thus preclude making reliable inferences about the population from which the animals were chosen.

Data on the occurrence of EBL have been published periodically in two sources (22-24). Different terminology, i.e. "bovine leukosis" versus "enzootic bovine leukosis" was used by the two investigators, and the incidence of EBL was reported for the calendar years 1979 and 1983 using semi-quantitative or discrete scales to classify a country, the experimental unit, as having had or not having had cattle with EBL. Thus, the quantitative assessment of the number of affected animals is very limited when such a classification system is used. Understandably, the most complete information about EBL was available from Western European countries which are member countries of the Common European Community, many of which have official EBL eradication programs (21). Consequently, information about the incidence of EBL was incomplete for most other countries. There have been numerous seroepidemiological surveys in which the AGID test as been used to study the prevalence of BLV infection (21). Many of the studies of BLV infection, like those of EBL, also suffer from inappropriate sampling techniques, and thus are not

indicative of the seroprevalence of BLV infection in the population. One of the earliest descriptive epidemiologic studies in which there was evidence for unbiased estimates was a study of BLV infection in Florida (25). Eighteen dairy and 28 beef herds were selected from four geographical regions by stratified random sampling procedures in which the various strata were type of production, (i.e. dairy versus beef), size of the herd, species, and breed. The antibody point prevalence as determined by the AGID test in 7768 dairy cattle older than 18 months was 47.8 per cent, and in 4911 beef cattle it was 6.7 per cent. The antibody prevalence was positively associated with age in both dairy and beef cattle, though much more so in dairy cattle, and the antibody prevalence of Jersey cattle exceeded that of other dairy breeds. There was no association of antibody prevalence with species or sex. Only the antibody prevalence of dairy cattle in Venezuela exceeded that of Florida (21,26).

A study of the antibody prevalence of BLV infection in Michigan dairy cattle was done using a combination of stratified, cluster, and simple random procedures (27). The state was stratified into agricultural districts and a density-weighted, proportionate sample of dairy cattle herds was selected from each district. The herds in each district were stratified based on herd size, and herds to be sampled were selected randomly from each stratum. Sixty-six per cent of cattle two years of age and older in each herd were sampled for BLV antibodies using the AGID test. Cattle from all seven districts were SP, and the age-adjusted BLV antibody prevalence of 3132 cattle from 82 herds was between 24 per cent and 36 per cent. The antibody prevalence increased significantly as the age increased and it was significantly greater in

open herds. The prevalence of antibodies was not influenced by the number of cases of EBL, the size of the herd, or the type of cattle housing.

In Canada, sera from 38,297 cattle from 990 (0.48%) of 206,900 dairy and beef herds were examined for BLV antibodies using the AGID test (28). The herd antibody prevalence was 40 per cent (n = 116) of 287 dairy herds and 11 per cent (n = 79) of 703 beef herds. The dairy cattle antibody prevalence was 9.3 per cent (95% CI = 8.9 to 9.7%), and the prevalence in beef cattle was 0.5 per cent (95% CI 0.4 to 0.6%).

In member countries of the CEC, countries in which there are BLV eradication programs, the entire cattle population is still being tested, and the antibody prevalence rarely exceeds 0.5 to 1.5 per cent. Results also have been reported from several other countries in which there was insufficient evidence about the entire bovine population (21). The data was usually reported as a herd point prevalence rate, i.e. the total number of herds having cattle + the number of herds tested, or as a cattle prevalence rate, i.e. the total number of cattle having antibodies + the total number of cattle tested. The sampling techniques were questionable or were not reported in many cases.

Epidemiologic studies of BLV infection in individual dairy cattle herds (29-32) have yielded prevalence rates as high as 95 per cent (33), though such a high prevalence probably occurs infrequently. Studies of these herds also have been conducted to investigate risk factors associated with the natural transmission of BLV-infection. Factors such as herd size, management procedures, type of production, breed, age, parity, population density and seasonal influences have been studied (34). Herd to herd variations with respect to these factors do not allow

accurate conclusions about the usefulness of the results in improving the control of BLV infection under natural conditions.

In addition to BLV surveys of large populations in which the endpoint was antibody point prevalence, several longitudinal studies of BLV infection in herds have been done (35-38). The cattle were usually stratified into four to five age cohorts and prevalence and incidence rates among cows and calves were determined repeatedly. The general conclusions from these studies were that the prevalence and incidence of BLV infection was greater in cattle 24 months of age and older, and that no more than six to 10 per cent of calves acquired prenatal infections (39). One of the greatest contributions of this series of studies was the repeated demonstration of low antibody prevalence in young cattle and an antibody-prevalence threshold beginning in cattle 24 months of age, the age at which they join the milking herd. These findings were significant in that they formed the basis for the hypothesis that the initiation of close physical contact as well as intensive management practices associated with the increased human intervention in cattle beginning at 1.5 to 2.0 years are important risk factors in the horizontal transmission of BLV infection to previously seronegative young cattle. Simultaneous reports were confirming the horizontal transmissibility of BLV infection (40-42), and investigations of the risk factors associated with human intervention would follow (see below). The seroprevalence of BLV infection has been summarized in figure 1.3.

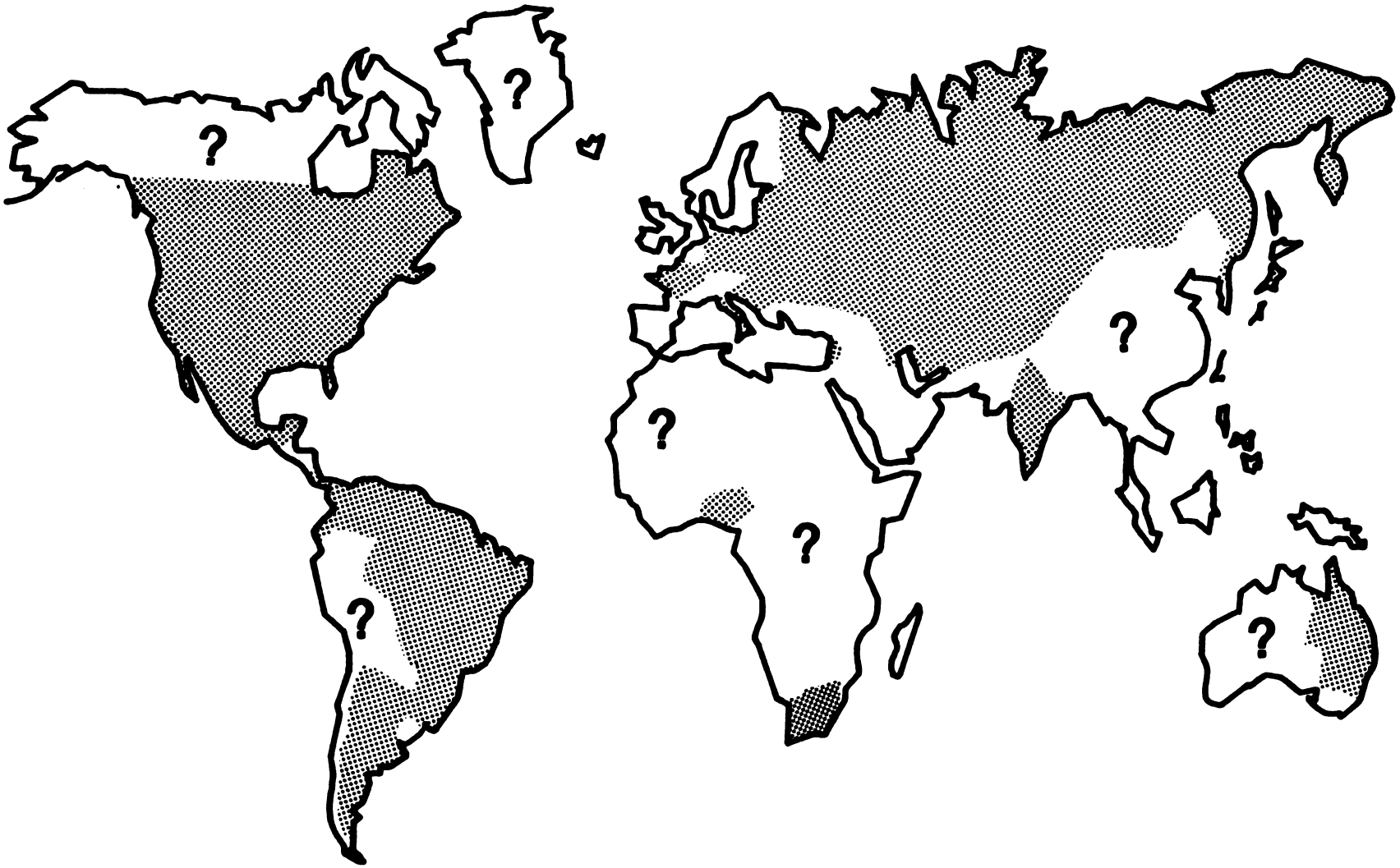

Fig. 1.3. Geographical distribution of BLV seropositive cattle. ?, no studies reported.

DISEASE CHARACTERISTICS OF EBL AND BLV INFECTION

The clinical symptoms (Table 1.1) associated with the deposition of solid tumors in cattle with EBL have been reviewed previously (43-45). Although the lymph nodes are the primary site (Fig. 1.4), tumors may be deposited in virtually every organ. Persistent lymphocytosis was defined by the International Committee on Bovine Leukosis as an increase in the absolute lymphocyte count of at least three standard deviations above the normal mean for a specific breed and age group of cattle in EBL-free herds (46). Additional criteria are that PL cattle have no detectable clinical manifestations of neoplastic lymphoproliferative lesions, and the lymphocytosis must persist for at least three consecutive months (47).

Lymphocytes from cattle with PL are infected with BLV (48,49), but cytogenetic studies have failed to reveal abnormal cells in cattle with PL when compared to cattle with EBL (50). In the absence of abnormal cells, objections have been raised to claims that PL is a pre-neoplastic phase, or a subclinical form of EBL. Instead PL is considered to be a benign proliferation of B lymphocytes. The prevalence of PL in BLV-infected cattle is 33 per cent (51). Thus, not all infected cattle develop PL; however, at least 95 per cent of cattle with PL are infected with BLV, and this supports an etiologic role of BLV in causing PL. Results of early epidemiologic studies of EBL herds indicated that both PL and EBL clustered along familial lines (52-55). Additional evidence was provided when three groups of 38 seronegative, colostrum-deprived calves obtained from cattle families with a history of being either EBL negative/PL negative, EBL positive/PL negative, or EBL positive/PL

Table 1.1. Prevalence of the common symptoms of EBL. †, from 298 hospitalized cattle; ‡, from 1100 field cattle. From references 43 and 44.

Symptoms	%†	%‡
Weight Loss		80
Agalactia		77
Lymphadenopathy (Enlargement)	58	58
Anorexia	62	52
Posterior Paresis/Paralysis	16	41
Fever		23
Exopthalmos	9	20
Labored breathing		14.3
GI obstruction	19	8.7
Myocardial abnormality	64	7
Abnormal blood lymphocytes	64	

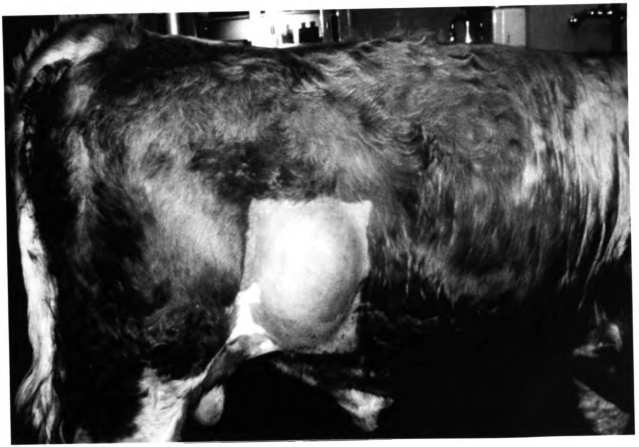

Fig. 1.4. Adult multicentric lymphosarcoma. Enlargement of the prefemoral lymph node in a cow with EBL.

positive were studied prospectively to determine the incidence of PL among the three groups (51). The mean antibody prevalence among the three families was approximately equal (86.6±5%), but the incidence of PL was 0 per cent, 21 per cent, and 88 per cent, respectively. Conclusions from these studies supported the hypothesis that the propensity to develop either PL or EBL was not merely a consequence of BLV infection, but was determined partly by genetic constitution. Since only 5 percent of all cattle with PL develop EBL, and 35 per cent of the cattle with EBL have no history of PL, the genetic basis for PL and EBL are thought to be different (51). Evidence for bovine lymphocyte antigen (BoLA)-linked resistance and susceptibility to the development of PL was reported recently (56). Tests of association between BoLA phenotypes/haplotypes and two categorical traits (infection by BLV and PL) were done using a panel of national and international bovine alloantisera. Cows with BoLA-w8 specificity were at increased risk of being seropositive. Resistance to PL among seropositive cattle was associated with BoLA-DA7, whereas susceptibility was associated with BoLA-DA12.3. In addition to supporting previous studies of genetic susceptibility, evidence to support the genetic selection for resistance to B cell proliferation and the development of PL in BLV-infected herds was presented.

The term bovine leukemia virus is somewhat misleading, since malignant lymphoma (Fig.1.5) is the rule and frank leukemia is the exception to BLV infection. However, leukemia does occur in about 10 per cent of all EBL cases (57). The cells have large, round undifferentiated nuclei with large nucleoli, basophilic cytoplasm, and mitotic figures.

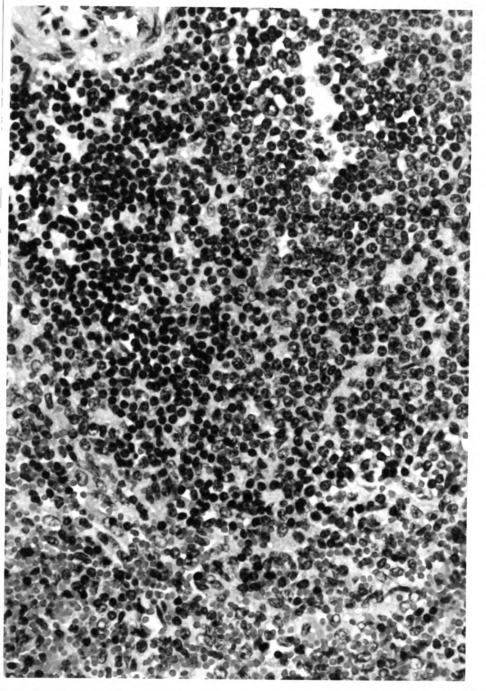

Fig. 1.5. Neoplastic lymphocytes from a lymphoid tumor in a cow with EBL.

Abnormalities of the karyotypes include aneuploidy and additional chromosomes, and the constancy of these features suggests that the tumors cells are monoclonal (58).

BLV DIAGNOSTIC TESTS

Agar Gel Immunodiffusion (AGID) Test. The first serological test for the diagnosis of bovine leukemia virus infection was the AGID test which utilized the internal protein p24 of the virus (16). The simplicity, practicality and economical advantages of the AGID test resulted in its rapid adoption and widespread usage in diagnostic settings. The development of the AGID test was extremely important for epidemiologists, because it was used to show that the prevalence of antibodies to BLV was higher than suspected based upon clinical and hematological observations (i.e. persistent lymphocytosis and tumor screening). Thus, it appeared that the AGID test was more sensitive as a detector of BLV infection. The hematologic and tumor screening tests were subsequently abandoned by many countries and the AGIDp24 test was adopted as the official screening test for BLV antibodies.

An ether-sensitive glycoprotein of the BLV virion was discovered in 1975 (59). AGID tests in which this glycoprotein, later named gp51, was used were found to be even more sensitive than the AGID gp24 test, and AGID gp51 has now replaced or is combined with gp24 as the antigen in the AGID test. Leukassay B (Pitman-Moore, Inc, Washington Crossing, NJ 08560), a commercially available serological test, is now used by most, if not all, diagnostic laboratories in the U.S. to detect BLV antibodies.

As stated earlier, the AGIDgp51 test indicates the presence of specific antibody induced by infection with BLV. This antibody was first thought to appear eight to 12 weeks after experimental infections. More recent studies (60) have shown that antibodies may be detected as early as three weeks post exposure. Obviously, like most infections, the initial appearance of the antibodies may depend upon the dose of virus received by the host as well as other features of the immune system unique to individual animals.

BLV infected cattle are considered to be permanent virus carriers. Any animal greater than approximately six months of age and having a positive immunodiffusion test result should be considered permanently infected. However, seropositivity cannot be correlated with presently existing or even subsequent tumor formation. Tumor formation is the exception rather than the rule as a result of infection BLV and also with some other oncogenic retroviruses viruses. A negative AGIDgp51 test indicates freedom from exposure to BLV for three to 12 weeks prior to collection of the serum sample.

The AGIDgp51 test is considered highly sensitive and specific when compared to the radioimmunoassay (61). In spite of the ideal characteristics, both false positive and false negative reactions may occur in some calves up to six or seven months of age. These calves usually will have consumed colostrum from their respective dams who are seropositive, or less frequently from some other seropositive dam. Much of the antibody in colostrum is due to a shift of immunoglobulins from the dam's circulation into the mammary gland during the late periparturient period. Thus, antibodies to any infectious agent to which the dam has been exposed may appear in the colostrum, be consumed and

absorbed by the calf, and then become detectable in the calf's serum within 12 to 24 hours postnatally. Calves having seropositive tests as a result of consumption of colostrum are rarely if ever infected with BLV at that time. These passive antibodies to BLV (62-64) and bovine respiratory syncytial virus (65) have been shown to gradually decay during the first six to seven months of life (Fig. 1.6).

Not all positive AGIDgp51 tests of calves' sera result from false positive tests. Four to eight per cent of calves from BLV seropositive dams in naturally infected herds are infected with BLV at birth (66). These infections probably occur as a result of BLV transplacental infection during the gestation period. This conclusion is based upon the fact that these calves were found to be seropositive when precolostral serum samples which were collected within minutes of birth were analyzed. Calves which are infected by other possible means, for example, traumatic injury in the birth canal, would not be expected to have positive serotests until at least two to three weeks postpartum, the minimum time required for a primary immune response to become detectable. Thus, these calves cannot be expected to develop a negative AGIDgp51 test at six to seven months of age. Though passively-acquired antibodies may decline, the calf's own active immune response to the infection causes the antibody titer to persist, thus giving rise to the persistently positive AGID test result (Fig. 1.6). False negative AGIDgp51 results may be obtained from calves and/or cows. Both calves and cows have undetectable antibody levels during the two weeks after their initial exposure to BLV. Cows may also have undetectable antibody in the presence of infection with BLV during the periparturient period (67). The reason for this is that the same shift of antibody from the dam's circulation into her

Fig. 1.6

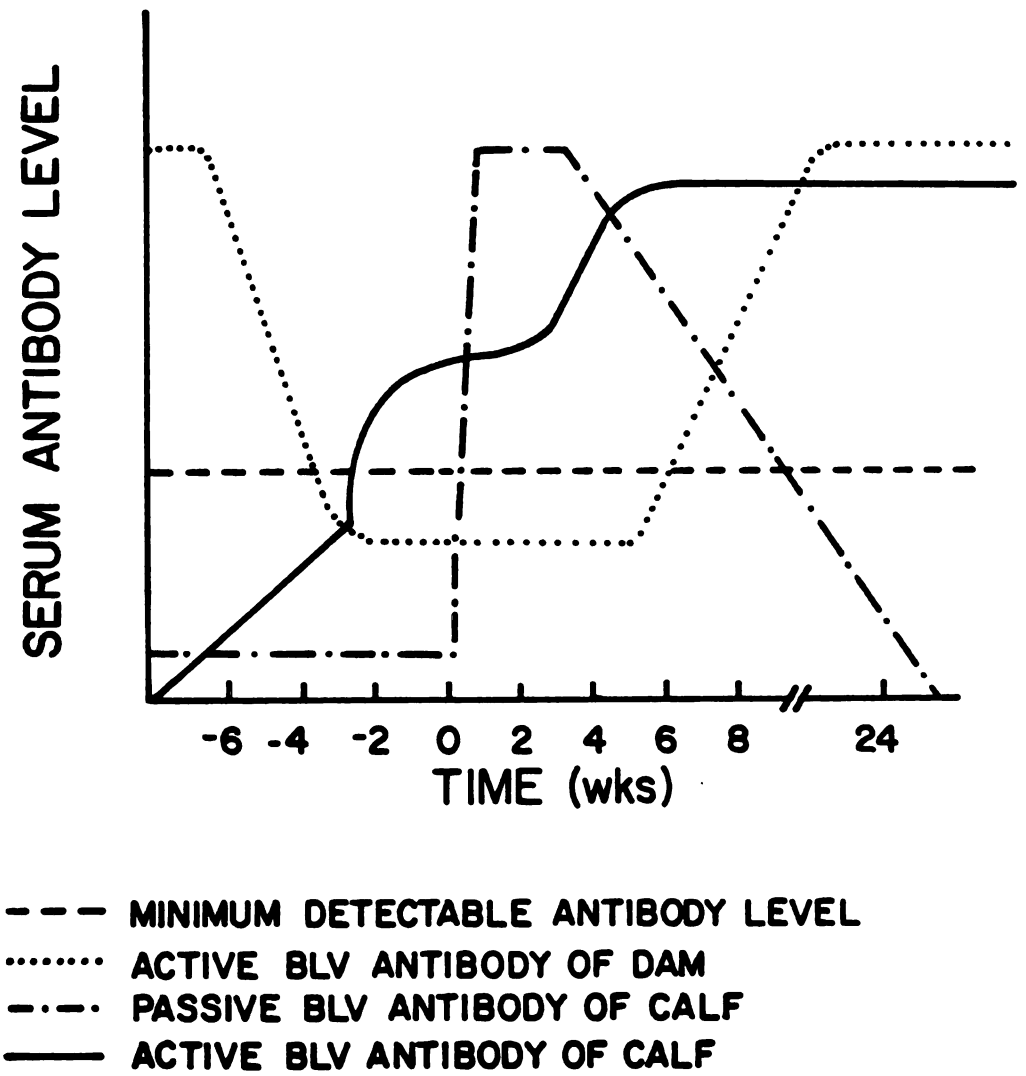

Fig. 1.6. Pre- and postpartum interrelationships of anti-BLV antibody in the dam, the fetus, and the calf.

colostrum, eventually giving rise to the false positive serotests in her offspring, may give rise to false negative serotests in the dams (Fig. 1.6). Thus serologic test results of dams sampled approximately two to six weeks pre- and postpartum should be interpreted with extreme caution. Our past experience suggests that a preponderance of seropositive dams who develop lymphosarcoma seem to do so during the periparturient period. Serum samples analyzed by AGIDgp51 during this period to support a clinical diagnosis of BLV-induced lymphosarcoma may frequently be negative, even when the animal is actually infected with BLV and has tumors. In dams without lymphosarcoma, these antibody levels usually return to detectable levels around six weeks postpartum. The high mortality rate associated with the development of lymphosarcoma has prohibited even short-term prospective studies of antibody patterns in periparturient dams with lymphosarcoma. A final reason for false negative serotests is that some cattle simply may not develop high levels of precipitating antibody after infection.

Radioimmunoassay. Radioimmunoassays employing both p24 and gp51 antigens have been developed (68-70) and have been used in epidemiological studies of BLV infection. The sensitivity of RIAp24 was found to be equal to that of RIAgp51 when undiluted serum was used (71). Though RIA is presently considered one of the most sensitive diagnostic tests, the added sensitivity has been found to be useful only for detection of BLV antibodies in cattle which had been exposed no longer than two weeks, in milk samples, and in serum samples from periparturient dams. Otherwise, the usefullness of RIA does not exceed that of the AGID test. Thus, considering the economic investment in expensive laboratory equipment and the expertise required for RIA, the AGID remains the

preferred test and the only official serological test for BLV.

ELISA. Considerable effort has been spent evaluating various technical modifications and practical uses of an ELISA test (72), and ELISA has also been compared with other serological tests. In one study, the sensitivity and specificity of ELISA equaled that of the AGID test, when sera from cattle from three BLV-infected herds was compared to sera from cattle from 19 uninfected herds(73). The sensitivity and specificity of ELISA in another study of several thousand cattle 98.5 per cent and 99.9 per cent, respectively. High reproducibility of ELISA was demonstrated when two consecutive tests on sera from 132 cattle in an infected herd were evaluated (74). ELISA has been used to classify entire herds of cattle as being infected or uninfected by evaluating pooled sera, i.e. combined sera from as many as 75 cattle. This procedure would result in a marked reduction of the work required if large scale studies were to be done (75,76). The superior sensitivity of ELISA on pooled serum samples allowed detection of antibodies in herds with a prevalence of less than one per cent, whereas the AGID test detected only 50 per cent of the herds detected by ELISA.

The capability of detecting antibodies in milk would be of extreme practical value since it not only would reduce animal stress during sampling, but also the requirements for laboratory personnel and equipment to process serum samples would be reduced (77). The sensitivity of ELISA on milk samples from individual animals equaled that of serum samples. However, due to a 27-fold reduction of antibodies in milk, the sensitivity on pooled milk samples was reduced, and herds with prevalence rates less than 10 per cent were not detectable. Another disadvantage of milk ELISA is that nonlactating cattle cannot be sampled

and, as with the AGID test, serum ELISA does not distinguish between active and passively-acquired antibodies in calves.

Tumor Associated Antigen (TAA)

Specific tumor-associated antigens have been detected on EBL tumor cells, as well as lymphocytes of BLV-infected cattle with no evidence of lymphosarcoma (78,79). Monoclonal antibodies to 13 antigens on EBL tumor cells were produced recently (80), and three of these MAB's specific for a 74000 MW polypeptide on EBL tumor cells were used to study cells from seronegative, seropositive, and cattle with PL (81). TAA was easily detectable in EBL tumor tissues. A double-labeling procedure used to detect B lymphocytes via surface immunoglobulins and to detect TAA via indirect immunofluorescence revealed a strong association between the two, and a complement dependent antibody cytotoxicity test of EBL tumor cells was strongly positive and specific for these cells. While only 10 per cent of the seropositive cattle were TAA-positive, PBL from PL+ cattle and both PBL and tumor cells from cattle with lymphosarcoma were TAA-positive.

Though no studies have been conducted, TAA may become a useful epidemiologic tool to detect cattle with subclinical leukemia/lymphoma since many of these cattle may have normal leucocyte counts, no detectable tumors, and may be seronegative occasionally. Although there have been arguments that PL is a benign lymphoproliferative condition not associated with leukemia/lympoma, the prevalence of cattle with PL and coexisting TAA suggests the contrary. Thus, TAA may be used in conjunction with PL or to replace PL as a tool for detection of cattle at increased risk of developing leukemia/lymphoma. TAA would be especially useful since the diagnosis of cattle with PL as part of a BLV control

program would require collection of at least two or three blood samples at bimonthly intervals in order to demonstrate a persistently high cell count.

TRANSMISSION OF BLV

Transmission of BLV in Various Body Fluids. BLV-infected lymphocytes rarely produce cell-free virus (82), viral RNA (83), or viral antigens (84,85) unless they are cultured in vitro while being stimulated simultaneously with the lymphocyte mitogens concanavalin A or phytohemagglutinin A. Since infectious BLV is rarely produced in vivo, most susceptible cattle are thought to become infected by exposure to virus-infected lymphocytes and not by cell-free virus,the latter of which is the case for many other viruses. Thus, any means by which BLV-infected lymphocytes can be transmitted from one cow to another is logically considered to be a potential means of transmission of BLV infection. This principle has formed the basis for many of the research studies on the transmission of BLV infection.

Since most evidence suggests that the transmission of BLV infection occurs horizontally and postnatally, studies involving transmission have been directed accordingly. Consequently, many of those studies have focused on evaluating the hypothesis that a variety of bovine tissues, body secretions and excretions harbor BLV-infected lymphocytes and consequently serve as suitable media for the transmission of BLV. It appears that prolonged, close physical contact is required for the transmission of BLV infection. However, logic dictates that since BLV has been found only in lymphocytes and there is no cell-free virus, close

contact alone is insufficient to transmit the infection. Exchange of contaminated biological materials would be required, and the degree of infectivity of these tissues, secretions and excretions would be limited by the quantity of infected lymphocytes in them.

Among the list of materials which have been studied are blood, saliva, nasal secretions, bronchoalveolar washings, colostrum, milk, urine, feces, semen, uterine lavage fluids and embryos. Under normal conditions, only blood and to a lesser extent colostrum and milk would be expected to have a relatively high cellular component and thus a higher degree of infectivity. The results of research studies closely coincide with these expectations. The medium that has been studied most frequently is blood. In one of the most recent experiments (86) either 10 ul (45,240 lymphocytes) or 1 ul (4,524 lymphocytes) of whole blood from a BLV seropositive six-year-old Holstein cow was injected into groups of four calves each by one of four possible routes, IM, IV, SC, or ID. Weekly serological tests using the AGID test revealed that calves receiving 10 ul of blood had seroconverted as early as three weeks and those receiving 1 ul of blood had seroconverted as early as four weeks post inoculation. Seven of the eight calves had seroconverted by week eight. All calves seroconverted eventually. Other studies conducted in a manner similar to this study produced similar results (87). Successful (88) as well as unsuccessful (89,90) attempts to transmit BLV by using saliva from seropositive cows has been demonstrated. In the latter case, transmission failure occurred in spite of the injection of large volumes of saliva. The infectivity of colostrum and milk (92-94) has been demonstrated repeatedly. Since the objective of these studies was to determine if there was any degree of infectivity, the recipient often was

exposed to the milk and colostrum via an unnatural route such as injection. Thus, exposure to these two media via a more natural route may actually result in reduced or even abolished transmission. Although bronchoalveolar washings (95) are infective, contents of nasal swabs (89,90,95) have not been shown to be so. Neither urine (88-90), semen (91,96,97), with one exception (98), or feces (88) have been shown to be infectious. The one exception with semen was associated with a collection procedure involving manipulation of the donor's urethra and accessory sex glands per rectum, a procedure which is associated with enhanced contamination of semen with blood. Although infectivity of uterine lavage fluids has been demonstrated(99) these fluids can be contaminated accidentally with blood subsequent to traumatic injury.

Transmission of BLV by Various Routes

Since the objective of the previous studies were to identify even remote sources of infectivity, the routes of exposure for the recipients often were unnatural in that substances which would have been encountered normally by the dermal or aerosol route were injected parenterally, thus guaranteeing receipt of a dose of inoculum which would rarely be encountered under normal conditions. After confirmation of the infectivity of some materials, the next approach to the transmission studies was to evaluate route of susceptibility to infection with BLV. The inocula, primarily blood cells or other virus-infected cells which had been shown to be infectious previously, were administered in relatively high doses via all traditional potential routes of exposure. Susceptibility to infection was confirmed via the intradermal (86,100), intramuscular (86), intravenous (86,87), intraperitoneal, intranasal (90,100), intrauterine (95,100), aerosol and oral (100) routes.

Risk Factors for the Transmission of BLV

Confirmation of the infectivity of certain biological materials and the high susceptibility of certain routes of infection would prove useful in the design of BLV control programs. Considering that BLV is cell-associated and that only some of the biological materials containing blood cells are infectious, it should be expected that BLV would not be transmitted as easily as some common respiratory microbes of cattle. Several epidemiologic studies have shown that the prevalence of BLV infection, particularly in dairy cattle herds, may be as high as 90 to 95 per cent (101). More importantly, recent studies (101,102) have shown that the incidence rate increases significantly in cattle approximately 16-24 months of age and older. The prevalence and incidence rates in younger animals are much lower than in older animals, and some of the few infections in younger animals probably arise from prenatal and not postnatal transmission of BLV. What then are possible explanations for this pattern of infection rates which seem to increase as cattle enter a more intensively managed phase of the life cycle involving increased human intervention? Even if cattle are closely contacting each other physically, and given that lymphocyte-contaminated media are essential as a source of BLV, how is this infectious medium transferred? Much attention has been given recently to the hypothesis that the intervention of humans with their livestock management and veterinary medical practices may influence the transmission of BLV greatly. Among these practices are injections of medications by any route, vaccinating, dehorning, castrating, eartagging, tattooing, supernumerary teat removal, rectal palpation, tuberculosis testing, feeding colostrum and/or milk, and blood transfusion. This hypothesis was initially based upon the

presence of clusters of leukosis in cattle subsequent to vaccination for piroplasmosis with Babesia-infected blood from leukotic cattle (82). It was later reported that cattle from whom blood samples were collected immediately after BLV-seropositive animals were sampled were at 8 times greater risk of subsequent seroconversion than cattle which were not sampled (103-105).

To evaluate the role of dehorning (106) in the transmission of BLV, 71 calves were divided into groups of 20, 30, and 21 and were dehorned using one of three procedures: 1) the gouge technique using a Barnes dehorner followed immediately by cauterization for 30 seconds with the electric dehorner, disinfection of the dehorner in chlorhexidine diacetate after dehorning each calf, followed by the application of a ferrous sulfate powder to each wound; 2) the gouge technique, followed by extraction of the cornual vessels; and 3) no dehorning (controls).The AGID test was used to detect seroconversion in the 14, 22, and 19 calves at risk of infection in each of the three groups prior to dehorning. The incidence of seroconversion in the three groups was zero per cent, 31.81 per cent, and five per cent, respectively. The relative risk of seroconversion for group two when compared to the control group was 6.04, thus suggesting that cauterization of the surgery site and disinfection of the instruments were protective, whereas failure to do so increased the risk of infection. The incidence rate of group two was significantly higher than the control group ($P = 0.003$) and group one ($P = 0.002$), but the incidence of group one was not different ($P = 0.56$) from the control group (Fisher's exact test). It was concluded that BLV could be transmitted during dehorning and that, at least in combination, sanitation and hemostasis prevented the infection of susceptible calves.

Tattooing is a requirement for calf-hood vaccination against brucellosis and it may be used also to permanently identify valuable livestock as a deterrent to theft. The sites frequently used for tattooing, the ears and muzzle, are highly vascular and the pressure applied by the instrument during the procedure may easily disrupt the integrity of the blood vessels. Lymphocytes from an infected animal may then be transmitted to seronegative animals on the contaminated instrument. To evaluate the ability of six tattoo dyes and pastes to possibly act as a virucide and prevent the transmission of BLV infection from BLV seropositive calves to sheep, six groups each consisting of four sheep were tattooed,the tattooing of each sheep being done immediately following the tattooing of a calf (107). The control groups consisted of sheep tattooed without the dyes. Eighty-eight per cent of the 24 treated sheep and 100 per cent of the six control sheep seroconverted after five weeks. The evidence suggested that not only do the dyes not prevent BLV transmission, but that tattoos may be contaminated with sufficient blood to infect cattle. Further support for tattoos as a means of transmission was provided when it was shown that 90 per cent of 30 seronegative sheep seroconverted within five weeks of being tattooed with an instrument which had been used to tattoo a seropositive calf immediately prior to tattooing each sheep (107). Though the recipients in this study were not bovine, the results are probably no less applicable. Similar studies using cattle as recipients have not been reported.

During rectal palpation of cattle, hemorrhage frequently arises from trauma of vessels lining the rectal mucosa and contaminates the examiner's obstetrical sleeve. Many, if not most, examiners also consider it to be impractical and expensive to use a new OB sleeve on

each cow, unless the bloody sleeve is torn. Failure to exchange sleeves between palpation of different animals could result in the transmission of BLV, especially if the sleeve is contaminated with blood. One of the initial studies (108) to test this hypothesis involved the rectal administration of 500 ml or 50.0 ml of pooled whole blood from three BLV seropositive adult Holstein cows to each of two adult Holstein cows and two yearling ewes. Rectal palpation of the cows was simulated before the infusion was done; the sheep were not palpated but the blood was infused with an infusion pipette. Seroconversion occurred as early as three weeks in the sheep and all sheep and cattle had seroconverted by five weeks. Negative control sheep which had been given 1.0 ml of blood intravenous from a seronegative cow remained seronegative. Although the volume of blood infused into these animals far exceeded that which contaminates OB sleeves, the study did demonstrate that transmission of BLV via rectal palpation is possible. Recently and in a timely fashion, the results of a second study (109) evaluating the role of rectal palpation were published. The rectums of ten, four-month-old, male BLV seronegative Holstein calves was evacuated of feces, 1.0 ml of whole blood (4620 lymphocytes per ul) was placed on the gloved palm and 1.0 ml on the forearm of the examiner, and the rectum was palpated for 30 seconds to simulate pregnancy detection or artificial insemination. This same procedure was repeated for three consecutive weeks at weekly intervals. All calves exposed had seroconverted by five weeks post exposure whereas controls remained seronegative. Not only did the results of this study support previous findings, but it also more accurately addressed the question as to whether contaminated OB sleeves contain sufficient blood to transmit BLV infection.

Restrictions on the international movement of BLV-infected cattle occurred concomitantly with the development of embryo transfer into a commercial industry. It became apparent that the export of embryos could serve as a practical alternative to the export of live animals if embryos were shown to be resistant to BLV infection. To evaluate the hypothesis that embryos could not be infected by BLV, embryos were collected from BLV seropositive donors, about half of which had been bred artificially with semen from BLV seropositive bulls and these embryos were transferred to BLV seronegative recipients (110); none of 50 live calves obtained from 57 conceptions developed antibodies to BLV by six months of age. Zona pellucida intact ova, morula and blastocysts exposed "in vitro" to BLV followed by lavage remained negative for BLV. Zona pellucida intact embryos and hatched embryos were also exposed "in vitro" to BLV, and transferred to seronegative recipients. Though no pregnancies resulted, the recipients had not developed antibodies to BLV four months post-transfer. Other studies have produced similar results (99,111,112). One hundred sixteen embryos transplanted from BLV seropositive cows into seronegative recipients resulted in the birth of 116 calves which were seronegative at birth based upon precolostral samples and samples taken at three months of age. Five of 29 calves arising from the transfer of embryos to seropositive recipients were seropositive at birth (precolostral sample) 3, 6,and 12 months of age. Neither of 30 embryos from seronegative donors transplanted into seronegative recipient nor six embryos transplanted into seropositive recipients gave rise to seropositive offspring. Thus, negative recipients, irrespective of whether their donors are seropositive, never gave rise to seropositive offspring. Though only seropositive recipients and donors were

associated (and inconsistently so) with seropositive offspring, the disparity of the sample size precludes adequate interpretation of the results. Nevertheless, the evidence favors embryo transfer as a poor means of transfer of BLV.

Though there have been several studies specifically investigating the role of semen (89,96,97,113) in the transmission of BLV, only one (98) has incriminated semen. Sheep inoculated intraperitoneally with semen from a bull which was positive via the AGID test, immunofluorescence, and virus isolation seroconverted and BLV was demonstrated via electron microscopy and virus isolation. Since manual massage of the genital tract, a technique which may result in diapedesis of leukocytes into seminal fluid, was employed to collect the semen,it has been argued that seminal transmission, though demonstrated here, probably occurs rarely if properly collected, leukocyte-free semen is used. The lack of transmission via semen (96) was subsequently demonstrated when large volumes of pooled, undiluted, leukocyte-free semen from seropositive bulls was inoculated IP into sheep. Since semen used for AI may be diluted 50-fold preceding insemination, a high degree of leukocyte contamination may be a prerequisite for successful seminal transmission of BLV. Other studies (113) support the failure of seminal transmission. Semen from 24 seropositive bulls accounted for 48.3 percent of 2110 units of semen inseminated into a herd averaging about 200 seronegative cows during a five year period (113). None of the cows seroconverted. There is evidence that semen may actually contain inhibitors of BLV. In simulated breeding trials, semen experimentally contaminated with either cell-free BLV or BLV-infected lymphocytes did not result in seroconversion of the inseminated dams and their offspring

or even sheep (114).

The practice of livestock therapeutic and prophylactic health care has always involved rapidly processing large numbers of animals. Part of this rapid processing procedure involves the repeated use of the same equipment, e.g. syringes and needles on numerous animals. This equipment often has been designed accordingly, thus setting the stage for iatrogenic transmission of BLV. The aforementioned experimental studies confirmed iatrogenic transmission as a potential major means of transmission of BLV, and a classic example of iatrogenic transmission in a natural setting along with its devastating economic consequences was reported recently (115). Blood-based, bivalent babesiosis and anaplasmosis (tick-fever) vaccines used in Queensland, Australia, have been obtained from AGID negative calves since 1981. The antibody prevalence in a herd of 329 cattle undergoing EBL/BLV accreditation-free status increased from 0.3 per cent three weeks prior to vaccination for tick fever to 27.98 per cent 16 weeks after vaccination with the bivalent vaccine. Repeated AGID tests, p24 immunoblots, and a sheep bioassay were used to confirm infection of the cattle with BLV. Though serum was not available from either of two donors (suspect A and suspect B) suspected to be the source of the BLV infection, an eliminative process indicated that 48 per cent of 27 cattle vaccinated with donor A blood (Anaplasma sp.) and donor B (Babesia sp.) were seropositive. However, 0.0 per cent of 27 donor B only (Babesia sp.) vaccinates were seropositive ($P = 0.00001$, Fisher's exact test). In a second herd, none of 25 donor B and donor C (Anaplasma sp.) vaccinates were seropositive. This suggested that blood from donor A could be infectious but blood from donors B and C was not. Both tick fever stabilates were eliminated as a source of

infection, since cattle exposed to the stabilates failed to seroconvert and were not infectious to sheep. Steers used to provide whole blood for transfusion into parasitemic donor calves A and B were not infectious to sheep, and destruction of BLV in the vaccine diluent, bovine serum, had already been demonstrated.

Approximately 14000 doses of vaccine from suspect donor A, each containing 50 times the minimum number of lymphocytes capable of producing infection by BLV, had been distributed. Since the population at risk for BLV infection was unknown, the number of cattle infected by the vaccine could not be determined. However, antibody prevalence rather than incidence between vaccinated and nonvaccinated dairy and beef cattle were compared and 62 per cent of 255 vaccinated dairy cattle were SP whereas 6.1 per cent of 575 nonvaccinated cattle were SP($P < 0.001$, chi-square test). Fifty-two per cent of 1866 vaccinated beef cattle were seropositive, but only 1.4 per cent of 1292 non-vaccinated beef cattle were seropositive ($P < 0.001$, chi-square test). Failure to detect the infected donor A was explained by 1) donor A was the offspring of a SN dam which provided no colostral antibody to protect the calf, 2) donor A was infected with BLV when it was fed milk from infected cows, and 3) donor A's active antibody response was below the detection level upon arrival at the center. Donor calf A was seronegative upon its arrival at the vaccine production center and was isolated immediately. Blood for the vaccine was collected 42 days later, but it was not stated whether the calf was serotested during the 42-day period to determine if it should have been given a retrospective reclassification as an incubationary carrier upon its day of arrival at the center. Economic losses were incurred due to compensation for the livestock owners, field

and laboratory investigations and further dissemination of BLV. The need for a sensitive, rapid, economical BLV antigen detection test was realized.

Insect and arthropod transmission

The impetus for investigating the role of insects in the transmission of BLV was probably derived from strong evidence that insects may serve as vectors for the transmission of equine infectious anemia virus, a macrophage-associated retrovirus of horses which exists also as a blood-borne, cell-free virus (116-119). Interruption of vectors feeding on horses separated by 109-146 meters has resulted in the return of the vectors to their original host rather than moving to a different host (120). These feeding habits suggested that vectors on animals in closer proximity to each other may alternate feeding on different hosts and serve as a source of infection for susceptible hosts. Freely roaming cattle and other domestic ruminant species, because of herd instinct, usually are not more than several meters apart, and in many types of livestock housing facilities, they may be no more than two to three meters apart for several consecutive years. The closeness of these animals provides ample opportunity for potential vectors to alternate their hosts and transmit infectious agents in the process. In one of the earliest studies of vector transmission of BLV (121), seven BLV-free cattle 21-26 months old were initially exposed during midsummer, the vector-high season, to 25 to 30 BLV-infected adult cattle followed by exposure of seven BLV-free cattle to the same group of infected cattle during midwinter. All cattle exposed during midsummer had seroconverted after four months whereas only one of the animals exposed for four consecutive months during the vector-free season seroconverted. When the

remaining six seronegative animals were further exposed during the succeeding vector season, an additional three animals seroconverted. BLV infection of lymphocytes which were collected from the midguts of horseflies (Tabanus nigrovitatus) that were allowed to feed on a BLV-infected cow was confirmed via a syncytium infectivity assay. Since these animals had remained free of BLV infection for approximately 24 months prior to exposure, the evidence suggested an association between blood-sucking vectors and seroconversion to BLV.

Empirical evidence suggested that the incidence of seroconversion of Japanese cattle was higher after the tabanid fly season (122). Vector transmission was studied by transferring flies which had fed on a persistently lymphocytotic, BLV-infected cow to each of three sheared lambs for 131 to 140 ad libitum feeding periods each during a four-day period. All exposed lambs had seroconverted by day 40 post-exposure. Though the design of the experimental protocol may have resulted in the transfer of an exceedingly large number of infected lymphocytes, the results do demonstrate that vector transmission of BLV should not be ignored. Mosquitoes have been used as a model vector for biting flies to study the transmission of BLV infection (123). Three species of mosquitoes (Anopheles sp.) were fed blood from a persistently lymphocytotic, BLV-infected cow, after which a mean of 92 mouthparts and heads from the mosquitoes were injected subcutaneously into sheep. Four of nine sheep had seroconverted after three months based on AGID and RIA test results. These investigators concluded that since the successful transfer of the infection occurred in spite of the small size of mosquito mouthparts, both tabanids as well as stable flies, the latter of which are biting flies whose painful bites often preclude their finishing

a blood meal on a single host, may serve as mechanical-vector candidates for the transmission of BLV infection. These investigators proceeded in much the same way to study the role of the stable fly (Stomoxys calcitrans), horn fly (Haematobia irritans), deer fly (Chrysops sp.), and tabanids (Tabanus atratus), all biting flies, in the transmission of BLV (124).Varying proportions of small numbers of lambs and calves given one, freshly prepared, large dose of the mouthparts of each potential vector seroconverted three to six months after exposure. Seroconversion did not occur if a smaller dose of the mouthparts were injected during a five-day period. Seroconversion also occurred after injection of the infected mouthparts of deer and horseflies, mouthparts which could be obtained only artificially by dousing them with infected blood. Transmission of BLV did not occur when stable flies were allowed to be fed first on an infected cow followed by feeding on non-infected calves. Only one of six cows seroconverted when hundreds of flies of each species, primarily stable and horn flies, were allowed to feed on the infected cows which was followed by feeding on the noninfected cows. The infected cows were separated from the noninfected cows only by a netwire barrier. The seroconversion was not attributed to infection by the flies, however. The duration of contact was three months, the time required for the one seroconversion was three weeks, and the five seronegative animals remained seronegative for at least seven months after their initial exposure. The latter two situations more closely resemble events occurring in the natural environment for livestock. Since the deer and horse flies, unlike the stable and horn flies, refused to feed not only on the infected blood in gauze pads but also not on the infected cattle, the results of the latter two experiments could be interpreted with

respect to stable and hornflies mainly.

The conclusions from these experiments were that the number of bites required to cause transfer of BLV was dependent on 1) the quantity of blood retained on the mouthparts after feeding, 2) infectivity of the donor cow, 3)the susceptibility of the recipient animals, and 4) the feeding habits and other characteristics of the vector which favor transmission.

Larger, engorging feeders, in contrast to the smaller, finicky feeders, genetically susceptible recipient cattle, aged SP donor cattle with persistent lymphocytosis, and vectors which frequently alternate hosts were considered to be important factors in the successful transmission of BLV by vectors. The investigators concluded that the ability of horn flies and stable flies to serve as BLV vectors was minimal due to 1) failure to infect sheep with mouthparts from stable flies dissected 1 hour after blood feeding, 2) the inability to infect calves with small numbers of simulated insect bites (50 horn fly mouthparts) over a period of five days, 3) the failure of bites from 75 stable flies transferred from a BLV-infected cow to cause infection in calves, and 4)the failure of horn flies and stable flies to transmit BLV under conditions as close to natural as possible (in a screened enclosure). It was also concluded that the important role for stable flies, horn flies and tabanids in the transmission of BLV may be limited to local areas of heavy fly infestation where numerous bites from insects interrupted from feeding on infected animals are common. Repeatability of the transmission of BLV to sheep by Tabanus atratus was recently demonstrated (125).

The latest study (126) of vector transmission of BLV was stimulated

by the immediately preceding conclusion and by the absence of reports of natural transmission due to high vector-density, i.e. reports where large numbers of potential vectors were allowed free access to cattle and also were allowed to move freely among BLV-infected and BLV-free cattle. The study was designed to answer two questions: 1) Can the stable fly (Stomoxys calcitrans) transmit BLV?, 2)Could early viremia in newly BLV-infected cattle result in successful natural transmission of BLV by this insect? The donor cattle were BLV seropositive cattle, some of which had persistent lymphocytosis, lymphosarcoma and all of which had high expression of BLV particles in their lymphocytes as determined by electron microscopic examination. Seronegative recipient calves were tethered one to three meters from infected donor cows which were either exposed or not exposed simultaneously to stable flies for 27 to 97 days. None of the recipient calves had seroconverted to the AGID test 90 days after exposure to the risk factor was terminated.

To explore the effects of viremia, calves were injected with $8x10^5$ or $2.8x10^8$ lymphocytes plus 10.0 ml of whole blood from a BLV-infected cow with 35 per cent VE. Each "viremic" donor calf was then tethered alternately between two BLV-seronegative recipient calves in either a fly-free or fly-infected compartment. All four injected calves seroconverted between 11 and 37 days post exposure, and none of recipient calves had seroconverted by 63 to 90 days post exposure. An experiment on manual transmission was done in which 50 starved stable flies were allowed to complete a meal on each of three BLV-SN calves after having been allowed to feed on a BLV-SP cow with 42 per cent VE. One calf seroconverted 84 days post-exposure. Scanning EM studies of the mouth-parts of the stable fly led to the conclusion that the maximum

estimated volume of its oral cavity was $29.4x10^6$ Ul. Since it had been demonstrated previously that approximately 2500 lymphocytes (5.0 Ul of whole blood) were sufficient to transmit BLV infection via the intradermal route, 3950 mouthpart-volumes of blood from an infected animal with a lymphocyte count of 10,500 per Ul would be required to transmit the infection. A preference of tabanids to feed on mares rather than on foals has been demonstrated also (127). Likewise, the ratio of cow:calf fly-bites was 3.9:1 to 9.3:1. Flies which were disturbed while feeding either returned to the same host or discontinued feeding. Thus, the small capacity of the stable fly's mouthpart, its feeding habits, and the failure to demonstrate seroconversion in calves exposed to flies which had fed on BLV SP cattle led the investigators to conclude that the transmission of BLV by stable flies is probably a rare event. The tick Boophilus microplus commonly parasitizes cattle in tropical climates (128) in which BLV infection is highly prevalent. Blood was aspirated from the midguts of engorged ticks taken from a seropositive cow and $4x10^6$ leukocytes were inoculated subcutaneously into each of four seronegative sheep. Two of the four sheep had seroconverted after four weeks postinoculation and type C viral particles in their leukocytes were demonstrated. In a second experiment of tick-borne transmission, Ixodes ricinus were allowed to feed on cattle positive for BLV and BLV-gp antibodies. BLV-p24 antigen and infectious virus were demonstrated in engorged ticks. Seronegative bulls exposed to starved adult ticks became positive when examined using an ELISA and an immunofluorescence test, but not with the AGID test. Though transmission of BLV by ticks under experimental conditions was successful, studies of the natural transmission by ticks have not been reported (129). Of this series of

vector studies, the most valuable ones are probably those which were designed to study transmission in an environment which mimics the natural environment. Nevertheless, the conclusions generally were the same, i.e. insect transmission of BLV probably occurs rarely under natural conditions.

The risk factors associated with the natural transmission of BLV have been summarized in table 1.2. To appreciate the importance of single risk factors, the degree of risk of each factor was assigned with the assumption that no single risk factor is interacting with or is being influenced by another risk factor simultaneously. However, cattle in a natural environment may be exposed, sometimes simultaneously, to virtually every risk factor listed during its lifetime,and the cumulative effects of exposure to several seemingly rather innocuous risk factors may result in infection with BLV.

Cattle are exposed to certain risk factors at very specific points during their lifetime, and there is a strong association between exposure and the antibody prevalence in various age cohorts (67). This association has been used to propose an epidemiologic model of the transmission of BLV in a herd of cattle (Fig. 1.7). The majority of infections are acquired horizontally rather than vertically. The model demonstrates the low antibody prevalence in calves which results from infrequent prenatal infections from SP dams, whereas SN dams always give birth to SN offspring. Managerial intervention by humans early in the calf's life initiates exposure to certain risk factors which continues throughout the cow's life. Infection by BLV may result from these exposures, and seroconverts subsequently can crossover from the SN to the SP population,thus contributing to the increasing prevalence of BLV

Table 1.2. Risk factors associated with the natural transmission of BLV. * means risk was confirmed (↑↑), or was empirically derived (↑), or is unknown(?), or is low (↓).

Risk Factor		Degree of Risk*
1	Dam's serologic status	↑ but rare if SP; ↓ if SN
2	Colostrum from SP dam	↓
3	Milk from SP dam	↓
4	Licking, e.g. to claim newborns	↓
5	Ear tagging	↑↑
6	Dehorning	↑↑
7	Teat excision	↑
8	Castration	↑
9	Tuberculosis (TB) test	↓

Table 1.2. Continued.

Risk Factor		Degree of Risk*
10	Tattoo	↑↑
11	Vaccination	↑
12	Palpation	↑↑
13	Insemination procedure	?
14	Insect/arthropod population	↓
15	Imported/exported cattle	↑↑ if SP; ↓ if SN
16	Close contact of SP and SN cattle	↑↑
17	Injections, e.g. medication administration	↑↑
18	Persistent lymphocytosis	↑↑
19	Embryo transfer	↓
20	Transfusion from SP donor	↑

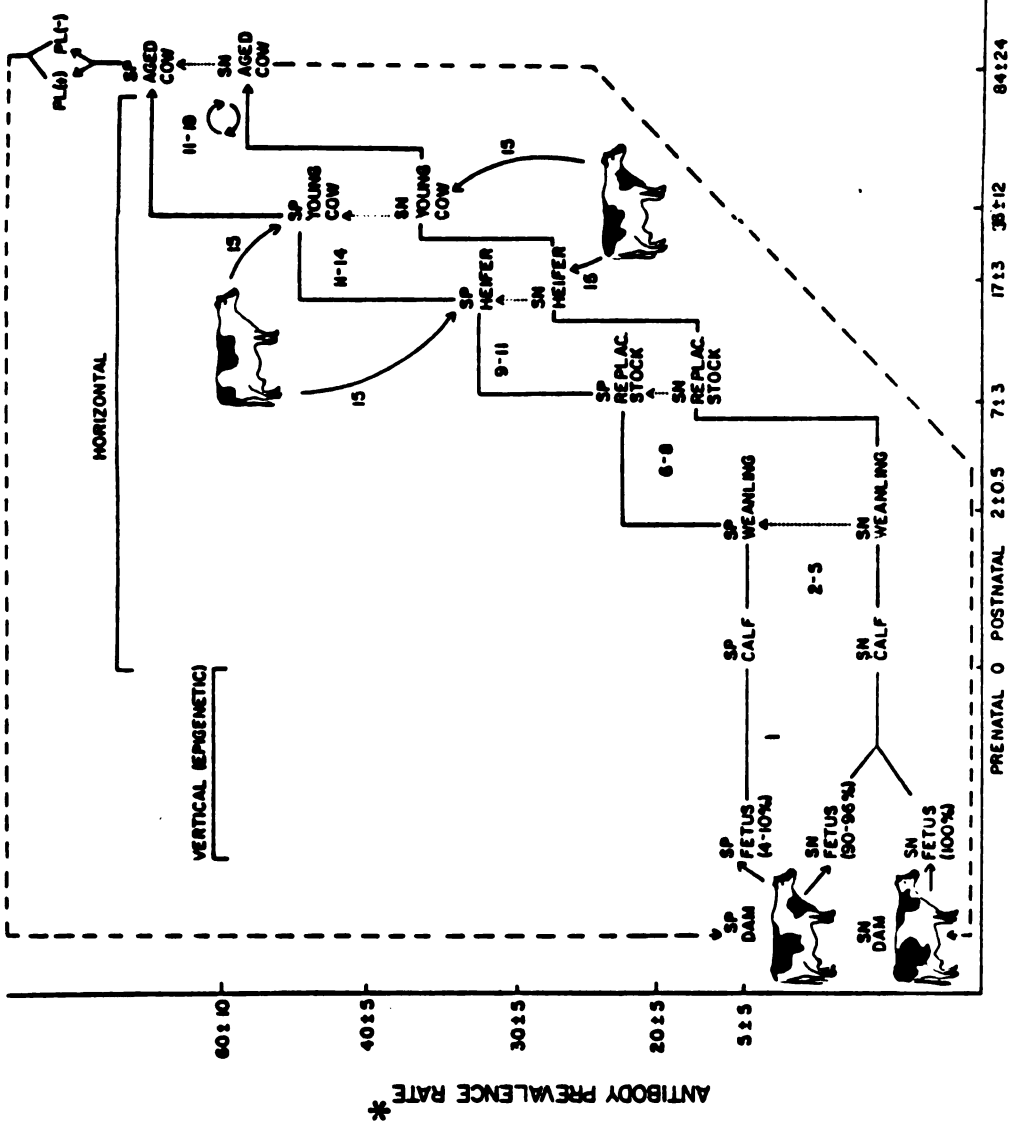

Fig. 1.7. Epidemiologic model of the transmission of BLV infection in cattle. SN, seronegative; SP, seropositive; PL, persistent lymphocytosis; 1-19, risk factors from Table 1.2; ....., seroconversion; *, scale applies to SP cattle only.

infection in a herd.. Once adulthood has been attained, cyclic exposure to risk factors 11 to 19 (Table 1.2) may occur, so that cattle which escaped infection due to exposure to the well-documented risk factors 1 to 10 (Table 1.2) may still become infected. Older seropositive cattle with PL may be a major source of infection for the younger lactating cattle. It is tempting also to hypothesize that the highly infectious cattle with PL may be the predominant source of the SP calves, but this hypothesis awaits investigation. Purchase of infected cattle may be the source of BLV in previously uninfected herds.

Cross Species Transmission of BLV

Although BLV was isolated initially and is thought to occur primarily in domesticated cattle of the species Bos taurus, infections have occurred in other cattle and nonbovine species as a result of both natural and experimental studies (Table 1.3). Naturally-acquired infections were found in various breeds of the cattle species Bos indicus, in Indian and Australian type buffaloes, in various breeds of sheep raised on a dairy cattle farm, and in wild capybaras (130). Experimental infections have been most successful in sheep, and sheep appear to be highly susceptible to the development of leukemia and solid tumors, possibly even more so than cattle. Although goats and pigs seroconvert, the pathologic effects of BLV infection are attenuated in these species. Infection of the chicken, chipmunk, deer, guinea pig, mouse,quail, domestic and wild rabbit, Sprague-Dawley rat, cat, and dog have also been attempted (151). During a 0.5 to 54 month observation period, persistent gp51 antibody was found in the deer, domestic rabbit and cat, whereas only one positive test was found in the dog, cottontail rabbit, and the rat.

Table 1.3. Cross-species infection by BLV. N,naturally acquired infection; E,experimentally acquired infection.

Species	Type Infection	Reference #
sheep	N	130
sheep	E	8,131,133,134,138-141,143-147
goat	E	134-136,138,144
pig	E	132,144
buffalo	N	130,150
rabbit	E	148
chimpanzee	E	137
monkey	E	149
capybara	N	130
antelope	E	142

N, natural; E, experimental

ZOONOTIC POTENTIAL OF BLV AND EBL

EBL is probably the most frequently diagnosed neoplastic disease of cattle,particularly dairy cattle. BLV particles were found in the milk of cows (152,153), and this finding has raised queries about the transmissibility of BLV to humans. The hypothesis that BLV may be involved in some human lymphatic leukemias was reproposed recently (154), and the identification of HTLV I antibody in the serum and spinal fluid of patients with multiple sclerosis prompted speculation about the involvement of BLV in multiple sclerosis (155). The close contact of humans with domestic cattle, and the consumption of milk and meat products raised the question of an association between human lymphoproliferative diseases and EBL. This question is most pertinent to livestock farmers whose cattle are infected with BLV and to persons, e.g. veterinarians and slaughterhouse personnel having close contact with the infected livestock. An investigation of livestock farmers revealed no cases of human leukemia/lymphoma in 4108 person-years at risk for occupants of 98 Michigan cattle farms on which at least 130 cases of EBL had been diagnosed (156) (Table 1.4). There was one case of human leukemia in 7968 person-years at risk for occupants of 212 control farms. Studies in Denmark (157,158) and Sweden (159,160) failed to demonstrate an association between human and bovine leukemia. The two Danish studies, the former of which was carried out in a geographical region in which the prevalence of EBL was high, included all human and bovine cases arising during a 16-year and also a five-year period. An 11 year study in Sweden (160) was done in an area with a high prevalence of EBL, and no association was found between human leukemia of farm and village dwellers

Table 1.4. Zoonotic potential of BLV. *,person-years-at-risk.

	Risk Factor	Experimental Unit(s)	Sample Size	Endpoint	Investigator's Result/Conclusion	Year	Investigator
1	EBL cows	Farm-inhabitant	entire population	lymphoid leukemia	Negative	1968	Jensen (157)
2	EBL cows	Farm-inhabitant	case=4108* controls=7968	"	Negative	1970	Priester (156)
3	EBL cows	Farm-inhabitant	1523 (find)	"	Negative	1968	Henricson (159)
						1971	Henriksen (158)
4	EBL cows	Farm-inhabitant	cases=47 controls=210	"	Negative	1975	Kvarnfors (160)
5	EBL cows; raw dairy products	Farm-inhabitant	Exposed=145; nonexposed=47	"	Negative	1976	Caldwell (162)
6	cattle	Farm-inhabitant	5	BLV antibody	Negative	1975	Olson (163)
		veterinarian	80	"	"	"	"
		lab. worker	15	"	"	"	"
		human leukemia patient	100	"	"	"	"
7	EBL cows; raw dairy products	Farm-inhabitant	45	"	Negative	1977	Donham (166)
		veterinarian	73	"	"		"
		human leukemia patient	30	"	"		"
8	undeterminable	human cancer patient	cases=203; controls=197	"	Negative	1975	Gilden (167)
9	BLV infected cows; raw dairy products	human lymphoid leukemia patients	cases=7; controls=14	herd antibody prevalence	Negative	1987	Donham (169)
	cattle	human lymphoid leukemia patients	"	cow density/100 KM^2			
10	Guardian with farm experience raw dairy products	children with acute lymphocytic leukemia and non-Hodgkin's lymphoma	cases=174; controls=136	integration of BLV provirus in lymphocyte DNA	Negative	1988	Bender (170)

* Person-years at risk

and EBL.

The discovery of serologic tests (161) for the detection of antibodies to BLV enabled studies of an association between BLV infection and human leukemia, just as it was done with BLV and bovine leukemia. A seroepidemiologic case-control study (Table 1.4) was conducted using 145 persons (cases) who were in contact with infected cattle and drank unpasteurized milk from cattle in 30 BLV-infected herds. The controls were forty-seven persons with the same two exposure factors in 13 BLV-free herds. The investigators failed to demonstrate an association between herds with seropositive cattle and leukemia in humans (162). None of the 192 persons had complement-fixing antibodies to the glycoprotein 51 antigen of BLV. Antibodies to p24, the core protein of BLV, were not found in 80 Wisconsin veterinarians with extensive contact with cattle, in 15 laboratory workers in a BLV research laboratory, in five dairy farmers with BLV-infected herds, and in 100 persons with a variety of leukemias (163,164), or in 50 other persons with varying degrees of exposure to BLV (165) (Table 1.4). Antibodies to p24 were absent in 73 Iowa veterinarians and in 30 persons with leukemia (166). There were no seropositive reactions to BLV gp antigen in 45 persons, of whom 75 per cent consumed raw milk, in contact with 21 dairy herds with a BLV antibody mean prevalence of 33 per cent and in which lymphosarcoma had been diagnosed (166). There were no seropositive reactions to the p24 antigen in a case-control study of an association between a variety of malignant human diseases and BLV infection (167). Blood from 50 humans with varying exposure to BLV were tested with a fluorescent antibody test, a radial imunodiffusion test for glycoprotein antigens, a syncytium infectivity assay and an immunodiffusion test for antibodies to

p24 and p51. None of the samples, many of which were examined repeatedly, gave positive results for either test.

An epidemiologic study was conducted in Iowa to compare the incidence of various leukemias and to determine if there was an association between high leukemia rates in rural dwellers and leukoses of farm animals, especially EBL. Lymphoid (168) leukemias were predominant in the humans, rural dwellers were at only a slightly higher risk for chronic lymphoid leukemia, as were older males, and younger males were at a slightly higher risk for acute lymphoid leukemia. There were significant correlations between the incidence of lymphoid leukemia in humans and cattle density (Table 1.4). However, the BLV antibody prevalence of these cattle, a more appropriate risk factor than mere cattle density, was not determined. There was a slight association between acute lymphocytic leukemia in human males and the incidence of EBL.

A case-control study (169) was conducted in Iowa to determine if there was an association between human acute lymphocytic leukemia and BLV via occupational contact (Table 1.4). Regular occupational contact was defined as 1) contact with cattle at least three times weekly for at least six consecutive months, 2) owner of the herd, 3) a farm employee, or 4) a consumer of raw milk from cattle in the herd. The associations which were examined were 1) the antibody prevalence of BLV infection in dairy herds with which the cases and controls had either occupational (i.e. regular) contact or from which they had consumed raw milk and 2) the density of dairy cattle in townships where affected persons and controls lived. Significantly fewer herds with which leukemia patients had contact were seropositive when compared with herds with which

nonleukemic control patients had contact. Dairy cattle density, defined as the number of dairy cattle per 100 km^2, was consistently higher in townships where the nonaffected persons resided. The results of this study are of limited value since the BLV antibody prevalence rates were available on only seven of potentially 243 herds with which the 243 cases of human leukemia were associated. Since this was a case-control study, many of the herds owned by the persons who developed leukemia, i.e.the cases, were no longer available for serological testing. Socioeconomic factors, and dispersion of the herds by the owners who contracted leukemia were explanations given for the poor availability of herds to be serotested. Since the prevalence rates were determined for only seven herds associated with seven cases which arose during an 11-year period, it was assumed that the antibody prevalence rates in those seven herds were constant during the period. Thus, to some extent, it was assumed also that the cases were exposed to the risk factor, i.e. regular occupational contact with cattle.

The most recent study of the zoonotic potential of BLV involved testing the association between genomic integration of BLV and childhood acute lymphoblastic leukemia/non-Hodgkin's lymphoma (170). DNA samples from the lymphocytes of 157 patients with ALL/NHL and 136 controls were studied by Southern blot analysis using a cloned BLV DNA probe (Table 1.4). BLV proviral DNA was not demonstrated in any of the case or control samples, whereas DNA in a BLV-producing bat cell line was readily demonstrated. The sensitivity of the technique, and therefore the statistical power of this study, was high. However, the study population was restricted to children with ALL/NHL from three midwestern states each with a relatively high density of dairy cattle. Though 50 per cent of

the parents of case children had farming experience which did not necessarily include contact with BLV-infected cattle, and 18 per cent of the cases consumed raw dairy products,the degree of exposure of the cases themselves to the risk factor was not clearly assessed. During the routine daily activities on livestock farms, adults generally have longer and closer direct physical contact with the cattle than children do. Consequently, the external validity of the study and the significance of the results may have been greater if the restriction procedure had not excluded the selection of cases from the adult population.

Though BLV particles have been recovered from milk of infected cattle and transmission of BLV infection has been demonstrated under experimental conditions, pasteurization of milk does inactivate BLV (171,172). The absence of any association between human lymphatic leukemia and BLV, of detectable BLV antibody in humans with vast exposure experience to BLV and the absence of genomic integration of BLV in humans with leukemia/lymphoma provides evidence that humans are at minimal to no risk of acquiring neither BLV infection nor the diseases classically associated with BLV infection.

IMMUNE RESPONSE TO BLV INFECTION

Humoral Immune Response. Cattle infected by BLV can be categorized into one of four groups based upon immunologic and hematologic responses. These are antibody positive (Ab+), antibody positive with persistent lymphocytosis, antibody positive with tumorous leukosis, and antibody positive with tumorous leukosis and concurrent PL. The majority of infected cattle remain in the Ab+ category without ever progressing to the three latter, more pathologic categories. Viremia occurs only during

the initial 10 to 12 days after infection (173). The disappearance of the viremia occurs simultaneously with the rise of neutralizing antibodies against both a glycoprotein (gp51) and a core protein(p24). Infected animals apparently undergo persistent antigenic stimulation, have persistent antibodies, and thus are classified as permanent carriers who are capable of transmitting the infection to susceptible animals in varying degrees.

The humoral immune responses to various specific BLV proteins of naturally infected, seropositive nontumorous cattle and seropositive tumor-bearing cattle were studied in order to identify the differences in expression of BLV proteins and to identify and quantitate the antibody isotypes produced (174). Each animal's serum produced the same Western blot profile when tested against the three viral isolates, thus suggesting homogeneity among the isolates. However, when profiles prepared with several different sera were compared to each other, the profiles were different, suggesting heterogeneity in different animals'responses to seven possible viral antigens. Generally, seropositive leukotic cattle reacted to more antigens than cattle which were seropositive only. All seropositive cattle had antibodies to p24 and gp51. Cattle with EBL also had antibodies to a 12K and a 15K protein, the functions of which have not been identified. These same cattle had antibodies to a 38K protein which is one of three protein products encoded by the tat gene, the mediator of trans-acting transcriptional activation and a key suspect in the leukemogenic process. Cattle with EBL had IgG and IgM antibodies to p24 and gp51. The IgG responses to gp51 were increased in cattle with EBL, and the total serum IgM responses were suppressed in these cattle. Whether the suppression

was a cause or a consequence of EBL was not determined, but it was speculated that decreased IgM levels may indicate a depleted or altered subpopulation of B cells, or an impaired B cell/T cell interaction.

In another study of naturally infected cattle, isotypes of cattle with EBL were not different from normal cattle (175). Other studies of humoral responses to BLV have centered on responses to the various BLV antigens (70). Antibodies were maximal during the tumor phase of the infection. The ratio of serum to milk gp51 antibodies was highly variable, thus confirming the need for a sensitive test, if milk samples were to be used in diagnostic tests. Antibodies to gp51 were shown to appear earlier, reach higher titers, and may exist alone when compared to p24 antibodies (176). A subsequent study confirmed humoral responses to several different viral antigens (177).

In an experimental study to test the effect of immunosuppressive drugs on BLV infection (178), calves were given the drugs either daily for 30 days followed by infection with BLV, or they were infected first with BLV and given one treatment of the drugs after 60 days. BLV was recovered from all exposed calves 18 days after inoculation. Infected animals not receiving the drugs, i.e. drug controls, developed antibodies to gp51 and p24 seven and 21 days after infection, whereas treated animals developed the antibodies two to four weeks later. Both IgG and IgM antibodies against viral antigens were produced, IgG remained constant, and IgM was increased immediately after infection followed by a slight decrease. Although immunosuppressive drugs were given, the dosage required to induce immunosuppression in calves was not predetermined. Consequently, drug-induced immunosuppression was never documented in either group of calves before or after they were exposed to BLV. Thus,

the conclusions that immunosuppression did not enhance the development of grossly diagnosible tumors and that immunological impairment was a consequence and not a cause of BLV infection, are invalid.

In a similar experimental study of BLV infection, BLV was isolated 18 days postinfection also (60). IgG and IgM isotypes to gp51 were detected after 21 days, and isotypes to p24 were detected 14 days after the calves were infected. IgM antibodies to viral antigens were consistently greater than IgG during the 36-week examination period. Weekly total serum IgM levels were reported to have decreased gradually when infected animals were used as their own controls. Though there were uninfected calves designated as controls, the IgM levels of uninfected calves were not compared over time with the IgM levels of infected animals. Their failure to do so did not consider normal biological fluctuations in the IgM levels of infected calves during the study period. A similar method of data analysis was used to compare the total lymphocyte count, sIg+ cells, and E rosette(+) lymphocytes. Thus, the lymphocytosis and the concomitant reversal of the T:B lymphocyte ratio may have been falsely elevated also, since preinfection values of the infected calves, rather than values from parallel control calves were used as a baseline for comparing all postinfection values.

Humoral immune responses of calves experimentally infected with BLV in utero have also been examined (175). Although BLV antibody was not detectable in precolostral sera, calves isolated immediately after birth did develop antibodies 25 to 85 days after birth, or 84 to 162 days after the in utero inoculation, and total serum Ig levels remained normal.

Cell Mediated Immune (CMI) Responses. The initial study of CMI responses in BLV infection tested the hypothesis that cows with PL would

have altered responses to T and B cell mitogens (179). By using the phytohemagglutinin A and pokeweed mitogen in the lymphocyte blastogenesis assay, background counts of 3 cows with PL were reported to be higher than counts from 14 normal cows, but the stimulation indices for these cows were considered lower. There was no statistical analysis of that data. In another study in which concanavalin A, a T cell mitogen, was used in the blastogenesis assay, CMI responses of five cattle with EBL were compared with five normal cattle (180). The stimulation indices of EBL cattle were reported to be significantly lower, thus suggesting the predominance of a B cell population in the EBL cows, but there was no statistical analysis to support that conclusion either.

The CMI responses of calves experimentally infected as fetuses were normal when determined by blastogenesis assay to nonspecific mitogens (175). The same was true for naturally infected cattle except when the mitogen lipopolysaccharide was used. Infected cattle with PL had increased responses to LPS, suggesting that there was an LPS-reactive class of lymphocytes in those cattle. Other investigators have claimed that sera from cows with lymphoma inhibited in vitro immunocyte function, but the results of those studies were difficult to interpret (181,182). There were few reports of the successful application of cellular assays for the detection of antigen reactive T cells, killer (K) cell,or natural killer (NK) cell activity in BLV infection. The results of the blastogenesis assay were not repeatable when a variety of BLV antigens were used to stimulate lymphocytes from infected and diseased animals (175).

The cytotoxic activity of lymphocytes, a function attributed to NK and Killer cells, has been examined during the progress of leukosis in

both cattle and sheep (183). Peripheral blood mononuclear cells (PBMC) obtained only by density gradient centrifugation of whole blood were exposed to a fetal lamb kidney cell line persistently infected with BLV, and a chromium release assay was used to measure NK and K activity against the infected cells. Cells from leukotic cattle and sheep and from persistently lymphocytotic cattle and sheep had significantly lower per cent-specific-lysis of the target cells when compared to normal controls, suggesting a reduction in NK and K activity. However, the PBMC fraction contains monocytes, NK cells, K cells, up to two additional subsets of T lymphocytes, and B cells. Assessment of the function of the various lymphocytes subsets would be achieved best by subfractionation of the mononuclear cells in order to obtain an enriched population of cells specifically responsible for a function being evaluated. Since leukemic cattle and cattle with PL certainly have increased percentages of B lymphocytes in their mononuclear cell fractions, the percentages and numbers of other lymphocyte subsets, e.g. NK and K cells, maybe reduced below those contained in an equal number of mononuclear cells from a normal animal. Thus, tests of hypotheses comparing two different populations in which the number of effector cells has not been established as being equal may lead to conclusions biased away from the null. The statistically significant reduction in the per-cent-specific-lysis of cells from the leukotic animals may be attributed simply to a reduced number of NK and K cells in the mononuclear fractions tested and not to altered NK and K cytolytic activity. Unfortunately, the absence of much needed markers for the various lymphocyte subsets of domestic species precludes alternative approaches to testing hypotheses in which pure populations of lymphocyte subsets are needed.

MOLECULAR BIOLOGY OF BLV

Although a detailed discussion of molecular biology exceeds the scope of epidemiology, molecular biologic tools are increasingly forming the basis for many diagnostic tests that are used being in epidemiologic studies. Thus, at least a superficial understanding of the molecular biology of infectious agents may be essential for proposing epidemiologic hypotheses and selecting diagnostic tools to investigate these hypotheses. The genomic structure of the provirus of BLV has been studied in great detail (184). Like some other retroviruses, the BLV provirus consists of a long terminal repeat at either end, a gag, a pol, and an envelope gene, and a short-open reading frame (185). These components contain all of the genetic information from which the various viral proteins are derived.

There are four major nonglycosylated proteins (p24, p15, p12, p10) and two glycosylated proteins (gp51 and gp30) encoded by the BLV provirus. The p24 was the first recognized BLV structural protein. It has been purified (186), sequenced (187) and was found to be distinct from the major core protein of other retroviruses. However, statistically significant amino acid sequence homology, i.e. 37 per cent, was found between BLV p24 and the major p24 core protein of human T cell leukemia virus I.

The major envelope glycoproteins of BLV particles are gp51 and gp30 (184,185). Monoclonal antibodies obtained in the mouse have been used to identify eight independent antigenic sites (sites A through H) on the gp51 molecule (188). Sites F, G, and H were shown to be involved in determining both virus infectivity and syncytia formation by

BLV-producing cells, and site G was shown to be a target for antibody-mediated, complement-dependent cytotoxicity (191). Whereas gp51 is the external envelope glycoprotein, the main function of BLV gp30 is to anchor the virion glycoprotein complex into the virus membrane, since it contains long stretches of hydrophobic amino acids and shares sequence homology to the anchor glycoproteins of other retroviruses (190,191). The protein p30 has 36 per cent amino acid sequence homology with the viral transmembrane protein of HTLV-I.

Two overlapping open reading frames are located 3′ to the envelope gene. The long open reading frame (LOR or XBL-I) encodes a protein which acts as a trans-activator of transcription (tat) of the BLV provirus (192-196). The LOR region also is presumed to activate transcription of a cellular gene(s) which, in turn, has been proposed to be a key event in the initiation of the transformation of cells. Molecular level analyses using BLV genome-specific probes have shown that all BLV-induced tumors as well as individual cells of these tumors, contain proviral genetic information (49,197,198). Thus BLV is indispensable for the initiation of tumor development. However, BLV may not be indispensable for maintenance of the tumor state in transformed cells, since a large percentage of the tumors have proviruses in which much of the 5′ half of the provirus is deleted. The consistent presence of the 3′ half of the provirus has led to the conclusion that a protein(s), specifically the tat protein, is mandatory to maintain cells in the neoplastic state (199). Minimal to no expression of viral antigen in both bovine and ovine tumor cells has led to the conclusion that BLV becomes dispensable after the neoplastic process has been switched on (200,201).The short open reading frame (XBL-II) encodes a protein of undefined biological

activity. An excellent discussion of further details of facts and hypotheses about BLV-induced cell transformation were reviewed recently (202). Additional discussion of these molecular-level events exceeds the scope of this paper.

EBL AND BLV: ANIMAL MODELS OF HUMAN DISEASE

Epidemiology of ATL and HTLV-I. Nationwide surveys in Japan in the late seventies showed that the proportion of T-cell malignancies was especially large in the perfecture of Kyushu, whereas the proportion of B-cell malignancies in western countries and the USA was larger than in the Kyushu district (203). The excessive rates of malignant lymphomas in the Kyushu district were due to a high incidence of T-cell malignancy. Among T cell lymphomas, adult T cell leukemia was limited to certain geographic areas of Japan (204). Clusters of ATL have been identified in both South Shikoku and Kyushu. The discovery of T-cell growth factor led to the establishment of a method for the long-term growth of human mature T lymphocytes in culture (205). It became feasible to grow human neoplastic T cells routinely in suspension, and this in turn permitted the isolation and characterization of the first human type C retrovirus from cultures of cells derived from individuals with T-cell malignancies, leukemias, and lymphomas consisting of mature lymphatic cells (206). The virus was designated as the prototype of a new class of retroviruses, since it was not related to any previously known animal retroviruses, and it was named human T cell leukemia/lymphoma virus. HTLV-I is not a genetic element in the human germ cell line and, like BLV, the proviral DNA of HTLV-I is not a genetic element incorporated in human normal T cells (207). Thus, HTLV-I is an exogenous retrovirus. Antigens against

the structural proteins p19 and p24 were used in international seroepidemiological studies to detect natural antibodies against HTLV-I in sera of inhabitants of endemic and nonendemic areas of Japan (208). The similarities between ATL and Sézary syndrome, the leukemic form of a cutaneous T-cell lymphoma/leukemia from which HTLV was isolated, suggested a viral etiology of ATL (209). The geographical distribution of ATL cases in Japan supported this suggestion. A controlled seroepidemiological study of patients from ATL-endemic and non-endemic areas of Japan indicated that approximately 90 per cent of ATL patients were seropositive. This finding added support for the suspected association between ATL and HTLV-I (208,210). An independent seroepidemiological study of Japanese patients confirmed the results of the initial study (211), and the virus isolated from cultured ATL cells was shown to be nearly identical to HTLV-I via sequence analysis of its nucleic acid (212). Geographical clusters of HTLV-associated malignancies have been found also in southeastern USA, Central and South America, and equatorial Africa (213). Similar results were obtained from Caribbean patients with T-lymphosarcoma cell leukemia, a disease that is morphologically indistinguishable from Japanese ATL and Sézary syndrome (214).

Comparison of EBL and ATL, and of BLV and HTLV. The initial observations of the biological and biochemical analogies between BLV and HTLV-I were considered to be striking (215,216). Thus, EBL was thought to have great potential for serving as a disease model of ATL, and likewise, BLV could serve as a virus model for HTLV-I (216). Further studies have been done to compare these two diseases and their respective etiologic agents, and the studies have shown that certain epidemiologic,

clinical, pathologic and molecular biologic characteristics of the two diseases and the agents are very similar (Tables 1.5). For example, studies of BLV had shown that the virus could infect cells in the milk, and that these cells could cause BLV infection under experimental conditions (92-94). Seroepidemiological studies of HTLV infection had suggested that horizontal transmission from parents to their offspring was a possible route (217). The BLV studies prompted the investigation and subsequent confirmation of HTLV-infected cells in breast milk of HTLV-carrier mothers (218), and a prospective study confirmed that offspring who consumed infected breast milk from their carrier mothers were at an increased risk of infection when compared to controls who consumed noninfected milk from HTLV carrier mothers (219).

Numerous studies of the transmission of BLV by insects have been undertaken (121-129). These studies have formed the basis for hypotheses about the transmission of HTLV-I by hematophagous insects in tropical areas (220,221). As with BLV, the hypothesis that transmission of HTLV-I requires a heavy insect burden along with interrupted feeding on a seropositive person with lymphocytosis has been proposed (220). This in turn may explain a need for close familial contact during a prolonged period in order for transmission to occur.

Several farm activities and health-care practices for cattle are known to result in the transmission of BLV (Table 1.2). These practices may be useful as a basis for proposing other hypotheses and designing models for epidemiologic studies of the transmission of the HTLV's. At the cellular level, the target cells of BLV and HTLV were thought to have different phenotypes originally, i.e. B lymphocyte versus T lymphocyte (222) which would suggest that the leukemogenic mechanisms could be

Table 1.5. Epidemiologic, pathologic, and molecular biologic comparison of EBL and ATL.

Characteristics	EBL	ATL
disease	enzootic bovine leukosis	adult T cell leukemia
infection:disease ratio	high	high
distribution of disease	pandemic	endemic
geographical clustering	rare	yes
familial clustering	no	yes/no
natural host	cow	human
median age at onset (yrs)	5	varies w/location; 34;46;56
median survival (mos)	weeks to months	months
neoplastic tissues:		
lymph nodes	yes	yes
skin	no	yes
thymus	no	no
organs involved	multiple metastases; thoracic and abdominal organs	hepatosplenomegaly

Table 1.5. Continued.

Characteristics	EBL	ATL
cytologic alterations	pleomorphic; lymphocyte nuclear pockets	pleomorphic
lymphocytic counts	persistent lymphocytosis; leukemia rare; normal frequently	leukemia
hypercalcemia	no	yes
osteolytic lesions	no	yes
immunoglobulin alterations	decreased IgM terminally	increased IgM and IgA; sometimes normal
immunosuppressive	generally not	yes
progressive nonmetastatic myelopathy	no	yes
interleukin 2 secretion	undetermined	increased
interleukin 2 receptors	undetermined	increased

Table 1.6. Epidemiologic, immunologic, and molecular biologic comparison of BLV and HTLV I.

Characteristics	BLV	HTLV-I
type of retrovirus	exogenous; type C	exogenous; type C
natural host	cow	human
transmission type	horizontal 1° = postnatal 2° = prenatal	horizontal 1° = postnatal prenatal?
transmission medium	blood products	blood products
potential transmission mode(s)	needles, surgical tools, etc; transplacental	sexual contact; transfusion; breast milk
antiviral antibody	p24; gp51	p24; p19; p15
target cells	1° = B lymphocyte; also T lymphocyte	T_4 lymphocyte; possibly B lymphocyte
oncogene	no	no
tat gene	yes; pxBL	yes; pxHTLV
provirus integration site: nontumorous cell tumorous cell	 polyclonal monoclonal	 same as BLV
proposed leukemogenic mechanism	transactivator of transcription	same as BLV

different. However, previous investigators have failed to distinguish between classifying the target cells which proliferate versus the target cells which become infected. Although B lymphocytes are the primary cell which proliferates in BLV infection, recent evidence suggests that BLV, like HTLV I, does infect T lymphocytes (223,224).

At the subcellular level, the absence of proviral integration in tumor cells, the monoclonality of these same cells, the absence of an oncogene, and the presence of the tat gene in the DNA of both viruses are unique features of these two viruses (Table 1.6). The presence of defective viruses not capable of replication in some ATL tumor cell lines indicate that viral replication is not required to maintain a state of malignant transformation (225). Therefore, as with EBL, there is no rationale for antiviral therapy of humans with ATL (226). Although the alternative of anticancer chemotherapy will not be used on cattle, cattle with EBL may become useful in clinical trials involving chemotherapeutic agents. All of these characteristics enhance the usefulness of EBL and BLV as a disease model and an agent model, respectively, for the HTLV's at the population, patient, cellular, and molecular level.

FIELD TRIALS TO ERADICATE EBL AND BLV

Nation-Wide Trials. Control and eradication programs for enzootic bovine leukosis have been conducted on a nation wide basis in several Western European countries. These control programs originally utilized the Bendixen Hematologic Key, a hematologic key constructed by Dr. H.J. Bendixen, when it was realized that some clinically healthy cattle in multiple-case leukotic herds constantly had abnormally high lymphocyte

counts (227). The key was used to classify the lymphocyte counts of cattle as normal, suspect, or lymphocytotic. It was considered necessary to make several assumptions (228) in order to use total lymphocyte counts and histologic studies as diagnostic tools for EBL in an official EBL control program. First, persistent lymphocytosis, a lymphocytotic blood picture for a duration of at least two consecutive months, was an indication of leukosis. Secondly, the appearance of animals either with persistent lymphocytosis or with tumorous leukosis in a herd within a two-year period was an indication that the herd was affected with EBL. A herd affected with EBL could not become "EBL-free" simply by removal of affected animals. Instead, the entire affected herd was slaughtered, and a new herd was derived from EBL-free herds. The first official EBL control program was introduced in Denmark in 1959 using these three criteria. EBL became a reportable disease and all adult animals from herds in which EBL cases originated underwent hematological examination. Affected herds were closed, and indemnity was offered to induce the owners to have their entire herd slaughtered. The herd-slaughter policy was continued until 1982.

The official control program was modified slightly on two occasions. Concomitant with the development of electronic cell counters, hematologic tests of all herds in Denmark were initiated between 1969 and 1978. Secondly, bovine leukemia virus was shown to be a likely etiologic agent of EBL in 1972. The AGID test became a valuable diagnostic tool because of its greater specificity and sensitivity relative to the Bendixen key, and it was introduced along with hematologic counts as part of the official control program in 1975.

The use of the hematologic test was discontinued in 1979, and only

the AGID test was used in the official eradication program between 1979 and 1982. A routine herd test consisted of serotesting all cattle older than two years and those between birth and six months of age. Routine testing of all herds was discontinued in 1982. Presently, monitoring involves testing random sera collected from every sixth adult cow to be slaughtered. This equals approximately 10 per cent of the cattle population at slaughterhouses. The results of the official Danish control program showed that the incidence of tumors in adult cattle at the start of the eradication program was at least 10 times greater than it is presently. Only 0.006 per cent of 149,300 cattle at slaughterhouses serotested randomly between 1982 and 1985 were seropositive.

A voluntary control program similar to the Danish program was established in the Federal Republic of Germany in 1964 (229). The discovery that newly infected herds arose from the importation of cattle from PL+ and/or EBL+ herds resulted in placement of trade restrictions on diseased herds. An official EBL/BLV program was introduced in 1973. The regulations of this program were based on the Bendixen Hematologic Key also, and it included yearly herd tests of all cattle two years and older, the introduction of prohibitive measures for infected herds, and the slaughter of affected animals in these herds. In 1978, the AGID test was introduced to the German program also. After the introduction of the AGID test, successful eradication of BLV-infection, based upon serological studies, was attained after five years. One of the reasons for the successful eradication of EBL and BLV in member countries of the EEC is that the prevalence and incidence of animals with persistent lymphocytosis and tumorous leukosis was low enough so that affected animals could be slaughtered without the owners and the state suffering

significant economic losses.

Herd-Based Trials. In other countries, for example the United States and Canada, it would be cost-prohibitive to slaughter seropositive cattle because the prevalence to BLV infection is high, the genetic pool is extremely valuable and highly sought, and neither federal or state indemnity programs are available. Thus all control programs for EBL and BLV are herd-based and are strictly voluntary. The livestock owners in most western countries who adopt these control programs do so 1) because of herd-level economic losses associated with exportation restrictions on their livestock to members of the EEC and other countries or 2) because of losses due to the occasional clustering of tumorous leukosis in their herds. There have been several attempts to establish guidelines to aid these farmers in reducing the incidence and prevalence of EBL and BLV in their herds. These guidelines are based upon a serotest and corrective management strategy rather than serotest and removal (i.e.slaughter). Thus, the serotest and corrective management strategy requires a thorough understanding of the transmission of BLV infection since many persistent carriers will remain in herds utilizing this strategy until removal by natural attrition has occurred.

One of the earliest reported herd-based studies of a BLV control program was conducted on a large stock farm at Kitasato University in Japan (230). A serologic survey of 227 cattle 24 months and older revealed a prevalence rate of 3.52 per cent. These eight seropositive animals were relocated, the herd was closed, and the adult cattle (median = 196; mean = 204+40) at risk of infection were tested on nine successive occasions at approximately a three-month interval. The seropositive animals were removed each time. The incidence rates during the following

36-month period were 2.12 per cent, 11.39 per cent, 5.61 per cent, 3.39 per cent, and 0 per cent for the last five serotests. Seroconversions were restricted primarily to cattle 48 months and older. A similar prospective epidemiologic study was conducted on a 130-cow farm in Canada(231). The animals were classified as leukotic, seropositive or seronegative; leukotic cows were culled and a number of empirical control practices were introduced. The herd was closed, attempts then were made to raise seronegative calves as herd replacements for cattle undergoing normal attrition, and practices to prevent the iatrogenic transfer of infected blood among animals were carried out. Infected calves were raised separately or were culled, BLV-free calves nursed seronegative cows only and were serotested regularly, and the entire herd was serotested biannually. Period antibody prevalence rates were compared for the 24-month period preceding and following the introduction of the control program. A comparison of the pre- and post-antibody prevalence rates showed that the control program was effective in significantly reducing the prevalence rates. Unfortunately, neither the incidence or risk rates, more valuable tools for assessing a reduction in the transmission of BLV infection, could be determined. Attempts have been made to establish a BLV-free dairy herd at the US Dairy Forage Research Center in Madison, WI (232). Only three of 133 cows seroconverted after entering a herd in which the prevalence rate was 12 per cent during the 20-month observation period. Ninety-four per cent of 104 embryo recipients remained seronegative during this same period. The Wisconsin control program closely resembled that used by Ruppanner. Yet another similar study involving nine herds and 867 cattle was conducted in Ontario, Canada (233). Each entire herd was serotested at approximately

six-month intervals for three years, seroconverters were immediately isolated at least 200 M from negative cattle and were culled within 30 days, replacement cattle were isolated and serotested immediately before and after purchase, and contact of experimental animals with animals from other herds was prohibited. The median point prevalence rate for serotest #1 was 22.0 per cent and the median incidence rate for the succeeding eight serotests was 0.56 per cent. Seropositive tests were attributed to naturally acquired infections, e.g. cows that were incubating BLV during the initial serotest, or to violations of the control protocol by the herd owners, e.g. failure to cull seropositive cows. Much of the success of this study was due no doubt to the policy of culling all seropositive cows and calves, even if the calves' positive test results were due to passively acquired antibodies. The policy of indiscriminate culling of seropositive cattle was used to prevent protraction of the control program. Though there is no evidence that anti-BLV sanitary practices were used during this study, the results indicate that success was achieved.

The average expenditure incurred by Agriculture Canada for this project was $1220 per seropositive animal. Even if indemnity were to be paid, some owners still may be resistant to culling valuable seropositive cattle. Thus, these cattle could continuously serve as a source of BLV infected lymphocytes. Failure to remove the seropositive animals has raised concern about retaining SP and SN cattle in close contact on the same premises and its effect on the incidence of BLV infection. Fortunately other investigators (33) simultaneously conducted a 36-month, prospective BLV control study in which a "serotest and segregation" rather than "serotest and slaughter" policy was used.

Ninety-five per cent of 114 lactating cows were seropositive initially. Unlike most previous studies,no seropositive cattle were culled, but they were separated physically from the seronegative cattle in order to preserve highly valued animals. Numerous anti-BLV sanitary practices also were utilized. Though the period antibody prevalence rates assessed at 12-month intervals decreased significantly for the three youngest of four different age cohorts, and the incidence rate for cattle six to 15 months of age was reduced, the incidence for the lactating cattle was not reduced. Possible explanations for the limited success of the study included 1) an extremely high prevalence pool in the older cattle, and thus a large source of BLV to infect younger cattle 2) noncompliance involving sanitary measures since the study was conducted in a university dairy herd which was a center of multiple research activities by many groups, 3) increased physical contact of seronegative and seropositive animals as the SN animals matured and crossed over into strata which were dominated by cattle with a high antibody prevalence, and 4) inadequate knowledge about additional anti-BLV sanitary measures, e.g. tattoos and eartags. Recent evidence confirmed that these materials are risk factors (234), but that information was not available when this study was conducted.

The latest study of the control of BLV infection was based also on a "serotest and segregation" policy (235) in which cattle from six commercial dairy herds were serotested and subsequently separated by a distance of 200 m into a seronegative and a seropositive group on each farm. A third group consisted of replacement cattle which were quarantined at another location on each respective farm until its seronegative status had been confirmed. Seronegativity for the

replacement cattle was defined as having two consecutively negative AGID tests, one within 30 days of purchase, and the second 60 days after the initiation of the isolation period. AGID testing of all cattle in each of the herd was conducted initially every three months, and seroconverts were removed promptly to the seropositive group. The only other reported anti-BLV sanitary practice was that of feeding colostrum and milk from seronegative dams or pasteurized products from seropositive dams. After the incidence in each group was reduced to zero, the interval of serotesting was doubled to six months. The median antibody prevalence among the six herds was 7.5 per cent, and the prevalence for all 539 cattle was 2.8 per cent. The median herd incidence rate determined by the second, third and fourth quarterly serotests was 1.9, 0.37, and 0.0 per cent,respectively, and the overall incidence for an average of 556 cattle at risk during these three serotests was 2.8, 1.08, and 0.0 per cent, respectively.Subsequent to the fourth serotest, seroconversions occurred only in one of the six herds, a herd in which the prevalence was four-fold that of the other herds. It would seem that a "test and segregation" policy would be particularly advantageous to herds in which the prevalence of BLV is high since seropositive cattle would not be slaughtered. However, this policy may meet with resistance since it would require the daily management of essentially two to three cattle herds on the same farm. The results of this study were favorable despite the fact that there was a stipulation for only one of several anti-BLV sanitary measures. The success in the absence of the anti-BLV sanitary measures may have been attributed to the 200 m distance which separated the cattle as well as the low prevalence in five of the six herds. Since seronegative status in the high prevalence herd was more difficult to

achieve, one must wonder about the effectiveness of "serotest and segregation" in high prevalence herds in which it would not be possible to segregate cattle by a long distance, and in which the anti-BLV sanitary measures would not be rigidly enforced.

PROPOSED GUIDELINES FOR THE ERADICATION OF EBL AND BLV

Based on numerous studies of the modes of transmission of BLV infection and on the results of the control studies in which the modes of BLV transmission were considered in the design of the anti-BLV transmission protocols, guidelines for eradicating BLV infection from cattle herds have been proposed. These proposed "corrective actions" have not been designated as official by the United States Animal Health Association, the organization normally responsible for such activities, since there are no official EBL/BLV control programs.

One plan requires the removal of all seropositive animals from a herd. To identify SP cattle, all cattle that are at least six months of age should be serotested using the AGID test. Serological tests of cattle less than six months of age may be false-positive, since their colostral BLV antibodies may not have decayed completely. Tests for the detection of BLV antigen in the lymphocytes of cattle less than six months of age are not available commercially, although there are reports of these from several research laboratories (236). The cost-effectiveness of screening animals less than six months old by using an antigen test instead of a serotest is probably negligible, since only about six per cent are infected. All SP cattle should be removed from the herd. All incoming cattle should be serotested and isolated for

30-60 days prior to their expected date of entry into the herd, and they should be serotested a second time at the end of the isolation period. After all the SP animals have been removed, the herd should probably be retested at least once if not twice at 30 to 60 day intervals to detect new SP animals, i.e. previously SN incubationary carriers. Afterwards, a single annual screen of the herd may be sufficient to detect any new reactors. Anti-BLV corrective actions should be instituted (228-235).

The second plan does not require that SP cattle be culled. SP and SN cattle are identified to distinguish them from each other and are segregated from each other. The anti-BLV corrective actions should be instituted also with the second plan.

CORRECTIVE ACTIONS

1. All animals at least 6 months of age should be serotested using the AGID test.
2. Annual if not biannual serotesting of previously seronegative animals of farmers who are interested in controlling the infection should be done. Though there in no current research to support it, a reduction in the prevalence rate may allow for a subsequent reduction in this serotesting interval.
3. Serotesting of pregnant animals should be done at least 6 weeks prior to parturition to prevent false-negative results due to immunoglobulin shifts from the dam's serum to her colostrum.
4. If the facilities will allow it, physically separate the BLV positive cattle from the negative cattle. Ideally, seropositive cattle should probably be culled, but this measure is too

impractical for most farmers.

5. Seronegative calves should be isolated from seropositive cows.
6. Young male calves should be isolated from seropositive cows.
7. Identify seropositive and seronegative animals with different colored ear tags.
8. A new sterile needle should always be used for injections, especially if the injections proceed from a seropositive to a seronegative cow.
9. A good quality insect control program should be instituted.
10. If young male calves are to be retained in the herd and require castration, a disinfectant should be used on the surgical instruments after each calf is castrated.
11. Calves can be fed colostrum fron BLV seropositive cows to obtain colostral antibodies, some of which are anti-BLV antibodies. However, they should be fed milk from seronegative cows to prevent the possibility of infection via infected lymphocytes in the milk.
12. Milk from the bulk tank may be safe to feed, if the infectivity of that milk is diluted sufficiently by milk from the seronegative cows. Pasturization of this milk may reduce its potential infectivity.
13. Seronegative cows should be milked first.
14. The milking procedures should prevent the contact of seropositive and seronegative cattle.
15. Cows with udder diseases which may lead to the deposition of blood on milking equipment should be milked at least temporarily by using different milking equipment.

16. Older, low-producing seropositive cows in commercial dairies should be culled.
17. Livestock owners involved in export or import should require the isolation of purchased cattle followed by having 2 consecutively negative serological tests conducted at least 4 weeks apart before the cattle are sold or purchased.
18. Cattle which are to be exported to countries which require negative AGID test results should probably be exported prior to 16 months of age, or they should be separated from seropositive cows until they are exported.
19. All farm personnel should be continously educated regarding the importance of following these guidelines.

PROSPECTS FOR A BLV VACCINE

Prior to the discovery of BLV as the etiologic agent of EBL, removal of cattle with persistent lymphocytosis from herds with EBL, as costly as it was, was effective in reducing the incidence of EBL (237,238). After the development of serologic tests, large scale epidemiologic studies revealed a high prevalence of BLV in some countries, for example, the USA. Eradication programs have never been considered as being economically feasible due to this high prevalence. Since the large prevalence pool increases the exposure potential of seronegative cattle, vaccine studies were initiated to provide some means of protection for these susceptible cattle. A BLV vaccine must meet at least the requirements of being noninfectious, nononcogenic, and it should not

interfere with the serological tests commonly used to detect BLV-infected cattle. In the initial study, four calves were immunized with BLV glycoprotein which was obtained from culture fluid from a fetal lamb kidney (FLK) cell line (239). The AGID and serum neutralization tests both were used to confirm seroconversion, and syncytium induction assay, radioimmunoassay for gp51, and a sheep bioassay were used to detect BLV infection in the vaccinates after they were challenged with BLV-infected lymphocytes. Since the sample size (N=4) and the challenge dose of 2500 lymphocytes was small, a second study (N=12; 4 million lymphocytes) was conducted (240). The glycoprotein vaccine was protective in 75 per cent and 16.0 per cent of the cattle in each respective study, and vaccine failure in study two was attributed to the increased dose of the challenge and possibly to failure to completely inactivate infectious virus produced by the FLK culture from which gp51 immunogen was obtained. A similar immunogen underwent extensive virus inactivation in a different study, and vaccine failure occurred in only 10.0 per cent of 20.0 animals vaccinated (241).

Glycoprotein 51 has been shown to have neutralizing activity against BLV, inhibitory activity on the release of BLV from infected cells, and complement-dependent antibody cytoxicity against infected cells (242), whereas p24 lacked all three activities. Thus, the purified soluble antigens gp51 and p24 were compared along with BLV-infected fetal lamb kidney cells and sheep fibroblasts transformed by BLV. The challenge consisted of 10,000 infected lymphocytes, antibodies to the immunogen were detected using a complement fixation test, and infection with BLV was detected with the syncytium induction assay only. Both gp51 and FLK immunogens induced gp51 antibodies and were protective, whereas p24 (59)

and SF28 immunogens induced only anti-p24 and anti-SF28. They did not inhibit infection upon challenge. The relationship between serum antibody titer and protection against infection by BLV was examined in sheep immunized with gp51 and a second protein (BLV-P) obtained from FLK cells (243). After challenge with 200,000 lymphocytes from a BLV-infected sheep, one group of immunized sheep and a second group of immunized/challenged sheep developed titers greater than 1:64 by two weeks postexposure, although the titers of the immunized-challenged sheep and immunized sheep were not different. The titers in both groups persisted for 44 weeks, and the syncytium infectivity assay of their lymphocytes for BLV infection remained negative. A second challenge administered at 45 weeks when the titers of both groups stabilized at 1:32 resulted in infection of all challenged sheep. Sheep which were passively immunized with serum from BLV-infected sheep as well as gp51 and BLV-P immunized sheep attained titers of up to 1:64. While this titer protected against challenge, lower titers provided no protection. The conclusion from this study was that titers of 1:64 provided some protection against BLV infection.

The very obvious drawback of these studies is that the immunogen which generally protected against challenge, i.e. gp51 is exactly the one which produces antibodies detected by the AGID and ELISA diagnostic tests. Thus, the tests would not discriminate between natural and artificial active antibodies in cattle. Several of the investigators used a battery of diagnostic tests to detect BLV infection in the vaccinates, whereas others (242) used only one relatively insensitive test. This, along with the somewhat unimpressionable results and the small sample size of most of the studies, suggests that BLV vaccine

trials have not been very successful. One alternative which warrants exploration is to develop a gp51 subunit vaccine and replace the gp51 diagnostic tests with a p24 diagnostic test (244). Since the antibody response to p24 is relatively low in infected animals, the new diagnostic test must have a high sensitivity and high negative predictive value. Tumor-associated antigens may provide an alternative to using gp51 as immunogen. Epitopes on the tumor cells rather than viral epitopes would be the source of the immune response. Thus, these antigens would be beneficial in that they may not interfere with the diagnostic test being used presently.

CONCLUSIONS

During the past 15 years, significant gains have been made in our understanding of EBL and BLV infection. The most significant gains for the animal health industry and the veterinary medical profession have been in the increased awareness of how the virus is transmitted and in the development of highly sensitive and specific diagnostic tests. Ironically, one of the greatest threats to cattle at risk of BLV infection was/is those persons most responsible for providing them with health care services. HTLV-I and HIV resemble BLV in this respect, since transfusion recipients were a high-risk group for contracting the agents during the early phases of those epidemics. Health professionals can now recommend measures to livestock owners as to how to reduce the incidence of BLV infection in their cattle without committing the economic suicide associated with the removal of seropositive cattle. These measures will also assist in retaining foreign livestock markets which insist that

imported livestock be derived from BLV-free herds. Failure to show an association between human lymphatic leukemia and EBL has suppressed and may continue to suppress the persisting hypotheses surrounding this proposed association. Not only is there a lack of evidence for an association between the two diseases, but evidence even for mere infection of humans by BLV is virtually nonexistent. The prospects for a BLV vaccine appear dim. Unlike both its close and distant human retrovirus counterparts, the devastating consequences of the absence of an artificial immunogenic agent for BLV may be tempered due to its high infection to disease ratio, a thorough understanding of its transmission, and the relatively short life span of the cow which is imposed by humans. Despite the massive gains in the knowledge of the molecular biology of BLV during the past seven years, this area of BLV research should probably be considered as one with great potential for future research. No doubt intensive searches for the molecular mechanisms of leukemogenesis of BLV will continue. This information may continue to contribute to the study of human lymphocytotrophic retroviruses from the epidemiologic to the molecular level, just as has other information about BLV.

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CHAPTER II

PROSPECTIVE SEROLOGIC, HEMATOLOGIC AND HISTOLOGIC STUDIES OF BOVINE LEUKEMIA VIRUS INFECTED SHEEP

ABSTRACT

The objective of this study to investigate the potential of BLV infection in sheep as animal model for studying the biology of leukemogenic retroviruses of animals and humans. A prospective study of the serologic, hematologic, and histologic changes of sheep infected with bovine leukemia virus (BLV) was conducted at 6-week intervals in conjunction with other studies of the leukemogenic mechanism of BLV in sheep. Whole blood from a BLV seropositive cow was used to infect 8 sheep with BLV. Antibodies to BLV were detectable in the sheep 3 weeks after exposure and have persisted for 120 weeks, whereas all control sheep have remained seronegative. There were no consistent statistically significant differences between the leucocyte counts, lymphocyte counts, and lymphocyte percentages of the infected and control sheep during the first 120 weeks of this study. However, one sheep did develop a leukopenia and lymphopenia 95 weeks after infection and died of histologically-confirmed lymphosarcoma 10 days later. The biological similarities between BLV and human T lymphocytrophic retroviruses, especially HTLV I, the susceptibility of sheep to the malignancies induced by BLV, the relatively short latent period for the onset of these malignancies, and the possibility that BLV infects both both T and B lymphocytes in sheep suggests that BLV infected sheep may be prove to be a useful animal model for studying mechanisms of leukemogenesis induced by both human and animal lymphocytotrophic oncogenic retroviruses.

INTRODUCTION

Bovine leukemia virus (BLV) is an exogenous type C retrovirus which causes naturally occurring lymphoid cell proliferations in cattle (Miller et al., 1969). The lymphoid cell proliferations may occur as persistent lymphocytosis which is nonpathogenic, or as lymphoma and leukemia, both of which are highly fatal malignancies (Ferrer, 1980). Type C oncogenic retroviruses have been incriminated as the etiologic agents of similar lymphoproliferative diseases in other domestic species such as the cat, chicken, mouse and gibbon ape (Gross, 1980). Human T lymphocytrophic virus type I (Poiesz et al., 1980) , Human T cell leukemia virus II (Kalyanaraman et. al., 1982; Robert-Guroff et. al. 1986) and Human Immunodeficiency Virus (Gallo et al., 1984) are recently discovered pathogenic retroviruses of humans, the former two of which are linked etiologically to adult T cell leukemia (ATL) and hairy cell leukemia. BLV (Lorenz and Straub, 1987) and HTLV (Ito, 1985; Reeves et al., 1988) both have a global distribution, and the viruses have strikingly similar biochemical and biological properties (Sagata et al., 1985). Although there is no form of therapy for enzootic bovine leukosis in cattle, epidemiologic studies (Johnson et al., 1985) have provided information about prevention of the transmission of BLV in cattle populations. The pandemic nature of the HTLVs lends support to the need for understanding these human viruses from an epidemiologic to a subcellular level. Consequently, considerable interest has arisen concerning the role of BLV

as a model virus for the HTLVs, especially HTLV I, because of the similarities between these two viruses and the diseases caused by them. Studies of this animal virus could provide information on the structure, function and biology of human retroviruses which in turn could be used to develop strategies to prevent, control and treat HTLV infections.

Although the cow is the natural host for BLV, experimental infections of BLV in sheep resulted in increased leucocyte counts and lymphoid tumors (Olson et al., 1972, Rogers et al., 1984). However, BLV infection in sheep differs from the infection in cattle. Only a small proportion of cattle naturally infected with BLV develop tumors during their lifespan, whereas a greater proportion of infected sheep develop tumors, and the latent period in experimentally infected sheep may be as short as 15 months (Rogers et al., 1984). This, along with the fact that T lymphocytes as well as the B lymphocytes in sheep may be targets for BLV (Horvath, 1982), suggested to us that the sheep may actually be a better host than other species for studying mechanisms of leukemogenesis induced by human T lymphocytotrophic retroviruses. Other domestic species such as the cat (Hardy, 1983) and the monkey (Lerche et al., 1984) do acquire natural retroviral infections other than BLV, but these infections are mostly associated with immune deficiencies due to ablation of T lymphocytes rather than being associated with neoplastic lymphoid cell proliferation. Consequently, they may be more useful for the study of infections induced by HIV and similar agents.

Persistence of antibody to an infectious agent is consistent with continual exposure to the agent. Cattle which become infected with BLV are classified as persistent carriers of the virus (Johnson et al., 1985). The usefulness of sheep as a substitute for cattle in

experimental models for retroviral malignancies would be enhanced by showing 1) that sheep, like cattle, become permanently infected after exposure to BLV, 2) that BLV infected sheep develop lymphocytosis which is an indicator of impending leukemia and/or lymphoma, and 3) that tumors develop and the presence of these tumors can be confirmed both grossly and histologically. These findings could be useful in the testing of antiretroviral and antileukemic chemotherapeutic agents in which BLV infected sheep are used , in studies of the phenotypic expression of the proliferating cells in sheep by using monoclonal antibodies, and in studies in which molecular probes could be used to examine the proliferating cells in sheep for infection with BLV.

A longterm prospective serologic and hematologic study of BLV infected sheep was undertaken in conjunction with other studies of the leukemogenic mechanism of BLV in sheep. The objectives of this portion of the studies in sheep were: 1) to develop a standard protocol for the infection of sheep with BLV. 2) to further confirm the previous findings that: a) BLV antibodies in sheep would persist, b) that abnormalities in the lymphocyte counts, i.e. lymphocytosis followed by leukemia would develop, and c) that lymphosarcoma would develop.

MATERIALS AND METHODS

Serum collection and storage

Ten ml of whole blood was collected into coagulant tubes (Becton-Dickinson) and was allowed to clot overnight at 26 C. The clots were removed, the remaining erythrocytes were pelleted by centrifugation at 400 g for 10 min, and the serum was collected and stored at -30 C until further use.

Serological test for BLV and BSV antibodies

The agar gel immunodiffusion (AGID) test was used to examine the cow's sera for antibodies to bovine leukemia virus and to bovine syncytial virus (BSV). The AGID test for antibodies to BSV were done by Dr.Janice M. Miller of the National Animal Disease Center, Ames, Iowa. All cattle and sheep which were BSV seropositive were eliminated from further study. The AGID test for BLV was done with a commercially available kit (Leukassay B, Pitmann-Moore, Washington Crossing, NJ). Briefly, 0.9 percent agar (Bactoagar, Difco, Detroit, MI) was prepared by dissolving 9.0 g of agar in 1.0 L of distilled water containing 85.0 g of sodium chloride. Fifteen ml of the agarose solution at 55 to 60 C was added to each 100 mm petri dish. The agar was allowed to solidify for one hour at 20 to 25 C. Wells were cut in the agar with a 28-hole template containing 4 seven-well patterns. The diameter of the holes was 5.0 mm, and the distance between the holes was 3.0 mm. The agar plugs and residual moisture in the wells were removed with a cannula connected to a low-pressure vacuum line. BLV antigen, control sera and test sera were added to the appropriate wells, and the plates were incubated in a humidified chamber at 26 C for 72 hr. The results were read against a black background in a dimly lighted room by using a beam of light to highlight the precipitin lines.

Peripheral blood leucocyte counts

Leukocytes were counted manually (Unopette, Becton-Dickinson, Rutherford,NJ).). Blood smears were stained with hematoxylin/eosin by using an automatic slide stainer. Differential counts were done by counting at least 100 to 200 leukocytes using an oil immersion objective.

Identification of seropositive and seronegative cattle

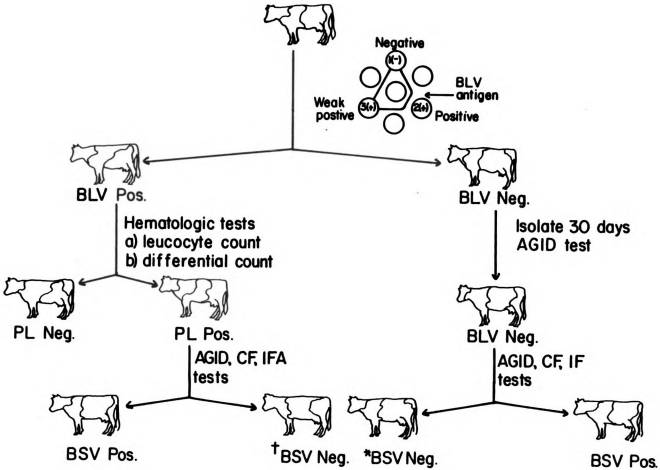
Seropositive and seronegative cattle from which the blood donors were eventually chosen were selected by screening adult cattle from the Michigan State University dairy herd for antibodies to BLV. The antibody prevalence to BLV in cattle in this herd had been between 70 and 95 percent during the previous six years.

Selection of cattle used to infect sheep

Several cattle whose initial AGID test results for BLV were negative were isolated for 30 days, and the test was repeated at the end of the isolation period to confirm that they had remained BLV antibody free. These cattle were then serotested for BSV antibodies by following the sequence in Fig. 2.1. Several cattle whose initial AGID test for BLV was positive were isolated for a 30-day period during which they were examined for persistent lymphocytosis. These cattle then were serotested for BSV antibodies also (Fig.2.1). The BSV antibody negative cattle whose initial AGID test for BLV was positive were also isolated for 30 days, and the test for BSV antibodies was repeated at the end of the isolation period. These latter cattle were tested for BLV antibodies only once during this screening process.

BLV seropositive cattle with persistent lymphocytosis

Several BSV seronegative, BLV seropositive clinically normal cattle were examined for lymphocytosis. A cow with persistent lymphocytosis was defined as one having a lymphocyte count at least two standard deviations above normal for 3 consecutive differential leucocyte counts using blood samples which were taken at 30-day intervals (Gross, 1983).



†"ideal" infectious donor; *"ideal" noninfectious donor; PL=persist. lympho; BSV=Bov. Syn. Virus

Fig. 2.1. Protocol for the selection of a bovine donor to infect sheep with BLV.

Selection of sheep to be infected with BLV

Fourteen, normal 8-month old mixed-breed ewe lambs were obtained from the Michigan State University sheep farm. The ewes were serotested for antibodies to both BSV and BLV by using the same AGID test as for the cattle. They were then isolated from all other animals for 30 days, and serotested again at the end of the isolation period by following the steps as shown in Fig. 2.2.

Infection of the sheep with BLV

A single BSV and BLV seronegative donor cow was selected to provide the blood which was used on the control sheep, whereas a single, BSV seronegative, BLV seropositive donor cow was selected to provide the blood which was used for the sheep which would be infected (Fig. 2.3). Blood samples were collected from the donor cows as well as all sheep immediately prior to the injection of blood from the donor cows. The sheep were randomized into groups of "controls" (N=6) or "infected" (N=8) sheep , and each group was housed in open lots 0.5 km apart during the remainder of the study. Blood was collected from the donor cattle into evacuated bottles containing the anticoagulant acid citrate dextrose. Tissue culture medium (RPMI 1640) at 38 C was added to 5.0 ml of blood at a ratio of 2.0:1.0, mixed by inversion, and equal volumes were injected intramuscularly into each sheep at 3 sites. Control sheep received 5.0 ml of diluted blood from the BSV, BLV seronegative donor cow only. Sheep to be infected only received blood from the BSV seronegative, BLV seropositive donor cow.

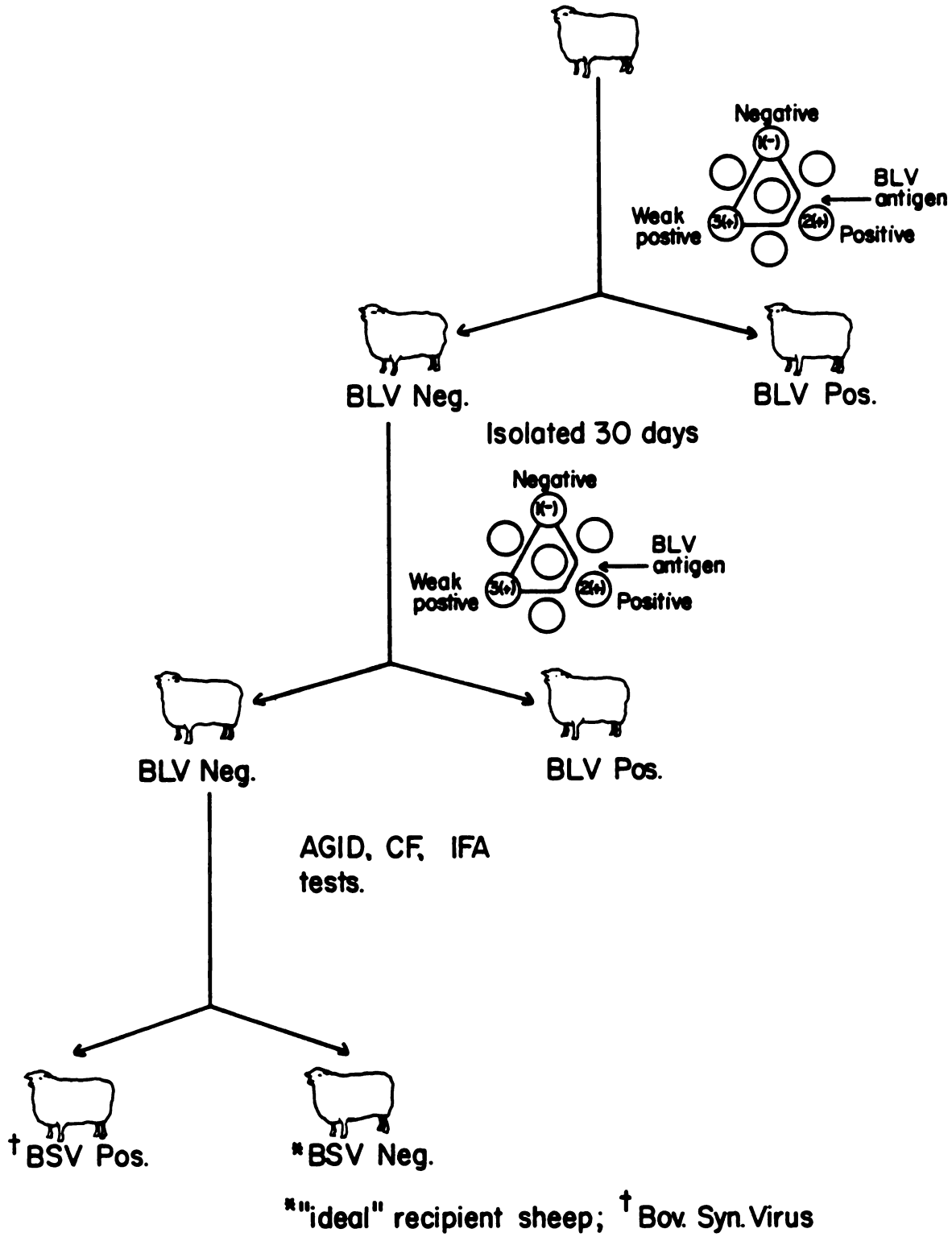


Fig. 2.2. Protocol for the selection of recipient sheep to be infected with BLV.

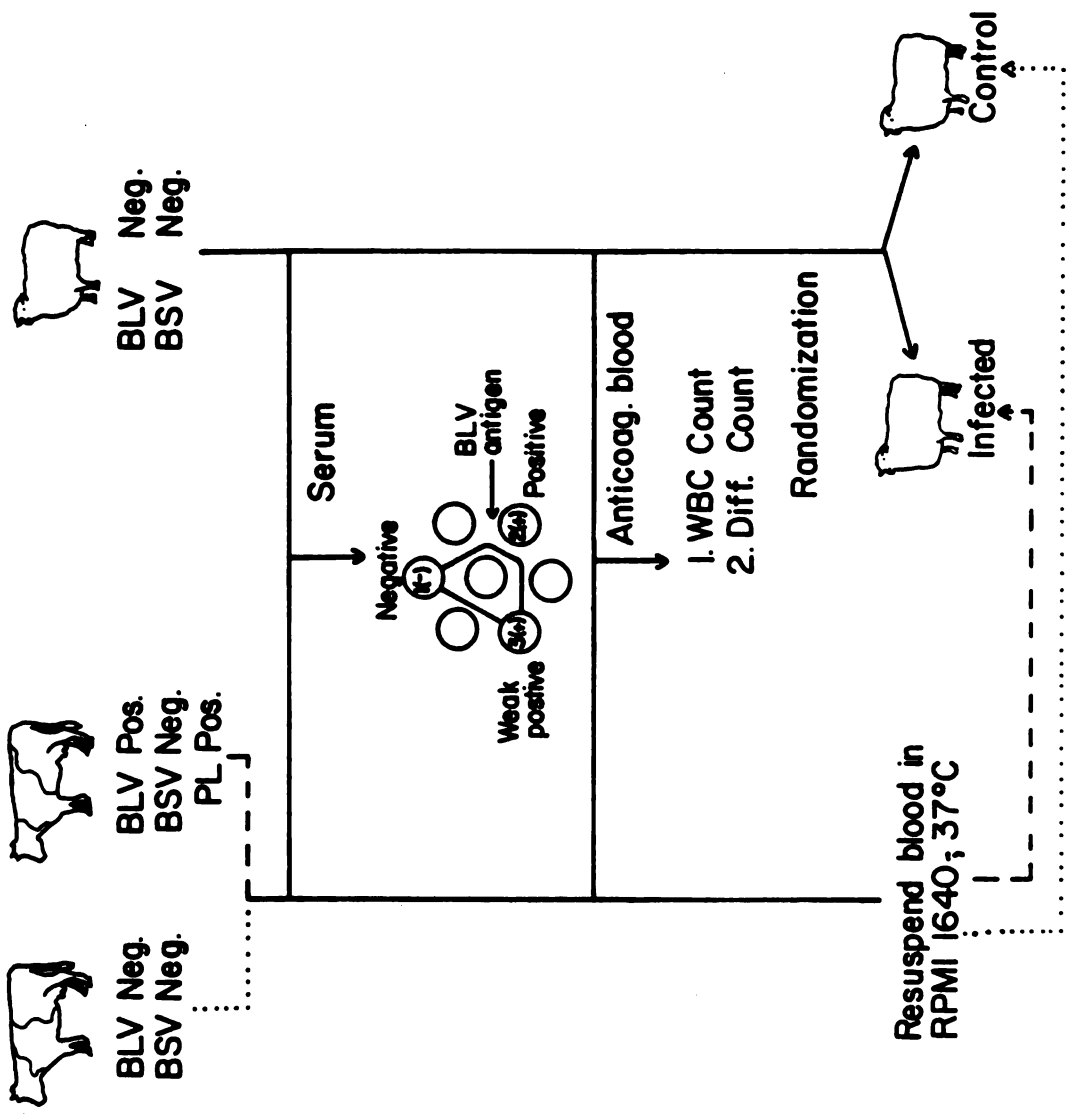


Fig. 2.3. Protocol for infection of the sheep with BLV.

Prospective evaluations of the sheep

AGID tests, leucocyte counts and differentials, and physical examination of the sheep's external lymph nodes were done on the day of infection and at 4 to 5 week intervals during a 27-month period.

Examination of the tumors

Tumors from the sheep which developed lymphosarcoma were fixed in 10 percent formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined via light microscopy.

Miscellaneous

Preventive medical care consisted of the administration of anthelmentics 4 times annually, shearing once each year, and trimming of the hooves as needed. Although the flow of traffic was always from the control sheep to the infected sheep, the instruments used during these procedures were always washed and disinfected in chlorhexidine (Nolvasan, Fort Dodge Lab., Ft. Dodge, IA) after having been used on each group of sheep to prevent the accidental transfer of infected lymphocytes to the control sheep.

Statistical analysis of the data

The general linear models analysis of variance for an unbalanced design (SAS, 1985) was used to analyze for differences between the leucocyte counts, lymphocyte counts, and lymphocyte percentages of infected and control sheep. All measurements were taken during 18 successive experiments. An unbalanced design was used because one ewe in the infected group was killed by a predator 60 weeks after infection with BLV, and one ewe in this same group died of lymphosarcoma 96 weeks after infection. Our hypothesis tested was the leucocyte counts, lymphocyte counts and lymphocyte percentages of the infected sheep would increase

concomitant with the development of tumors. The statistical model was:

$Y_{ijk} = u + a_i + b_j + c_k + (a_ic_k) + e$, where

Y_{ijk} = an observed cell count or percentage during a given examination period,

u = the mean value of all cell counts or percentages,

a_i = the effect attributed to BLV infection (treatment),

b_j = the effect of an individual experiment,

c_k = the effect of an examination period,

a_ic_k = the interaction effect between treatment and examination period,

e = random error.

RESULTS

BSV and BLV antibodies before exposure

Cows which were known to be BLV seropositive based upon serial AGID test results from a prospective epidemiologic studies were tested again, and all were still seropositive. However, only one of the 12 BLV seropositive cows was BSV seronegative, and this cow served as the blood donor for the 8 sheep which were infected with BLV. All three of the BLV seronegative cows which were tested for BSV antibodies were seronegative. None of the sheep had antibodies either to BLV or to BSV prior to exposure.

Prevalence of persistent lymphocytosis in the donor cattle

None of the BLV seropositive cows had persistent lymphocytosis. The lymphocyte count of the BLV infected donor was 7950 per ul, whereas that of the BLV free donor was 6500 per ul. Thus, each sheep in the infected group received 19.9×10^6 bovine lymphocytes, and each control sheep

received 16.25 x 10^6 bovine lymphocytes.

Prevalence of BLV antibodies in sheep after exposure

All 8 sheep which were exposed to blood from the BLV seropositive donor had developed antibodies to BLV within 3 weeks of exposure (Fig. 2.4). The AGID test results were always strongly positive during the entire 27-month observation period. None of the 6 control sheep developed antibodies to BLV.

Blood cell counts of the sheep

As a group, there were no consistently statistically significant differences between the leucocyte counts (p=0.143; Fig. 2.5), lymphocyte counts (p=0.70; Fig. 2.6), and lymphocytes percentages (p=0.074; Fig. 2.7) of the infected sheep and the control sheep during the 18 different experiments in which the values were determined. Both the lymphocyte count (519 per ul) and the lymphocyte percentage (17%) had decreased dramatically in one sheep 10 days prior to her death due to lymphosarcoma (Figs. 2.8, 2.9, and 2.10). There was no cytologic evidence of malignant cells in the peripheral blood based upon the microscopic examination of peripheral blood smears examined 10 examined days preceding the death of this sheep. There were significant differences in the counts and percentages among individual animals and among experiments.

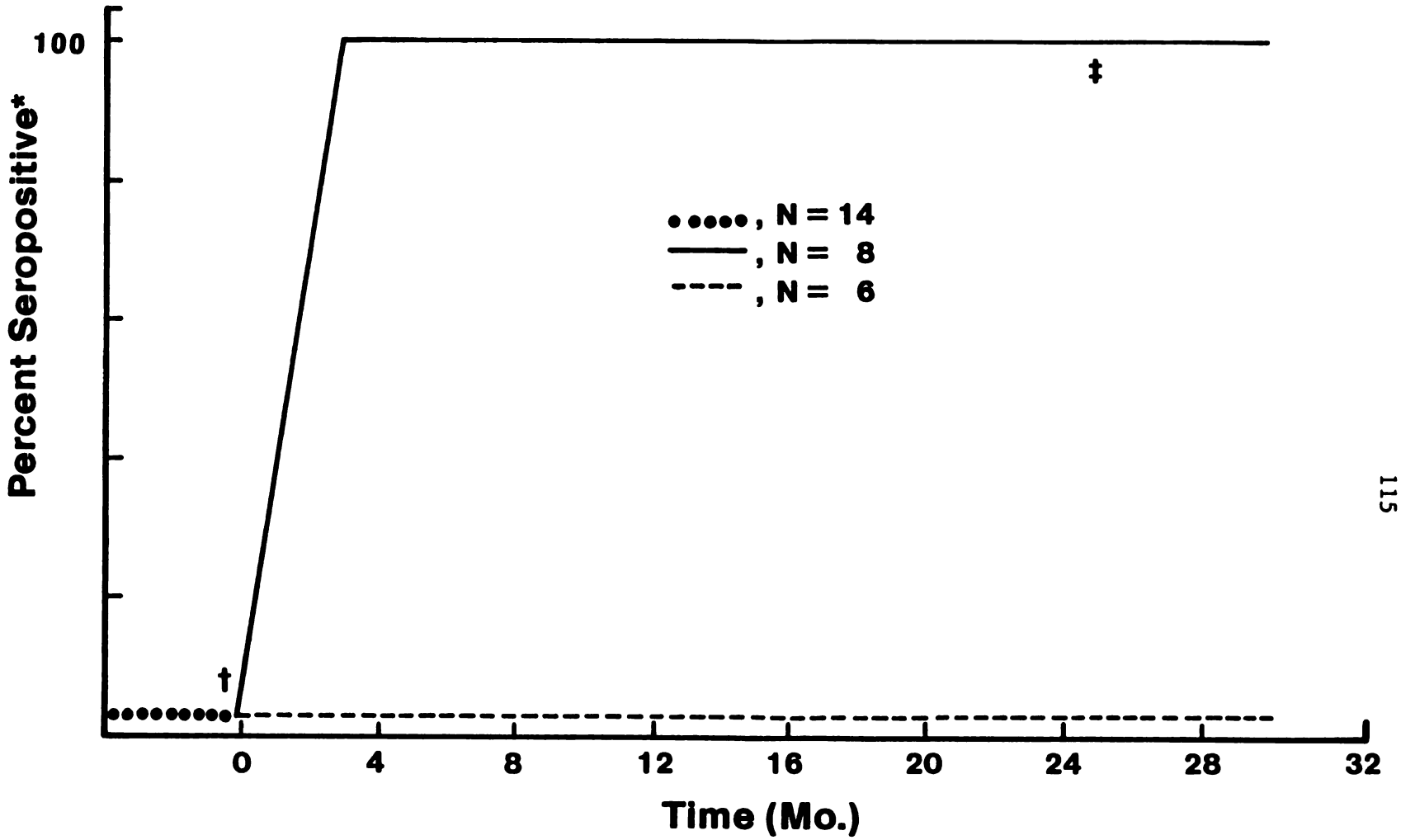


Fig. 2.4. A prospective serologic study of BLV infected sheep.

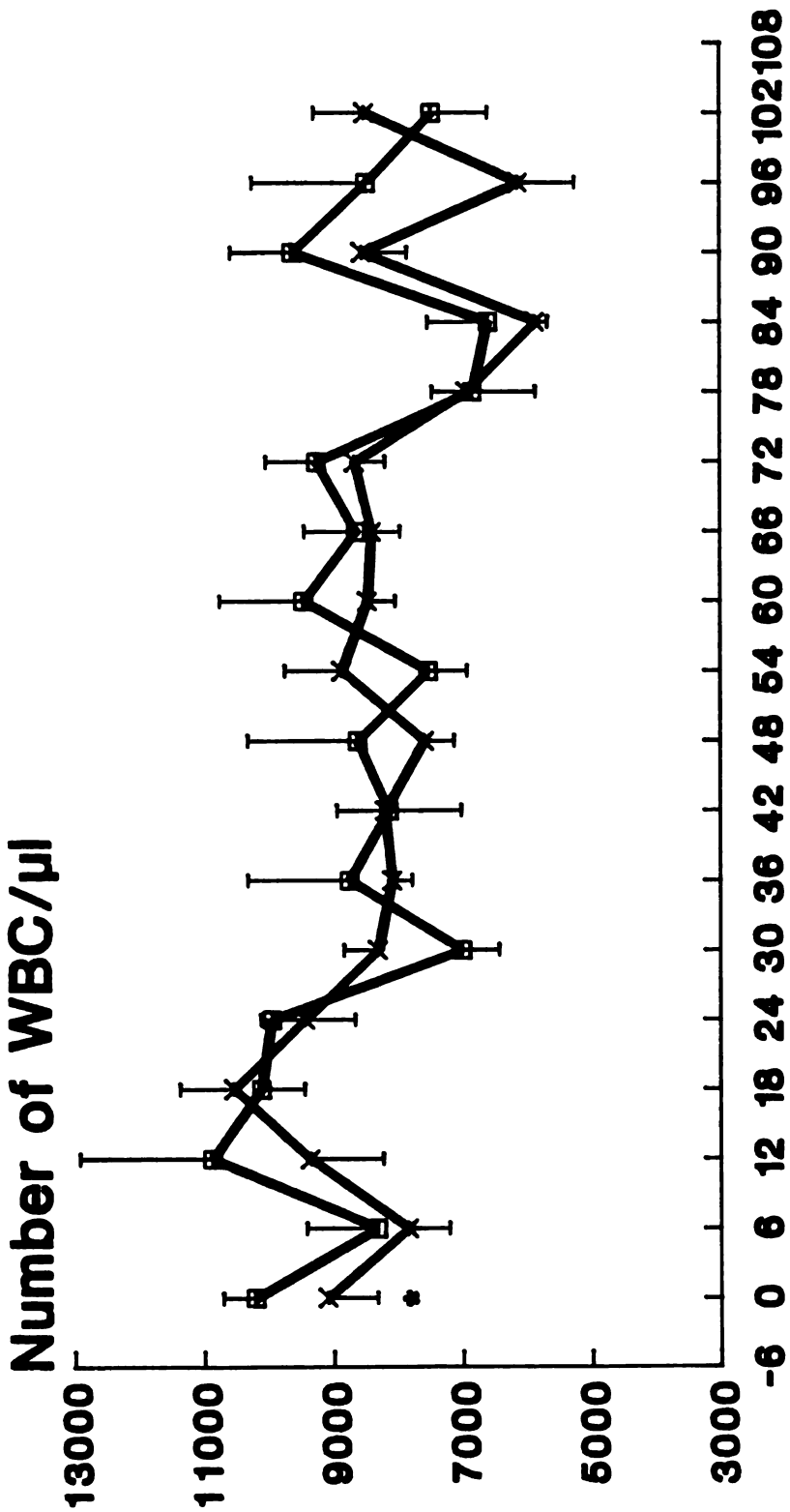


Fig. 2.5. A prospective comparison of peripheral blood leucocyte counts in BLV infected and control sheep. *, exposure.

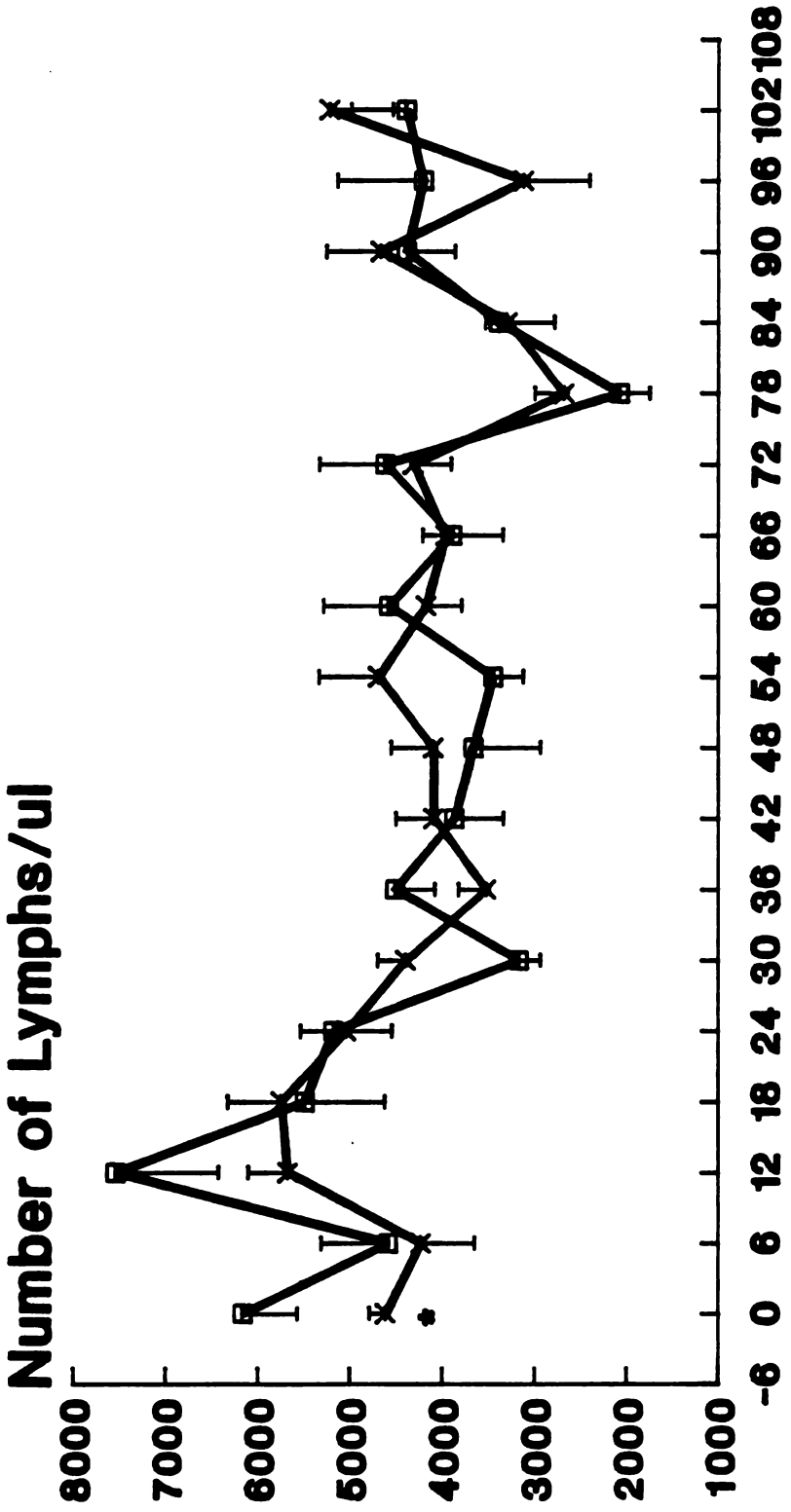


Fig. 2.6. A prospective comparison of peripheral blood lymphocyte counts in BLV infected and control sheep. *, exposure.

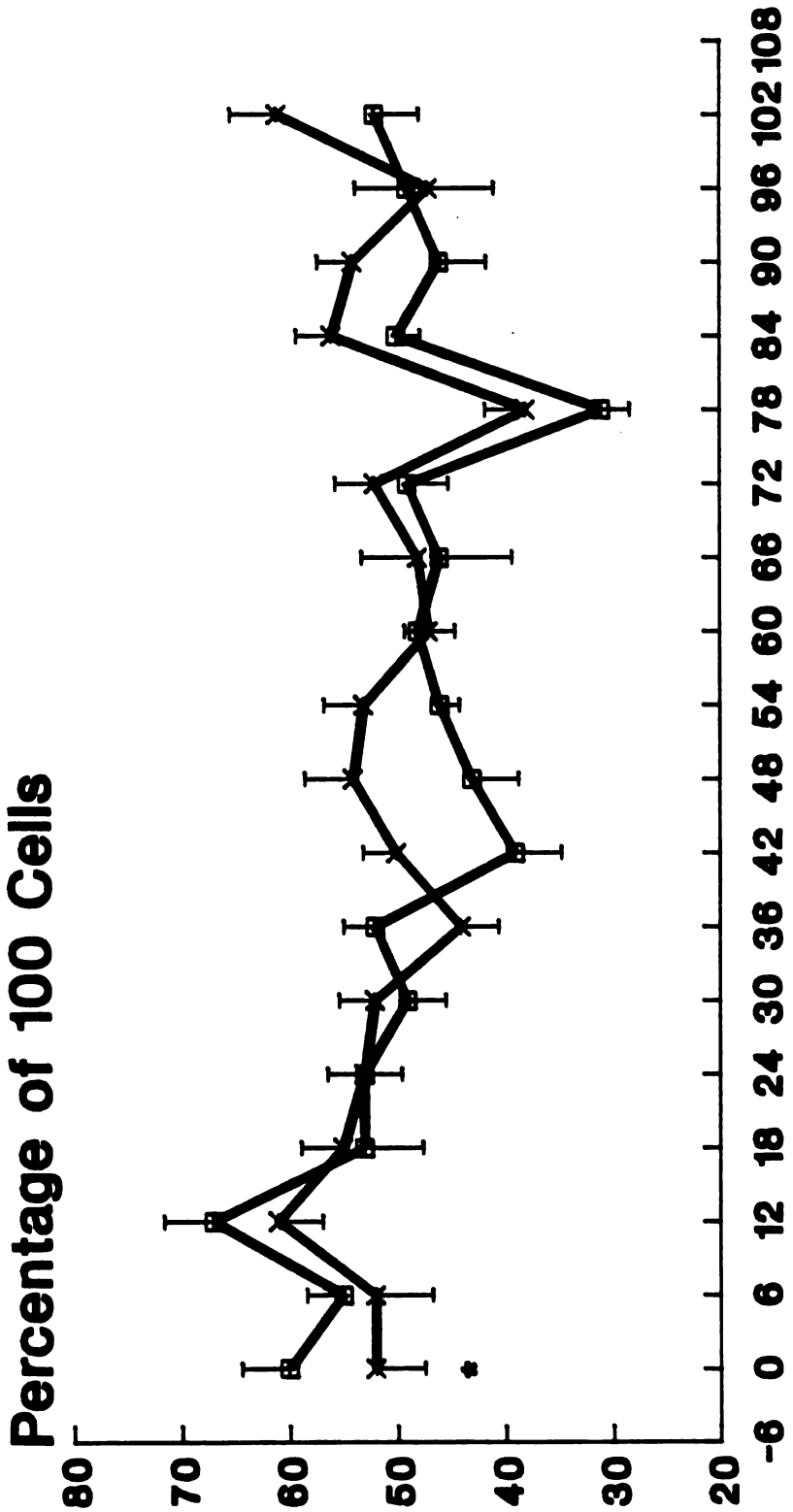


Fig. 2.7. A prospective comparison of peripheral blood lymphocyte percentages in BLV infected and control sheep. *, exposure.

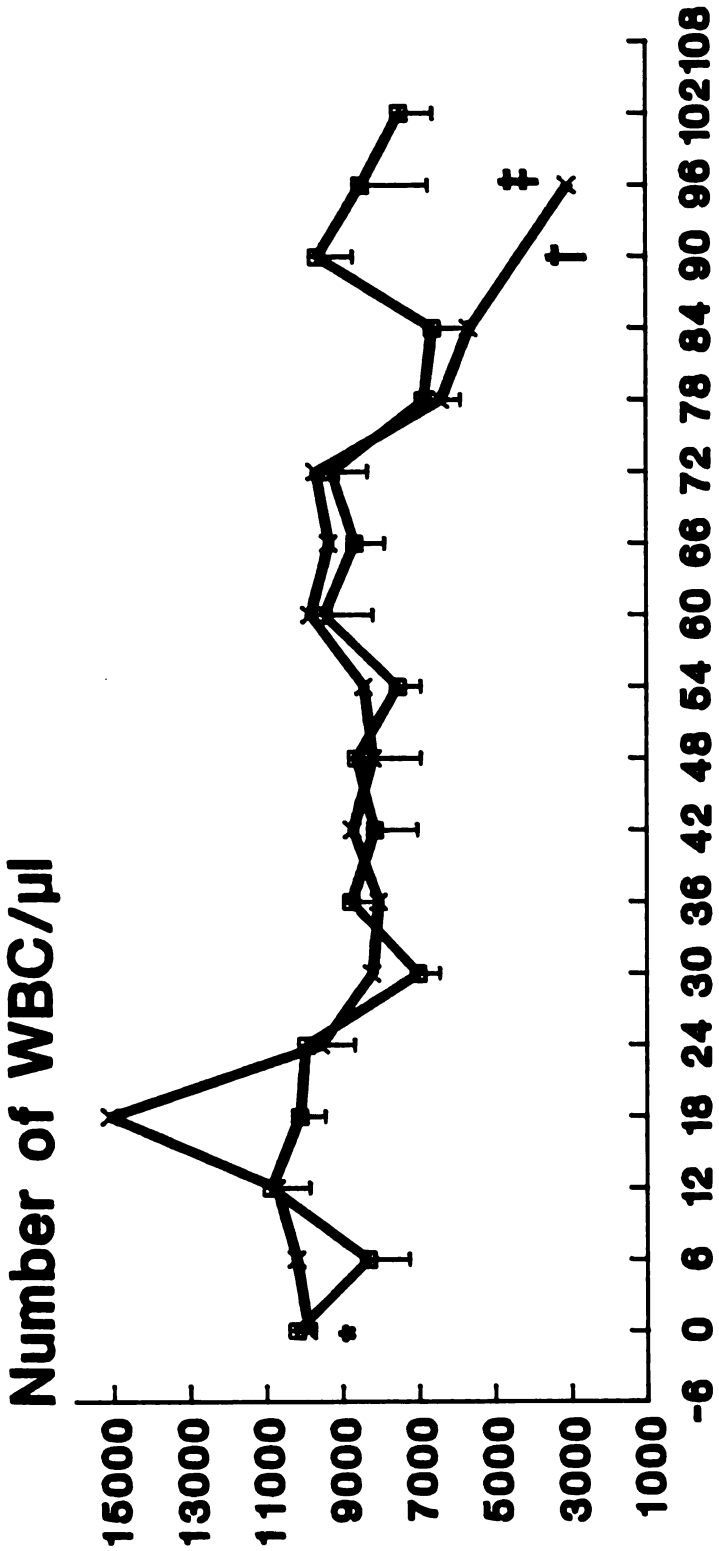


Fig. 2.8. A prospective comparison of peripheral blood leucocyte counts in one leukotic sheep and control sheep. *, exposure; †, no data; ‡, death.

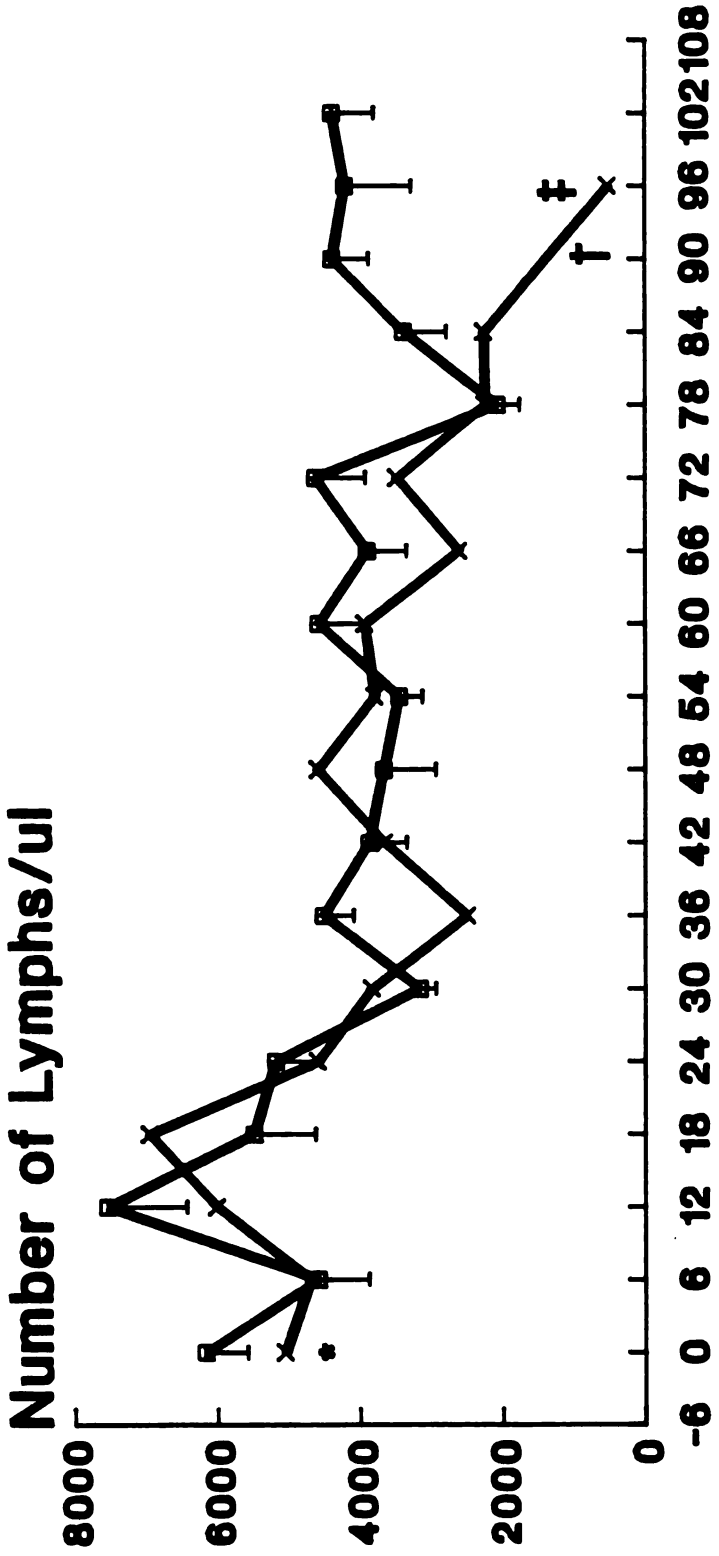


Fig. 2.9. A prospective comparison of peripheral blood lymphocyte counts in one leukotic sheep and control sheep. *, exposure; †, no data; ‡, death.

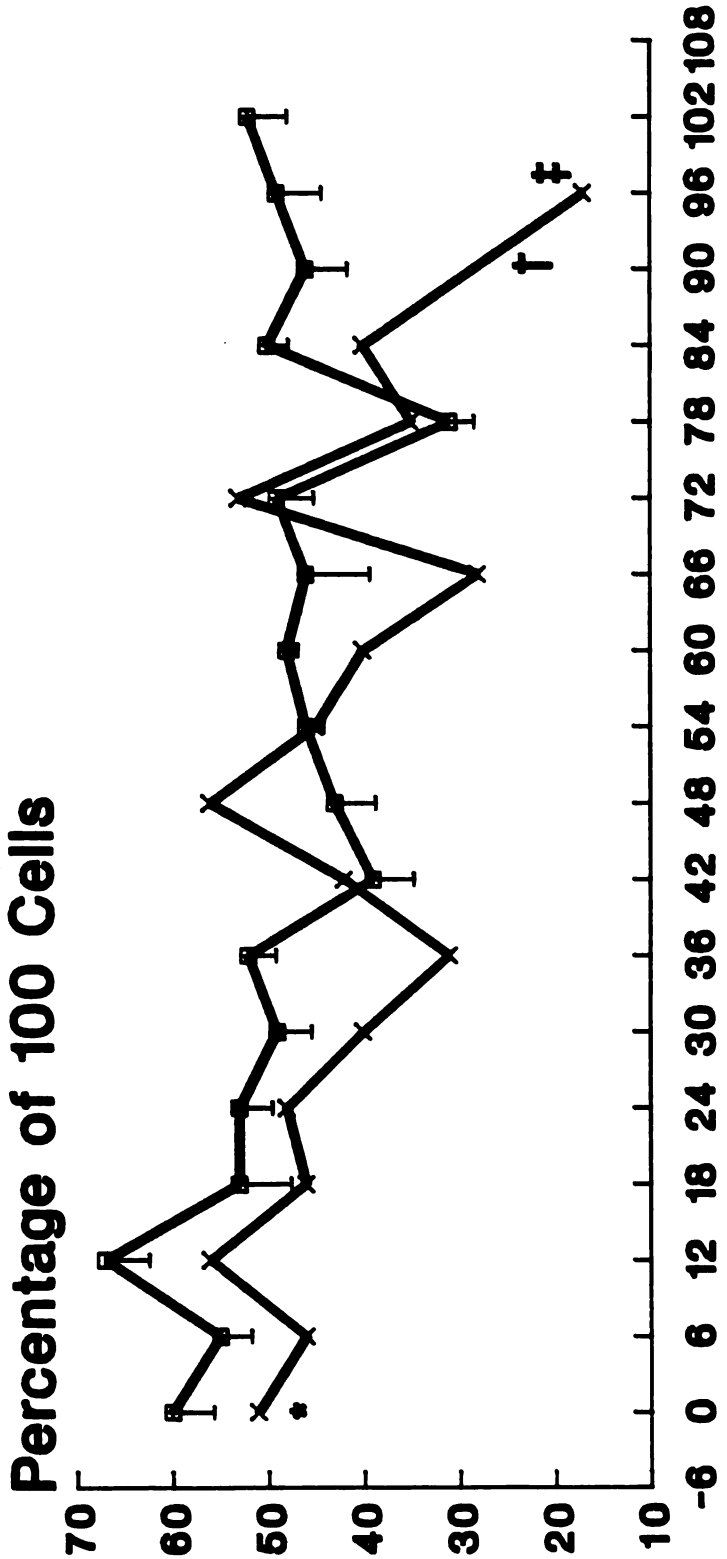


Fig. 2.10. A prospective comparison of peripheral blood lymphocyte percentages in one leukotic sheep and control sheep. *, exposure; † , no data; ‡ , death.

Tumors in the sheep

Only one of the infected sheep had developed tumors 96 weeks after exposure to the virus. Tumors between 3.0 mm and 70.0 mm in diameter were located in most of the abdominal organs including the liver, kidney, ovary (Figs. 2.11A, B, C), stomach, diaphragm, and mesenteric lymph nodes. Histologically, the highly cellular and compact tumors were comprised of multiple and sometimes single large nodules that compressed the surrounding normal tissue (Fig. 2.12). The tumor cells were round to ovoid with distinct cytoplasmic borders, and hyperplastic nuclei within the cells contained 2 to 3 large nucleoli. There were as many as 10 mitotic figures per 40x field. The normal lymphoid follicular architecture of the lymph nodes and spleen was completely destroyed due to the proliferation of large lymphoblastic cells, and approximately one half of the normal parenchyma in the liver, kidney, adrenal glands and uterus was destroyed.

Fig. 2.11 A, B, C. Infiltration of tumors in the liver, kidney, and ovary of a sheep with lymphosarcoma.

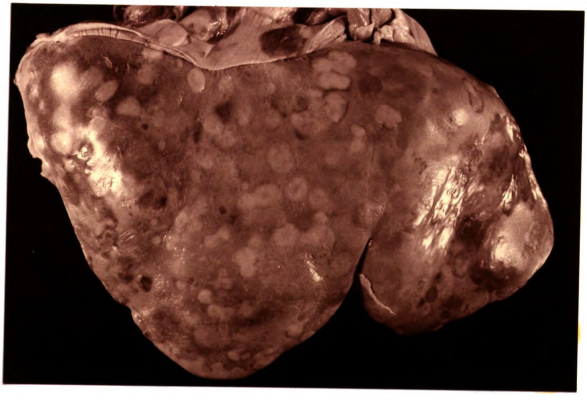


Fig. 2.11 A. Liver.

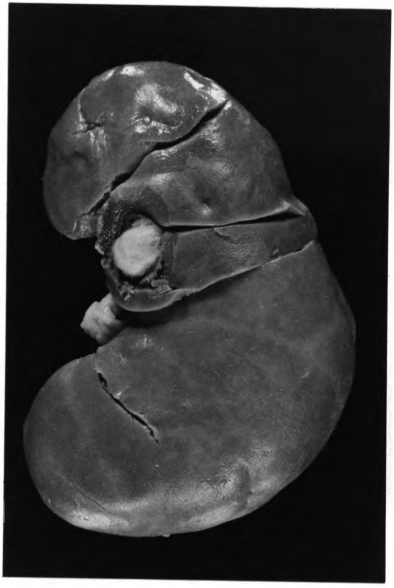


Fig. 2.11 B. Kidney.

Fig. 2.11 C. Ovary.

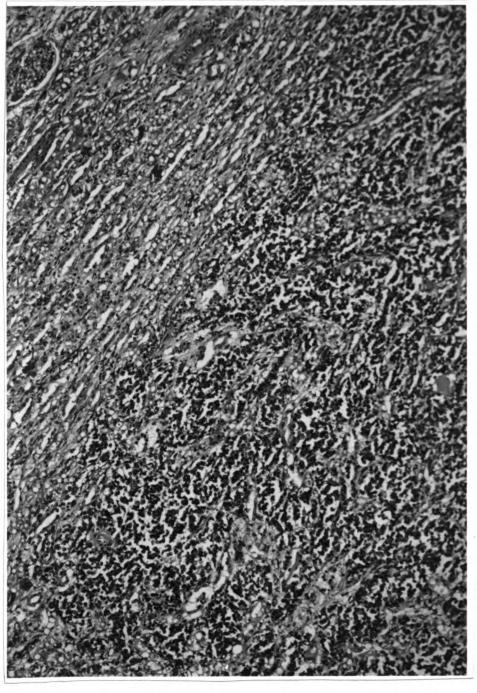


Fig. 2.12. Neoplastic lymphocytes disrupting the normal renal parenchyma in a sheep with lymphosarcoma (40 X).

DISCUSSION

Several procedures have been used to infect sheep with BLV, and we used the procedures as a basis for establishing our own method for infecting sheep. Cell-free BLV particles were first demonstrated by using mitogen-stimulated, short-term cultures of lymphocytes from cows with lymphoma (Miller et al., 1969). These findings may have lead to the belief that infection of recipients with BLV would occur only if the inoculum from cultured donor cells was used. Experimental infection of sheep subsequently was done by using mitogen-stimulated, short-term lymphocyte cultures from BLV infected cows with (Olson et al., 1972) and without (Hoss and Olson, 1974) lymphoma, by using supernatant fluid from cultured lymphocytes collected from infected cows (Van Der Maaten and Miller, 1975), by using cell lines infected with BLV (Van Der Maaten and Miller, 1975; Kenyon et al., 1981; Rogers et al., 1984; Suneya et al., 1984) and by using cells derived from tumors from cows with lymphoma (Olson et al., 1972).

Epidemiologic evidence strongly supports horizontal transmission of BLV infection, especially by blood contaminated instruments (Johnson et al, 1983). This suggested to us that it may not always be necessary to use inoculum from cultured lymphocytes or from BLV-producing cell lines to successfully infect sheep with BLV. As few as 1.0 ul (4,524 cells) to 10.0 ul (45,240 cells) of whole blood from a seropositive cow have been shown to cause seroconversion in calves as early as 4 weeks after exposure (Evermann et al., 1986) . Since the infection procedures other than those in which whole blood was used could be more technically difficult, time-consuming, and expensive, we attempted to infect sheep by

simply using whole blood from a seropositive cow in a herd with a high prevalence of BLV antibodies. The whole blood was very effective in causing rapid and persistent seroconversion to BLV. The effectiveness of any of these inocula to induce infection is probably as much a function of dose as it is the type of inoculum. Increasing the dose of cultured lymphocytes injected into both sheep and cattle has resulted in an increased frequency of seroconversion, more rapid seroconversion, and increased frequency of isolation of BLV from lymphocytes collected from the recipients (Hoss et al., 1974). A recent study (Stirtzinger et al., 1988) of the response of sheep to varying doses of lymphocytes from another BLV infected lamb resulted in an increased frequency both in seroconversion and in the isolation of BLV from the recipients as the dose of the inoculum increased.

The appearance of BLV gp51 antibodies in the serum of our sheep as early as 3 weeks after exposure is consistent with the recent findings of others (Cockerell et al.,1986; Walker et al., 1987; Stirtzinger et al., 1988). Persistence of the antibody also has been documented in studies which were conducted for at least 5 years (Bex, 1979; Kenyon,1981). Though conversion back to a seronegative status rarely occurs , it has been reported to occur in one sheep that received a "low dose" of inoculum (Stirtzinger et al., 1988). The only other report of seroreversion in a sheep was subsequently followed by reseroconversion (Suneya, 1984), thus suggesting that those sheep probably were never free of the infection in spite of the fact that antibodies were not detected. Thus most sheep, like cattle, become permanent carriers of BLV upon exposure, and all sheep probably become permanent carriers if the dose of the inoculum is sufficient.

Bovine Syncytial Virus (BSV) is a nonpathogenic exogenous lymphocytotrophic retrovirus of cows (Bouillant and Ruckerbauer, 1984) which induces formation of syncytia when cocultured with bovine embryonic spleen, embryonic lung, and ovine embryonic spleen cells (Marshall and Gillette, 1983). BSV also expresses detectable antigens in cultured bovine lymphocytes. Thus the presence of BSV in lymphocytes may cause false positive test results in some in vitro assays such as the syncytium induction assay and other cellular immunoassays, both of which have frequently been used to detect the infection of cells by BLV. Widespread epidemiologic studies of the prevalence of BSV have not been done. We serotested potential BLV donors for antibodies to BSV, and only one was seronegative. In retrospect, this should not have been surprising since the modes of transmisson of the two viruses in cattle populations are probably the same because they both infect lymphocytes. The antibody distribution of BLV and BSV will probably be similar in cattle herds in which both viruses are found. Contamination of bovine anti-BLV antisera with BSV antibodies should be considered when immunoassays are being used to detect BLV antigens in lymphocytes. Since BSV does not cause natural infections in sheep, sheep infected with BLV, rather than cattle, may be better donors of the virus on a longterm basis, if the sheep are initially infected with BLV by using blood from BSV free cows.

We initially sought a BLV donor with persistent lymphocytosis because in vitro studies had suggested that cows with persistent lymphocytosis could be more infectious than other cows (Itohara et al., 1985). The high degree of infectivity of both seropositive, antigen-positive and seropositve, antigen-negative donor cows has been determined by using microquantities of blood in a bioassay in which calves were the

recipients, but since the peripheral blood lymphocyte counts of the donors were not reported (Miller et al., 1985), it is not known if they had PL. We infected all of our sheep by using blood from a donor without PL, albeit a large number of lymphocytes was used. The in vivo infectivity of cows with and without PL was recently confirmed (Mammerickx et al., 1987) by showing that only 926 lymphocytes from a donor cow with PL and a high titer to gp51 were infectious to sheep, but 325,000 lymphocytes from a cow without PL and a low titer to gp51 were required to infect sheep. Thus, we have concluded that it not necessary for a BLV donor to have PL, especially if a large number of cells are used.

The peripheral blood lymphocyte numbers in sheep infected with BLV were highly variable among the studies reported previously and among infected sheep within a given study. Lymphocytosis (Olson et al., 1972; Paulsen, 1976; Rogers et al., 1984;, Suneya et al., 1984), leukemia (Rogers et al., 1984; Dimmock et al., 1986), lymphopenia (Olson et al., 1972); and normal lymphocyte counts (Cockerell et al., 1986) have all been reported in these studies, the duration of which was 30 to 46 months. Abnormally increased peripheral blood lymphocyte counts have been detected by others 2 to 24 weeks before the death of sheep which developed tumors. The only ewe in our study which had developed tumors at the time that this data was reported was slightly lymphopenic 10 days before her death. Additional changes in the other leucocyte parameters indicated that the hematologic response of that ewe was consistent with a stress triad response which may be seen in cancer victims. The significant differences in the counts and percentages among animals and among examination periods were attributed to biological flucuations which

are known to occur, especially since the changes were not consistently altered in either direction. Analysis for an interaction effect between infection with BLV and the examination periods was done because mere infection with BLV does not result in the immediate development of detectable neoplasia or clinical disease. Therefore, other undetermined biological events, which themselves may be a function of time, must play a causal role in the development of tumors in the infected animals.

We established a flock of BLV infected sheep by directly inoculating them with blood from a BLV seropositive cow. Although previous attempts to insure that the BLV donors were free of BSV have been rarely reported (Kenyon et al., 1981), we felt that it was essential to use donors free of other adventitious viruses that could interfere with immunoassay procedures such as the syncytium induction assay. If sheep are inoculated with BLV by using cells from a BSV free cow donor, the process of screening donor cows for previous exposure to BSV during subsequent inoculations of sheep could probably be eliminated, because the sheep already infected with BLV could then replace the cattle as donors.

Our primary objective for establishing the flock of infected sheep was to investigate the leukemogenic mechanisms of BLV, and the results reported here were obtained in conjunction with our other studies. A protocol was established which could prevent the simultaneous infection of the sheep with known adventitious lymphocytrophic retroviruses. BLV antibodies have persisted in all the infected sheep, and tumors developed in one sheep. The lymphocyte counts of the sheep had not increased by 108 weeks post infection. To our surprise , the lymphocyte count of the sheep which developed the tumors was decreased rather than

increased. The preliminary results from this ancillary study of BLV infection add further support to the previous findings of antibody persistence, tumor development and lymphopenia, the latter of which has rarely been reported in cattle and never in sheep.

Retroviruses have long been known to cause a variety of hematopoietic cancers in animals, but oncogenic retroviruses of humans have been discovered only recently. Irrespective of the species, the viruses and the diseases caused by them are very similar, and the animal model systems have already contributed much to our understanding of the diseases in humans. Research studies leading to a further understanding of leukemogenic mechanisms, host susceptibility, chemotherapeutic agents, and effective vaccines for retroviruses are in infancy. Thus, a sheep model for studying BLV may be a useful tool for scientists in a variety of disciplines to develop therapeutic and prophylactic measures against retroviruses in both animal and human species. This model is being used presently to investigate hypotheses of the leukemogenic mechanism of BLV in sheep which could be useful in enhancing our knowledge of retrovirology.

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CHAPTER III

STANDARDIZATION OF A T COLONY ASSAY FOR THE CULTURE OF SHEEP LYMPHOCYTES

ABSTRACT

A technique for culturing sheep peripheral blood lymphocytes in soft agar was standardized according to several technical parameters, which included T lymphocyte mitogens, mitogen doses, cell number, distribution of the cells in the culture vessel and the duration of the culture. Although both concanavalin A (CON A) and phytohemagglutinin A (PHA) stimulated T lymphocyte colony formation, CON A consistently produced more and larger colonies. A statistical analysis was done with a quadratic regression model to derive an equation to predict responses to varying doses of 1) mitogens and 2) cell number. The doses of CON A and PHA which yielded maximal responses were 35 ug per ml and 61 ug per ml, respectively, and the optimum number of cells per well was 1.36×10^6. Adherence of the cells to the floor of the culture vessel was essential for the formation of colonies. The number of colonies reached its maximum between day 5 and day 6. The T lymphocyte colony assay may be a useful adjunct to other immunologic assays for studying cell-mediated immunity of domestic animals.

INTRODUCTION

Several techniques for growing lymphocyte colonies in semisolid media from human peripheral blood cells have been described (Rosenszajn et al., 1975; Fibach et al., 1976; Riou et al., 1976; Claesson et al., 1977). The cells comprising individual colonies were shown originally to be predominantly of a mature T cell phenotype as determined by E rosette formation (Shen et al., 1977; Foa et al., 1979; Goube de LaForest et al., 1979; Klein et al., 1981). Additional studies (Triebel et al., 1981) using monoclonal antibodies suggested that the colonies may originate from immature T cell precursors which later develop into mature T cells. Recent evidence (Mossalayi et al., 1984) further confirmed the T cell nature of the colonies, and subpopulations of T lymphocytes bearing CD2, CD3, CD4, and CD8 antigens according to current lymphocyte antigen nomenclature have been identified (Goube de LaForest and Rosenszajn, 1984).

The T colony assay's potential applications include the study of the different steps of T-cell differentiation and their control mechanisms, providing a source of clones for the production of functional cells and lymphokines, and providing a method for the induction and initial cloning of functional subsets of mature T cells before subsequent expansion of these cells by using liquid cultures (Goube de Laforest and Rozenszajn, 1984). The T colony assay has been used to study the cell-mediated immune responses of humans with a variety of

nonhematopoietic diseases such as cancer (Wilson, 1976), rheumatoid arthritis (Winkelstein, 1982), and extrinsic bronchial asthma (Carvajal et al., 1986). It has been used to study the effectiveness of bone marrow T cell depletion as a method to prevent graft versus host disease, after allogeneic bone marrow transplantation in humans (Farcet, 1986), to study hematopoietic disorders such as B cell chronic lymphocytic leukemia (Foa and Testa, 1982) and to evaluate treatment outcomes in acute nonlymphoblastic leukemia (Browman et al., 1983). The lymphocyte colony assay has been used to study the effects of immunosuppressive drugs (Winkelstein, 1984), cytotoxic drugs (Taetle et al., 1983), and irradiation (Woods and Lowenthal, 1984) on lymphocyte colony formation.

Contradictory results of studies of lymphocyte colony formation by human lymphocytes have frequently been reported (Goube de LaForest and Rozenszajn, 1984). The differences in the results were attributed primarily to differences in techniques used for growing T cell colonies. Thus, the need for standardization of techniques for growing human lymphocyte colonies has been realized. Technical parameters which may account for differences in the T colony assay include: 1) the culture system, e.g. single vs. double-layer technique, cell concentration, contents of the culture medium, 2) cell sensitization in a liquid medium prior to transfer to an agar medium, 3) source of the cells, e.g. peripheral blood vs. bone marrow, 4) and addition of colony-enhancing factors, e.g. conditioned media.

Although there are no previous reports of the lymphocyte colony assay being used for immunologic studies of peripheral blood cells from domestic animals, the assay could be used to investigate numerous aspects of normal and abnormal cell-mediated immune responses of animals. An

attempt was made to standardize a lymphocyte colony assay for sheep lymphocytes as a prerequisite to studying the cellular immune responses of bovine leukemia virus infection in sheep. The specific objective of this study was to investigate the effects of different lymphocyte mitogens, mitogen doses, the cell number, the distribution of the cells in the culture vessel, and the duration of the culture upon lymphocyte colony formation by sheep peripheral blood lymphocytes.

MATERIALS AND METHODS

Animals

Eight, 30-month-old healthy crossbred ewes served as blood donors.

Collection of blood

Twenty to fifty milliliters of blood was collected from each sheep into sterile, 10-milliliter, heparinized (14.3 IU per ml), prewarmed (37 C + 3 C) evacuated tubes (Becton Dickinson, Rutherford, NJ) after aseptic preparation of a jugular vein. The blood was transported in a 37 C portable incubator.

Normal autologous sheep serum (NASS)

A volume of whole blood equal to twice the volume of the NASS desired was collected from each sheep into 10.0 ml coagulant tubes from an aseptically prepared jugular vein. The blood was allowed to clot overnight at 26.0 C. The clots were removed using aseptic technique, and the remaining erythrocytes were pelleted by centrifugation at 400 g for 10.0 min. The serum was aspirated, filtered through a 0.20 micron

syringe filter (Millpore Prod., Bedford, MA), heat-inactivated at 56 C for 30.0 min and frozen immediately in 1.5 to 4.0 ml aliquots at -30.0 C.

Storage of phytomitogens

Concanavalin A (CON A) and phytohemagglutinin A (PHA); (Sigma Chemical, St. Louis, MO) were dissolved in sterile Dulbecco's modified Eagle's medium (DMEM, pH 7.4, Gibco, Grand Island, NY) containing 200 IU per ml of penicillin g, 0.2 ug per ml of streptomycin and 0.6 ug per ml amphotericin B (Antibiotic/Antimycotic, Sigma Chemical). Serial two-fold dilutions of CON A (0.55 ug per ul to 0.068 ug per ul) and PHA (1.1 ug per ul to 0.138 ug per ul) in DMEM were aliquoted and stored at -18 C.

Separation of mononuclear cells

Whole blood was diluted in warm isotonic phosphate buffered saline (PBS; pH 7.4; 37 C) at a ratio of 1.5 parts PBS to 1.0 part blood, mixed gently for 5.0 minutes, and layered onto 4.0 ml of a 60% percoll (Sigma Chemical, St. Louis, MO) gradient at a ratio of 2 parts of the blood and PBS mixture to one part 60% percoll (Fig. 3.1A). One hundred percent percoll was prepared (Miyasaka, 1985) by mixing undiluted percoll with Hanks balanced salt solution (HBSS) 10X (Gibco, Grand Island, NY) at a ratio of 9 parts percoll to 1 part HBSS 10X (100% percoll). Sixty percent percoll was prepared from 100% percoll by mixing 100% percoll with HBSS 1X at a ratio of six parts 100% percoll to four parts HBSS 1X (specific gravity 1.075). The diluted blood was centrifuged at 500 g (Jouan CR412, Jouan Inc., Winchester, VA) for 26.0 min at approximately

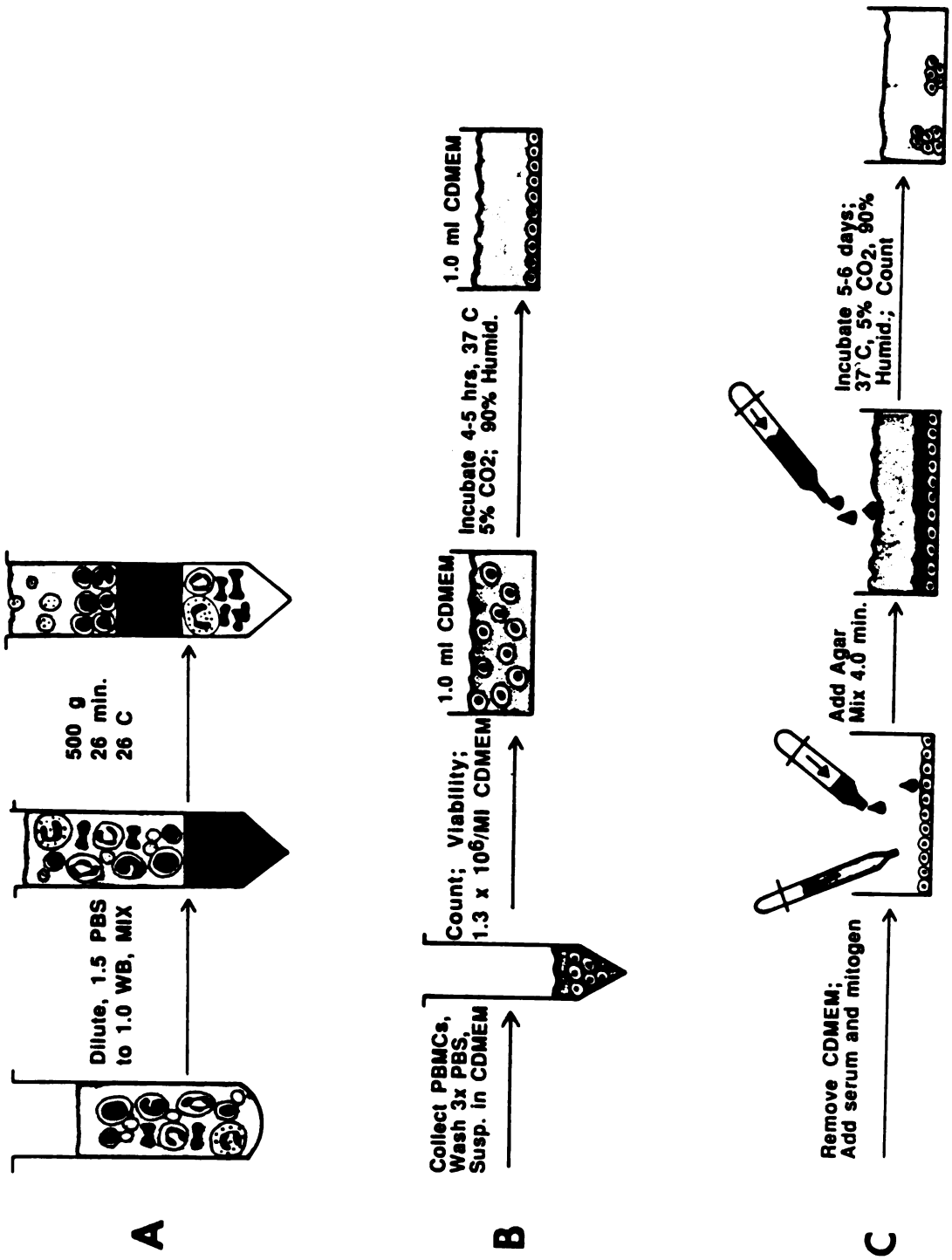

Fig. 3.1 A, B, C. Steps involved in the lymphocyte colony assay for sheep lymphocytes.

37 C. After removal of the plasma-PBS layer, the mononuclear cell layer was aspirated, resuspended in 5.0 ml of warm PBS, mixed thoroughly to reduce spontaneous agglutination, and washed three times in PBS at 50 to 75 g for 5 minutes. The pellet was resuspended in 1.5 to 3.0 ml of warm DMEM. The cells were counted, viability (94 to 100%) was assessed using 0.5% trypan blue dye (Sigma Chemical, St. Louis, MO), and the cell concentration was adjusted to not exceed 10.0 to 12.0 x 10^6 per ml (Fig. 3.1B). These stock PBMCs were incubated at 37 C, 5% CO_2 and were mixed by gently pipetting them every 25.0 min to prevent autoagglutination, if they were not used immediately.

Adherence of PBMC

A volume of the stock PBMC was adjusted to the final desired cell concentration per ml of DMEM and 1.0 ml of the PBMC suspension was seeded into each well of a 16.0 mm diameter, 24-well, flat-bottom, polystyrene tissue culture plate (Costar, Cambridge, MA; Corning Glass Works, Corning, NY). These cells were incubated (Stericult 200, Forma Scientific, Marietta, OH) for 4.5 to 5.0 hours at 37 C, 5% CO_2, 90% humidity to allow the PBMC to settle and adhere to the floor of the wells of each plate (Fig. 3.1B).

Preparation of DMEM-agar

One hour prior to the end of the PBMC-adherence period a 0.4% stock-agar (Bactoagar, Difco, Detroit, MI) suspension was prepared by adding a volume of sterile DMEM (39 C) equal to 20.0% of the final desired DMEM-agar volume to agar in a 50 ml polypropylene centrifuge tube (Corning, Corning, NY). This concentrated agar suspension was gradually dissolved by heating it in 4 to 5 second bursts in a microwave oven at

reduced power, along with vigorous mixing of the solution between each cycle of heating. Additional DMEM equal to 10% of the final DMEM-agar volume was then added, and 2 to 3 additional heating cycles were carried out to completely dissolve the concentrated agar solution. The agar solution was brought to its final volume with warm (39 C) DMEM to give a final concentration of 0.4% stock-agar. The 0.4% DMEM-agar solution was mixed thoroughly and held in a waterbath (39 C) until further use (see below). Thorough dissolution of the agar was critical. Although the DMEM-agar solution was not sterilized during its preparation, microbial contamination of the cultures was never a problem.

Dose response to phytomitogens

The DMEM was aspirated from each well after the 4.5 hour incubation period during which the PBMC adhered to the wells of the plates. One hundred ul of NASS was added to the cells in each well followed by the addition of 50 ul of mitogen (Fig. 3.1C). Control wells received 50 ul of DMEM. After addition of the NASS and mitogens, the contents of the plates were gently manually mixed for 4.0 minutes. The final serum concentration was 18.0 percent, the final CON A concentrations were 6, 12, 25, and 50 ug per ml of DMEM-agar and the final PHA concentrations were 12, 25, 50, and 100 ug per ml of DMEM-agar. Preliminary studies using doses outside these ranges indicated that lower mitogen doses were nonstimulatory to the PBMC, and higher doses were toxic. The final PBMC number per well and concentration per ml of DMEM-agar used to study the responses to the mitogen doses was 1.0×10^6 per well and 1.81×10^6 per ml, respectively. An average of 5 measurements were made at each dose of mitogen by using cells from each of 6 sheep.

Addition of DMEM-agar

Four hundred microliters of the 0.4% stock-agar was added to each well and the liquid contents were gently mixed either manually or by using a rotator (American Dade, Miami, FL 33152) for 4.0 minutes. Vigorous swirling of the plates as a means of mixing their contents resulted in detachment of the adhered PBMC. The agar-overlays were allowed to harden by incubating at 26 C for at least 30.0 to 45.0 minutes. The plates were then gently shaken by hand to confirm solidification of the agar and were incubated at 37 C, 5% CO2, 90% humidity for 5.0 to 6.0 days.

Optimum number of mononuclear cells

The effect of mononuclear cell number upon lymphocyte colony formation was determined by culturing 0.25, 0.50, 1.0, and 1.5 x 10^6 PBMC per well. No colonies formed when 2.0 x 10^6 cells per well were cultured. The final PBMC concentrations per ml of DMEM-agar were 0.45, 0.90, 1.81, and 2.72 x 10^6 PBMC. An average of 6 measurements were made at each dose of cells by using cells from each of seven (7) sheep.

Adhered PBMC versus suspended-PBMC cultures

For the adhered-PBMC cultures, 1.3 x 10^6 PBMC in DMEM were allowed to adhere to the culture plates during a 4.5 to 5.0 hour incubation period. After removal of the DMEM, a mixture of 100 ul of NASS (18% final concentration) and 50 μl of CON A (33 ug per ml final) were added followed by the addition of 400 ul of 0.4% DMEM-agar. The suspended-PBMC cultures were prepared by first adding 150 ul of serum and mitogen combined to the wells followed by the addition of 400 ul of DMEM-agar containing PBMC at a concentration of 3.25 x 10^6 per ml. The final PBMC

concentration per well was 2.36 x 10^6 per ml of DMEM-agar, and the final concentration of agar was 0.29%.

Colony counts

The lymphocyte colonies were counted after 5 to 6 days in culture by using an inverted microscope (Biostar, Cambridge Instruments, Buffalo, NY) at 100X magnification. A colony was defined as a group of 30 or more large, blast-like cells. These criteria were used in order to distinguish colonies from small groups of more mature cells which arose infrequently as a result of cellular aggregation or clumping of the PBMC prior to the PBMC adherence-step. Photographs of the colonies were made with a 35 mm automated camera (Photostar, Cambridge Instruments, Buffalo, NY) and ektachrome 160 tungsten film (Kodak, Rochester, NY).

Duration of the culture

Visual observation was used to assess the optimum duration of the culture. The plates were scanned microscopically on days 3 through 8 to determine the approximate day on which new colonies ceased to form.

Plating efficiency

Plating efficiency (PE) was determined using the following formula: PE = no. of lymphocyte colonies $\div$ [(0.6) x no. of PBMC per culture vessel] X 100, since only about 60 per cent of the sheep peripheral blood mononuclear cells have a T cell phenotype (Ezaki et al., 1985). PE was estimated by using the colony and cell counts of wells in which 1.3 x 10^6 PBMC were cultured, since this cell count provided optimum growth of the colonies.

Statistical Analysis

The effects of CON A and PHA doses and PBMC number upon colony formation were analyzed by using regression analysis (MicroTSP). The independent variables were mitogen dose or cell number per well, and the dependent variable was the number of colonies formed. A comparison of the adhered-PBMC and suspended-PBMC culture techniques was done using a two-factor crossed analysis of variance (ANOVA) model in which the treatments "adhered-PBMC" and "suspended-PBMC" were repeated for each sheep (Petersen, 1985). The data were analyzed using the general linear models procedure from the computer program SAS (SAS User's Guide, 1985). Plots of the data were made using the computer program, PLOT IT (Eisensmith, 1986).

RESULTS

Colonies

The colonies contained several hundred to several thousand cells (Figs. 3.2A, B, C, D).

Dose Response to lymphocyte mitogens

Preliminary studies using CON A doses exceeding 50 ug per ml and PHA doses exceeding 100 ug per ml resulted in no colony formation, probably due to toxic doses of the mitogens. Thus, the range of the doses used to generate the dose-response curves were 6 to 50 ug per ml for CON A (Fig. 3.3) and 12 to 100 ug per ml for PHA (Fig. 3.4). The regression model

Fig. 3.2 A, B, C, D. Lymphocyte colonies in a 5-day-old soft-agar culture of sheep lymphocytes stimulated with 33 ug/ml of CON A. A,40X; B,100 X; C,200X; D,450X. Colonies consisted of several hundred to several thousand cells.

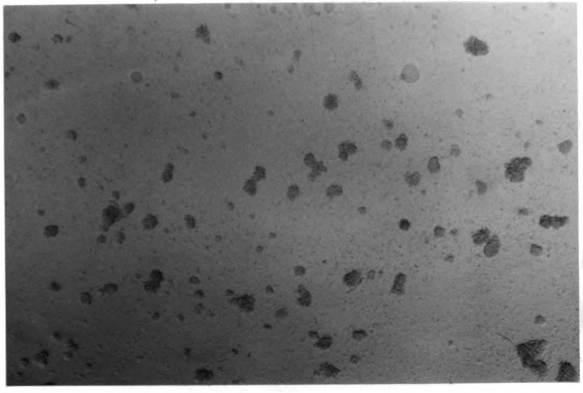

Fig. 3.2A. Colonies at 40X.

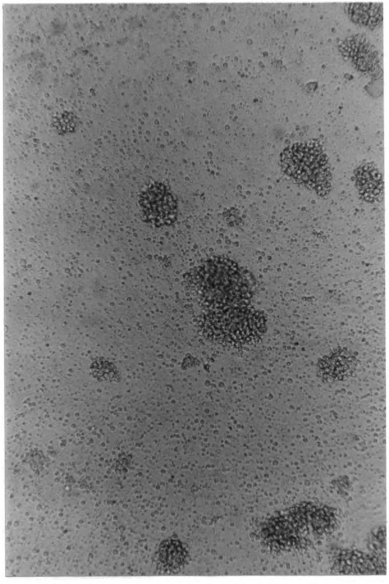

Fig. 3.2B. Colonies at 100X.

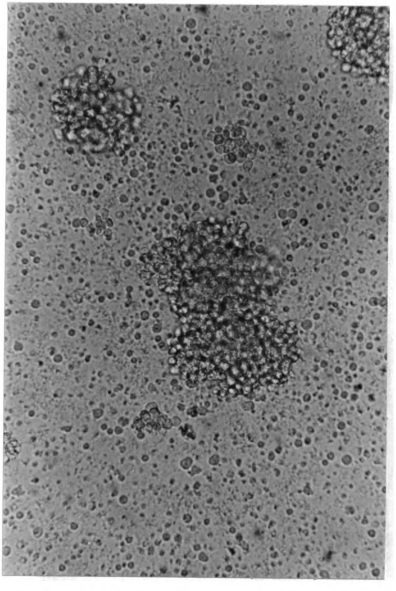

Fig. 3.2C. Colonies at 200X.

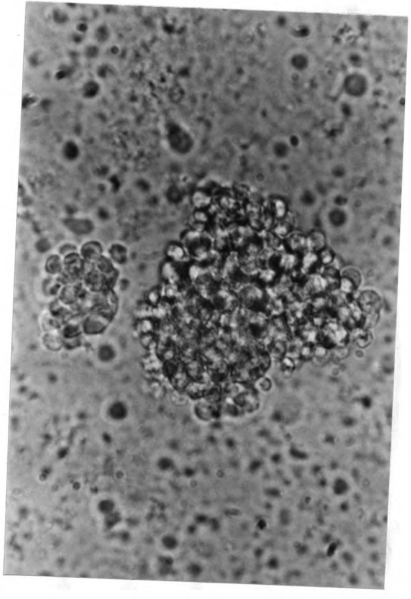

Fig. 3.2D. Colonies at 450X.

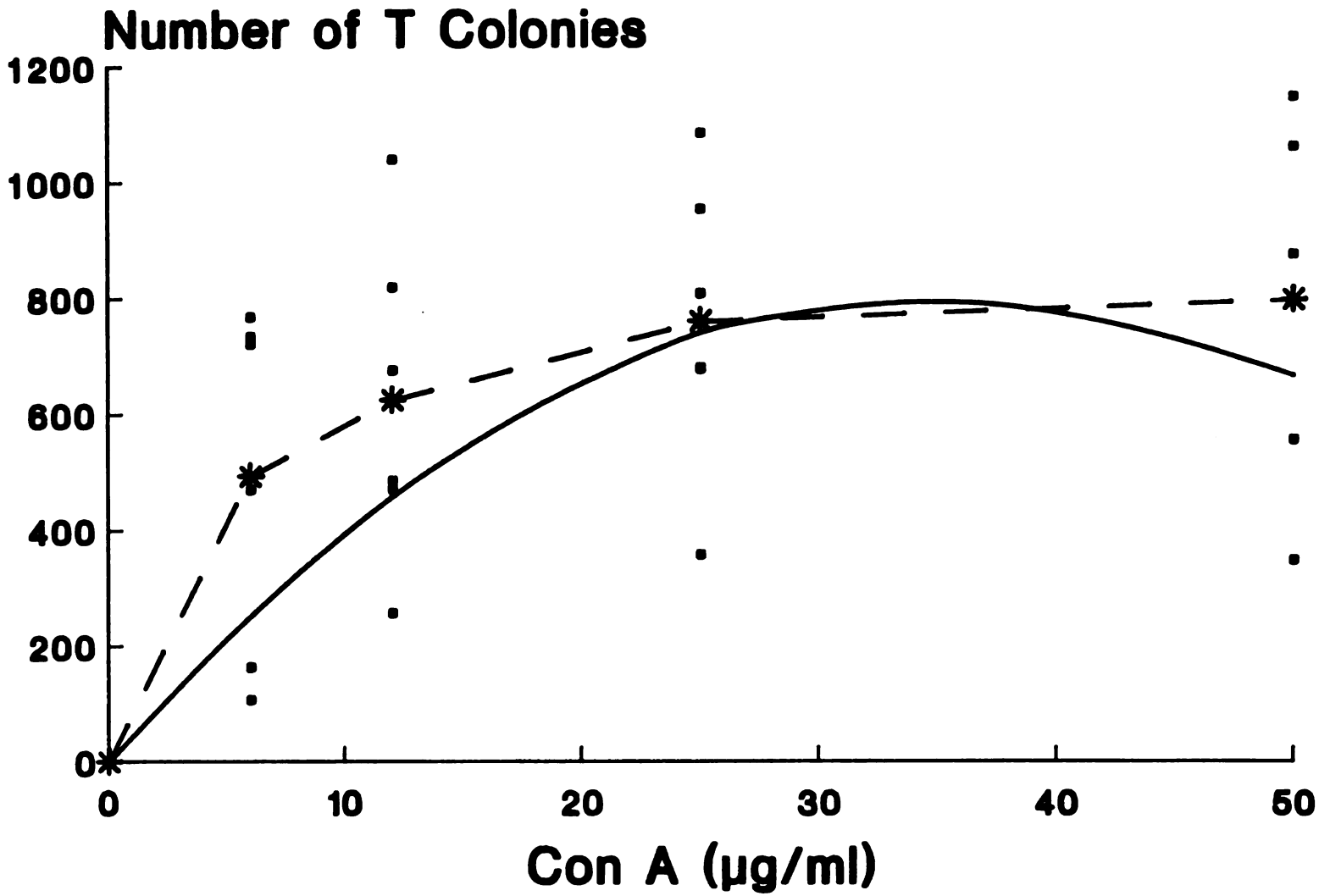

Fig. 3.3. Dose-response of lymphocytes from 6 sheep to the phytomitogen concanavalin A (CON A). -- * -- = observed mean number of colonies; ———— = predicted mean number of colonies; ■ = mean response of 5 measurements from each of 6 sheep; p =0.001; R^2 = 0.65.

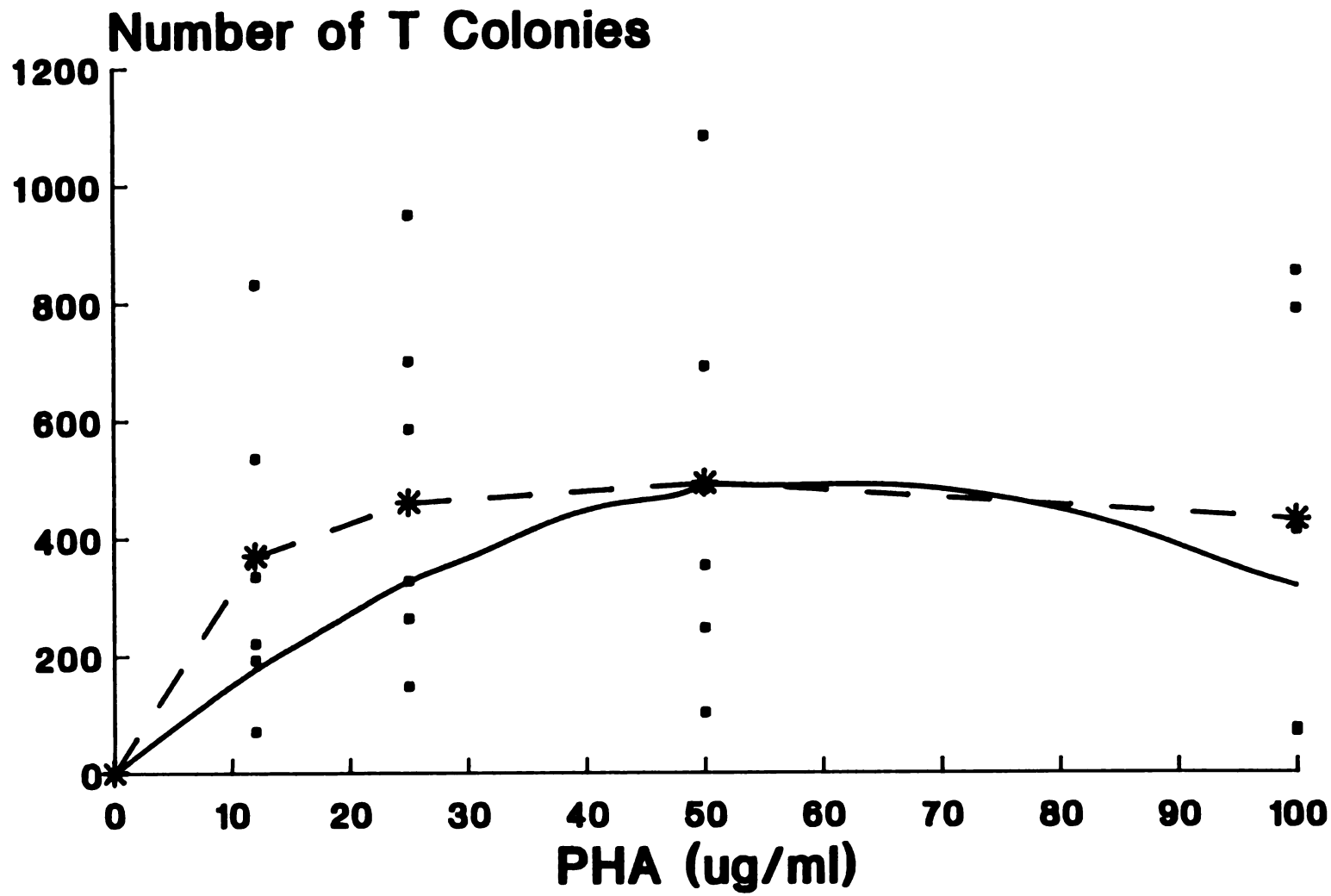

Fig. 3.4. Dose-response of lymphocytes from 6 sheep to the phytomitogen phytohemagglutinin A (PHA). -- * -- = observed mean number of colonies; ———— = predicted mean number of colonies; ■ = mean response of 5 measurements from each of 6 sheep; p =0.001; R^2 = 0.65.

which best described the responses to both CON A and PHA was a quadratic model which included dummy variables for each sheep. The regression model was:

$Y = \beta_0 + \beta_1(X_1) + \beta_2(X_1)^2 + e$, where

Y - the number of lymphocyte colonies formed,

X_1 - the dose of mitogen,

β_0 - a constant,

β_1, β_2 - coefficients for the independent variable,

e - error term.

The number of the colonies formed by CON A-stimulated cells always exceeded the number formed by PHA-stimulated cells when the doses of CON A and PHA were the same, i.e. 12, 25, and 50 ug per ml doses. The size of the colonies formed in CON A-stimulated cultures was greater, and the cells forming these colonies appeared to be larger, more blast-like structures, thus suggesting that CON A was a better stimulant.

The effect of mitogen dose on colony formation was statistically significant for both CON A ($p = 0.001$) and for PHA ($p = 0.001$). The coefficient of determination (R^2) for CON A and PHA respectively was 0.70 and 0.65 respectively, suggesting that the majority of the variation in colony-formation was due to the changes in the dose of the mitogens and to differences between individual sheep.

The actual regression equations used to predict the lymphocyte colony formation for any dose of mitogen for 1) CON A and for 2) PHA were:

1) Number of colonies formed = 106 + 46 (CON A dose) - 0.66 (CON A dose)$_2$.

2) Number of colonies formed = -43 + 16(PHA dose) - 0.13(PHA dose)^2.

The dose of mitogen at which maximum proliferation of the colonies occurred was determined by computing that dose at which the slope of the prediction line became zero, i.e. the first derivative of the prediction equation for each mitogen. The dose of CON A at which maximum colony proliferation occurred was 35 ug per ml and it was 61 ug per ml for PHA (Fig. 3.3 & Fig. 3.4). No colonies formed in the negative control wells.

Optimum number of mononuclear cells

Preliminary studies using 2.0 x 10^6 PBMC per well resulted in no colony formation. Thus, the doses of cells between 0.25 and 1.50 inclusive were used to determine the optimum number of PBMC per well (Fig.3.5). The actual regression model which best described the relationship between colony formation and the number of PBMC per well was the same as that for the mitogens, except that the number of PBMC was substituted in the equation for the independent variable mitogen dose, and seven sheep were used for these experiments. The effect of PBMC number on colony formation was statistically significant ($P = 0.001$), and the coefficient of determination was 0.49, indicating that approximately one half of the variability in colony formation was due to the changes in the number of PBMCs cultured and to differences between individual sheep. The actual regression equation used to predict the number of colonies

formed for a specified number of PBMC was:

$$\text{No. of colonies} = 66 + 1092\,(\text{No. of PBMC}) - 402\,(\text{No. of PBMC})^2.$$

The approximate dose of PBMC at which maximum colony proliferation occurred was 1.36×10^6 PBMC per well (Fig. 3.5).

Adhered versus suspended PBMC

PBMC which were first allowed to adhere to the bottom surface of the well before being overlayed with agar produced as high as a 33-fold increase in the number of colonies when compared to an equal number of cells which were suspended in the agar. The number of colonies obtained using the adhered-cell technique was statistically greater ($p = 0.0001$) than the number obtained using the suspended-cell technique (Fig. 3.6).

Duration of culture

The colonies became visible by day 3, and the maximum number of colonies was attained between day 5 and day 6 based upon visual observation. The size of individual colonies continued to increase, however, often leading to coalescence of the colonies and increased difficulty in counting them.

Plating Efficiency

The PE was approximately 0.144 per cent, indicating that only about 144 of every 10^5 peripheral blood T lymphocytes had colony-forming capacity.

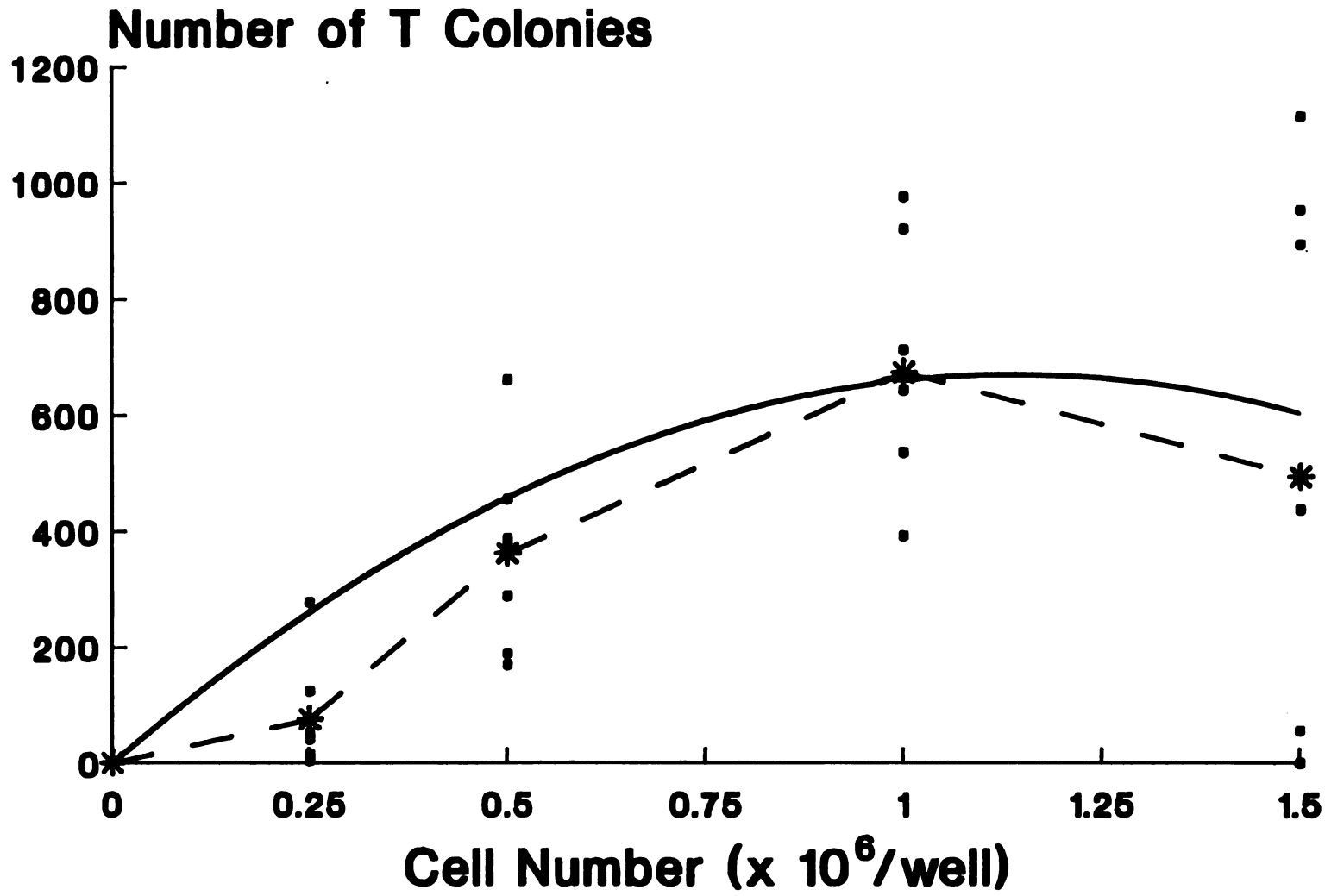

Fig. 3.5. Dose-response of lymphocytes from 7 sheep to varying numbers of cells per well. -- * -- = observed mean number of colonies; ——— = predicted mean number of colonies; ■ = mean response of 6 measurements from each of 7 sheep; p =0.001; R^2 = 0.49.

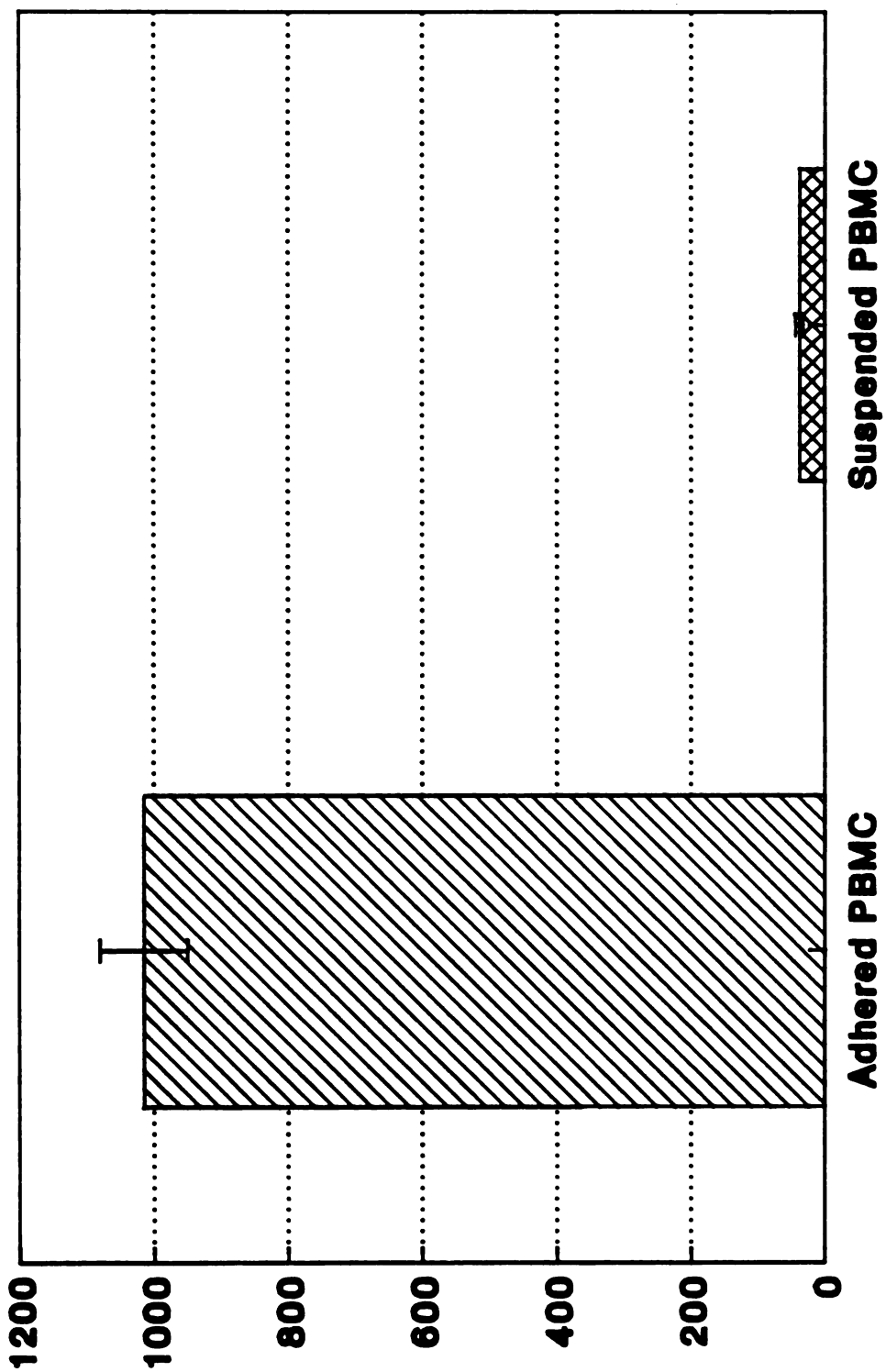

Fig. 3.6. Comparison of lymphocyte colony formation by PBMC which either were adhered to the floor of the plate or were suspended in the agar layer. The results were statistically different ($P = 0.0001$).

DISCUSSION

The doses of the mitogens that have been reported for the lymphocyte blastogenesis assay, a standard assay of cell-mediated immunity of sheep cells, was between 2 ug per ml and 100 ug per ml (Rai-El-Balhaa et al., 1987; Staples et al., 1981). Dose-response curves were rarely reported, so the basis for the selection of the doses by these and other investigators is unknown. Thus, these studies were done to determine the optimum responses to two T lymphocyte mitogens, not only due to the technical differences between the two assays but also to the wide range of differences in the doses reported by others. The T colony assay and the lymphocyte blastogenesis assay are similar in that the proliferative responses varied both between mitogens and among doses for a given mitogen.

A double-layer agar system, usually consisting of a 0.5 percent lower-agar layer overlaid with a 0.33% upper layer of agar in which the cells were suspended, has been used to culture human lymphocytes (Rozenszajn et al., 1975; Fibach et al., 1976; Gerassi and Sachs, 1976). However, the sheep lymphocytes failed to produce colonies when the double-layer technique was used in these experiments. Human lymphocytes used in the double-layer cultures usually were preincubated in a liquid medium containing PHA, and the cells often agglutinated during the incubation period. Although the agglutinated human cells could be dispersed into single cells by vigorous pipetting, the sheep cells agglutinated irreversibly, so the liquid preincubation step was subsequently abandoned. Substitution of methyl cellulose for agar has been used to prevent the clumping and reduced viability associated with

liquid preincubation of human cells (Shen et al., 1977), but methylcellulose was not used as a medium for culturing sheep lymphocytes in our experiments.

Both the distribution of the cells in the wells as well as the number of cells per well affected the formation of colonies by sheep cells. Sheep cells appear to require closer contact than human cells in order for colonies to form, and the only method by which this close contact could be obtained was by allowing the cells to adhere to the floor of the wells in the plates. Colonies did not form when an equal number of cells was suspended in the agar. Attempts were made to increase the contact between the cells suspended in the agar by increasing the concentration of the cells, but this procedure also failed to increase colony formation. In spite of the fact that a very large number of cells were used, thus requiring a prohibitively exhaustive PBMC-fractionation and collection process, colony formation was not substantial in the suspended-PBMC cultures.

Colony proliferation did not occur at low cell concentrations, even when the cells were allowed to adhere to the wells. Human cells have been cultured at concentrations between 1.0×10^3 per ml to 1.4×10^6 per ml of the final culture volume. The effects of varying the concentration of human cells were rarely studied (Shen et al., 1977; Rozenszajn et al., 1981), and the cell concentration which resulted in maximum colony formation was reported in only one of those studies (Shen et al., 1977). Attempts to culture sheep lymphocytes at concentrations approximately equal to those used for human cells failed, necessitating the generation of a dose-response curve to determine the optimum number of cells per well. The minimum number of cells, approximately 2.5×10^5 per well or

4.5 x 10^5 per ml, required for the generation of colonies by sheep lymphocytes was at least twice the numbers commonly used in the human lymphocyte colony assays. Also, the diameter of the wells of the culture plates used for sheep cells was usually two to four times smaller than the wells of plates used for the human cells, thus providing less surface area over which the sheep cells could spread. The optimum number of sheep cells per well was 34 times greater than the number of human cells which were successfully cultured in the same 16 mm-diameter well (Mossalayi et al., 1986). This suggests that much closer contact is required for colonies to be generated from sheep cells.

Proliferation of normal resting T lymphocytes requires presentation of an antigen or mitogen to the T lymphocytes in association with the major histocompatibility complex on the surface of an antigen presenting cell (Male et al.,1987; Milanese et al., 1987). The concomitant release of interleukin 1 increases the expression of interleukin 2 receptors by T cells, and the IL 2 released from T helper cells binds to the IL 2 receptors and provides a final proliferative signal for many types of T cells. The necessity of the APC, e.g. monocytes and macrophages, in the formation of colonies from resting human lymphocytes has been documented (Goube de LaForest et al, 1980; Winkelstein, 1983). Cell cooperation between mitogen-induced colony-forming cells and accessory cells was thought at the time to be mediated by unidentified diffusible soluble mediators. Others (Klein et al., 1983; Winkelstein et al., 1986) confirmed that IL 1 and IL 2 were among these mediators by showing that either monocytes or IL 1 released from activated monocytes induced T colony formation, that IL 2 and lymphocyte colony promoting activity were related entities (Klein et al., 1983), and by showing that both

recombinant (Jourdan et al., 1985) and human lymphocyte-derived IL-2 were able to enhance the clonal growth of PBMC (Winkelstein et al., 1986). Furthermore, a monoclonal antibody to the IL 2 receptor completely inhibits T-colony formation in PHA-stimulated PBMC, when directly added to the culture system (Jourdan et al, 1985).

Evidence for the importance of cell-to-cell contact also has been demonstrated by showing a strong linear relationship between the number of accessory cells plated and the number of T colonies formed (Farcet et al., 1984). The relatively high concentration of sheep PBMC which were required for colony formation to occur along with the failure of colonies to form when PBMC were suspended in the agar suggest that the plating efficiency of sheep cells is lower than that of human cells. Lower number of cells with colony-forming capacity and lower production and diffusibility of the various interleukins may explain the reduced plating efficiency of sheep cells relative to human cells.

The T lymphocyte colony assay is proving to be a useful tool for studying aberrations in cell-mediated immunity of humans. Although techniques for this assay have not been described previously for common domestic species such as sheep, cattle, cats and dogs, its application also may be particularly useful for studying diseases involving immunologic disorders restricted to animals as well as those disorders which resemble diseases of humans. The T colony assay is now being used in immunologic studies to investigate the feasibility of using bovine leukemia virus, the etiologic agent of enzootic bovine leukosis, as an agent and a disease model for the human T lymphocytotrophic retroviruses.

The steps involved in the T colony assay for sheep lymphocytes have been summarized in Figs. 3.1A, B, C. The results of this study suggest

that the technical parameters for the culture of sheep lymphocytes in soft agar are quite different both qualitatively and quantitatively from the techniques used for human cells. The reduced plating efficiency required the use of a larger number of cells, the colony size, shape and number varied with the type and dose of mitogen used, adherence of the cells to the plate was essential for colony formation to occur, and fresh autologous serum seemed to have given better responses than either commercially obtained sheep or bovine serum. These technical parameters should be considered during the standardization of the lymphocyte colony assay for cells of other animal species.

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CHAPTER IV

LYMPHOCYTE COLONY FORMATION BY ALEUKEMIC SHEEP INFECTED WITH BOVINE LEUKEMIA VIRUS

ABSTRACT

The current interest in using BLV as a model virus for human lymphocytotrophic retroviruses stimulated an investigation of the leukemogenic mechanism of BLV. Colony formation by lymphocytes from bovine leukemia virus-infected, aleukemic sheep was assessed as a means of evaluating possible abnormalities in the interleukin 2 (IL 2)/interleukin 2 receptor system in these sheep by using a lymphocyte colony assay in which autologous sheep serum was used as the nutrient. There was no significant difference in the number of lymphocyte colonies formed by BLV infected and control sheep. Nor was there a significant difference in the number of colonies formed by lymphocytes from the BLV infected sheep when autologous serum was replaced with either pooled serum from the infected sheep or from the control sheep. Thus, we were not able to demonstrate abnormalities in IL 2 activity in aleukemic sheep infected with BLV by using a lymphocyte colony assay. The effects of human recombinant interleukins on colony formation and IL 2 production by cells from these sheep will now be investigated.

INTRODUCTION

Retroviruses have long been been known to cause a variety of naturally occurring diseases in several animal species (Gross 1983, Varmus, 1988). The diseases can be categorized into (a) diseases characterized by uncontrolled growth of cells of various types and origins, e.g. leukemias and lymphomas; (b) diseases characterized by the loss of certain cell types, e.g. immunodeficiencies and anemias, and (c) diseases characterized by inflammation and autoimmunity, e.g. encephalitis and arthritis. Although retroviruses were thought to be involved in human cancers, evidence for this was long missing due to the lack of biological and biochemical markers, since the human viruses had not been shown to share antigenic cross-reactivity with known animal retroviruses (Gallo et al., 1977). Retroviruses could not be easily isolated from tissues or fluids of human patients, unlike in most animal models in which viral replication and viremia are usually present (Hardy 1985, Robinson et al., 1986). Failure to isolate the viruses generated skepticism about the involvement of retroviruses in human malignancies.

Bovine leukemia virus is an exception to most animal retroviruses in that viremia has been rarely found. BLV was discovered only after lymphocytes from cattle with leukemia were cultured in vitro (Miller et al., 1969). Human T cell leukemia/lymphoma virus Type I (HTLV I) is similar to BLV in this respect, and this knowledge of BLV was used to isolate HTLV I from human lymphocytes. A second major contribution to the first isolation of HTLV I was the discovery of interleukin 2 (IL 2), a lymphokine specific for the long-term growth of T lymphocytes in vitro

(Morgan et al., 1977). The isolation of the prototype virus from cultured lymphocytes of patients with lymphoma (Poiesz et al,.1980) was followed by the availability of specific probes (Hinuma et al., 1982) which enabled the establishment of a link between the human virus and adult T cell leukemia (ATL), a rapidly progressing cancer of T4 lymphocytes, first described in southeastern Japan (Yoshida et al., 1982).

The proviral genomes of HTLV I, HTLV II, and BLV each have the obligatory gag, pol, and env retroviral genes which code for structural proteins (Fig.4.1). The 3' parts of the proviruses are unique from other retroviruses , however, because they contain an unusual sequence termed "pX" between the env gene and the 3'long terminal repeat (Fig 4.2). The pX region of the genome encodes a nuclear transcriptional activator of protein, Tax (previously referrred to as the tat protein) (Felber et al., 1985) that activates the expression of genes directed by the viral long terminal repeat (LTR) sequences. Likewise, expression of the tax gene is thought to activate expression of several cellular genes, including those for IL 2 and the interleukin 2 receptor (IL-2R) (Greene et al., 1986; Greene et al., 1987).

Upon exposure to antigens and mitogenic signals, normal T lymphocytes express IL 2 receptors (IL 2R) (Robb et al., 1981) (Fig. 4.3 and Fig. 4.4). These activated T cells produce IL 2 which, after interaction with high-affinity IL 2 receptors, stimulates the proliferation of these cells. Unlike normal T cells, cells of ATL patients do not require prior activation to express IL 2 receptors (Poiesz et al., 1980). HTLV I infected leukemic T cell lines express 5 to 10 fold more receptors per cell than do maximally PHA-stimulated T lymphoblasts, and unlike normal

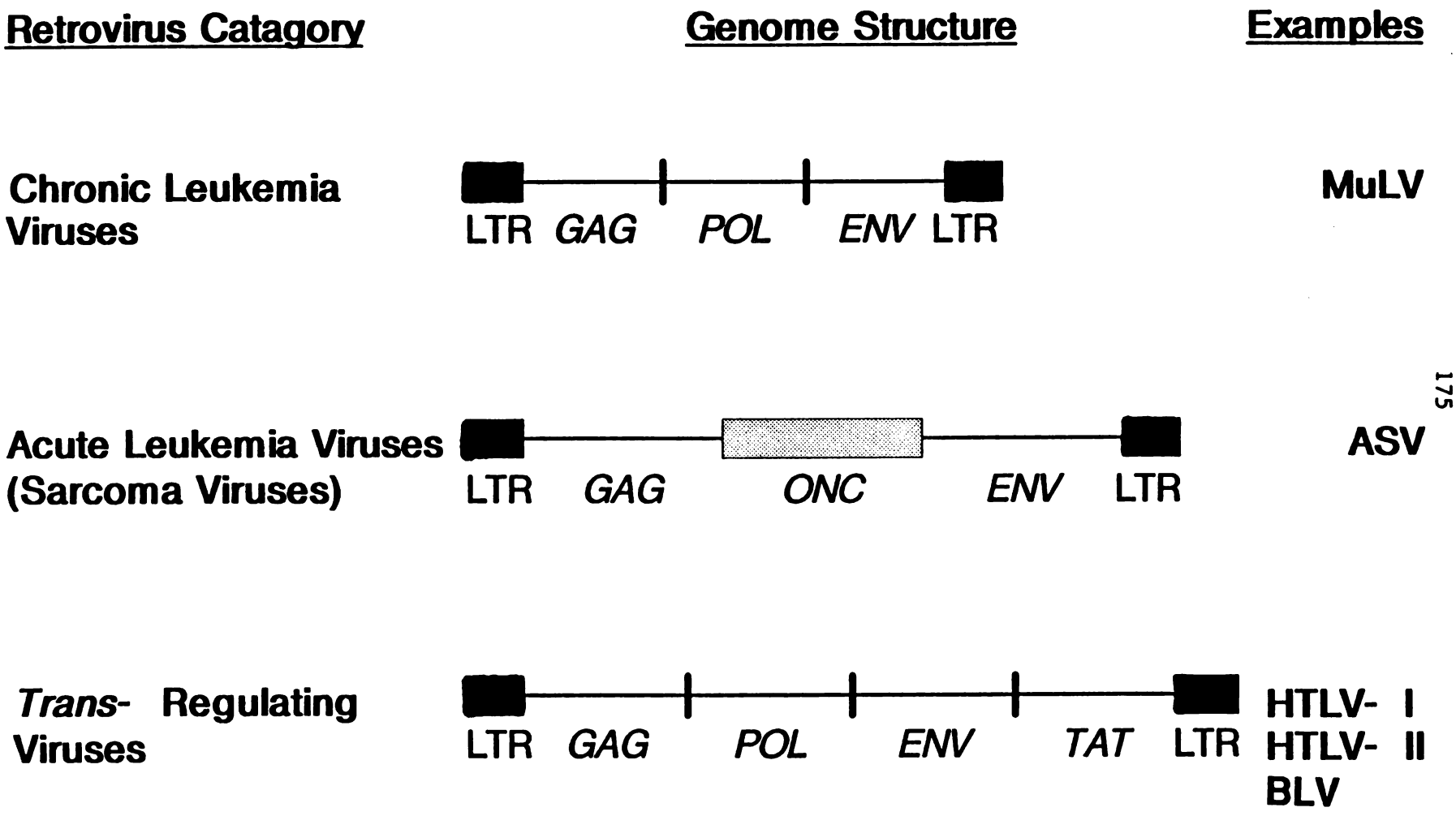

Fig. 4.1. Genome structure of some leukemia-causing retroviruses.

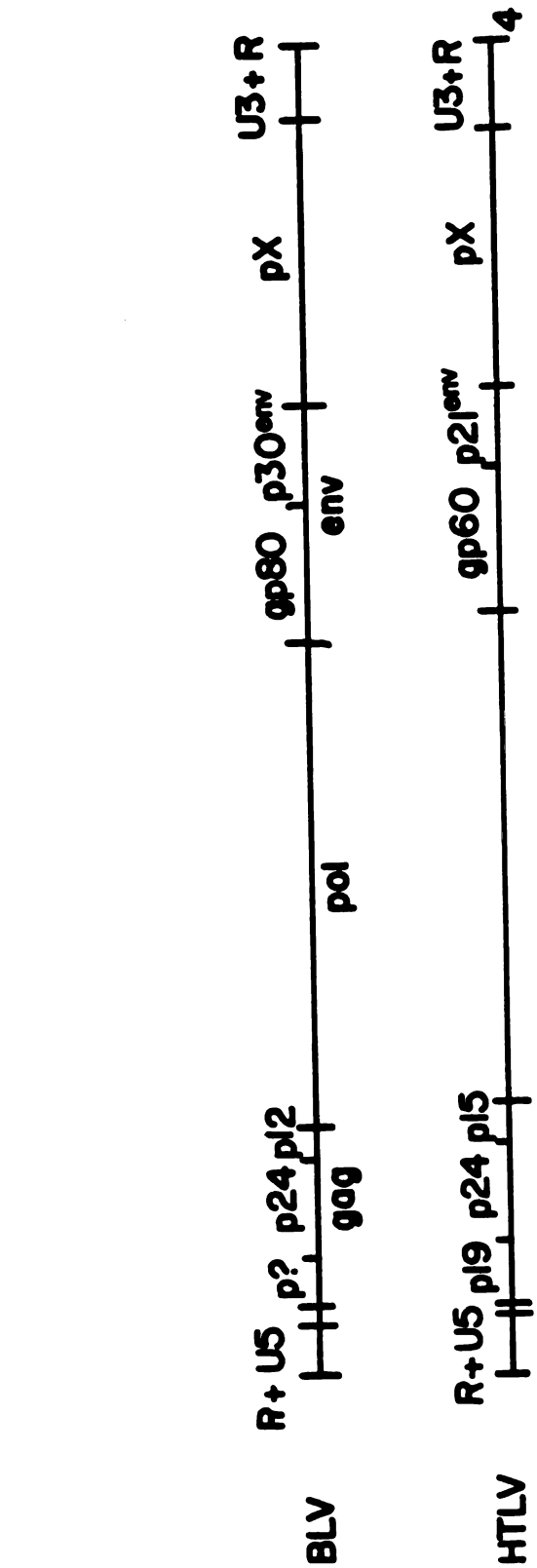

Fig. 4.2. Comparison of the genome structure of BLV and HTLV I.

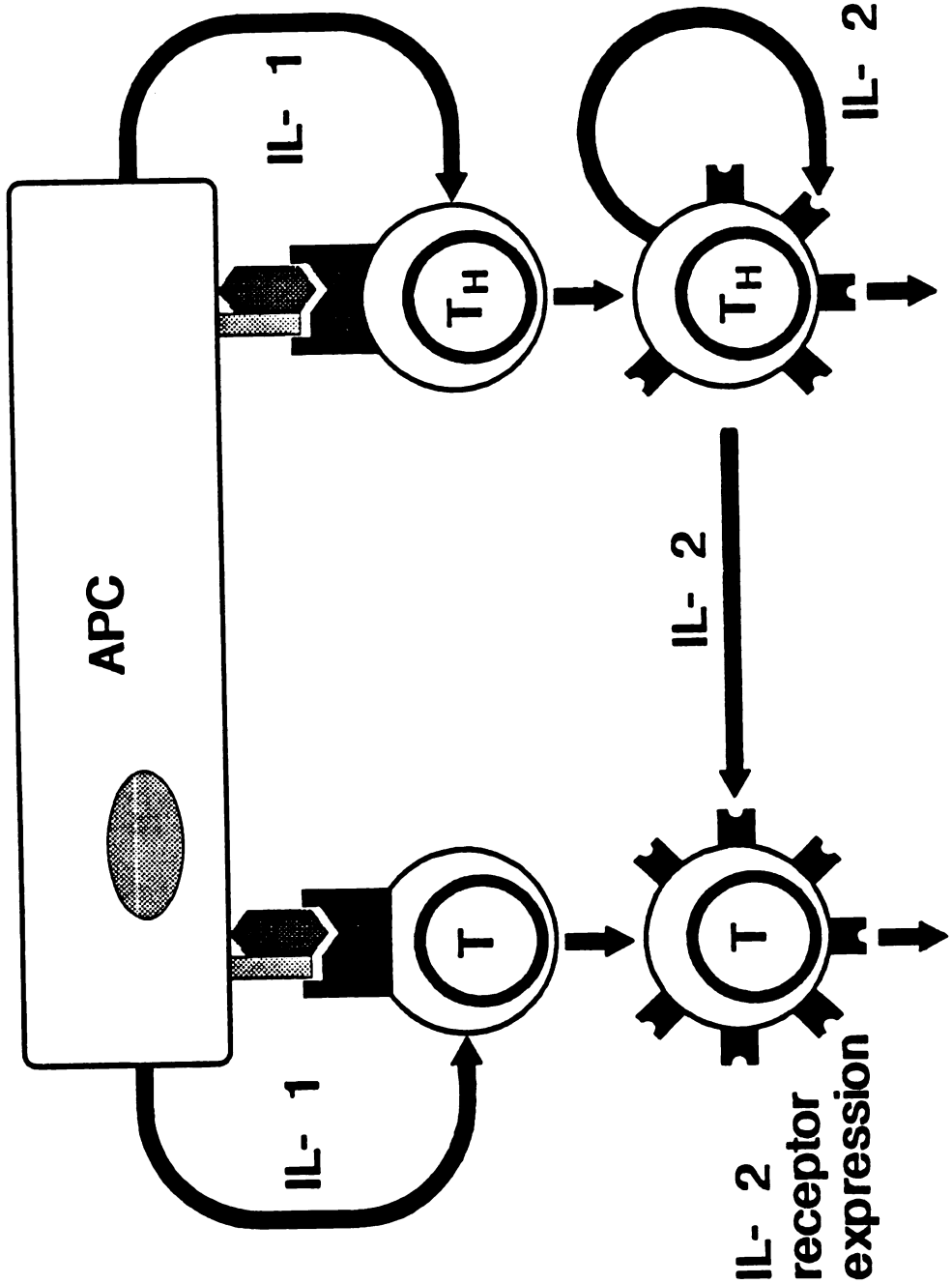

Fig. 4.3. T helper, T suppressor, and T cytotoxic response to IL 2 (revised from Male et al., 1987).

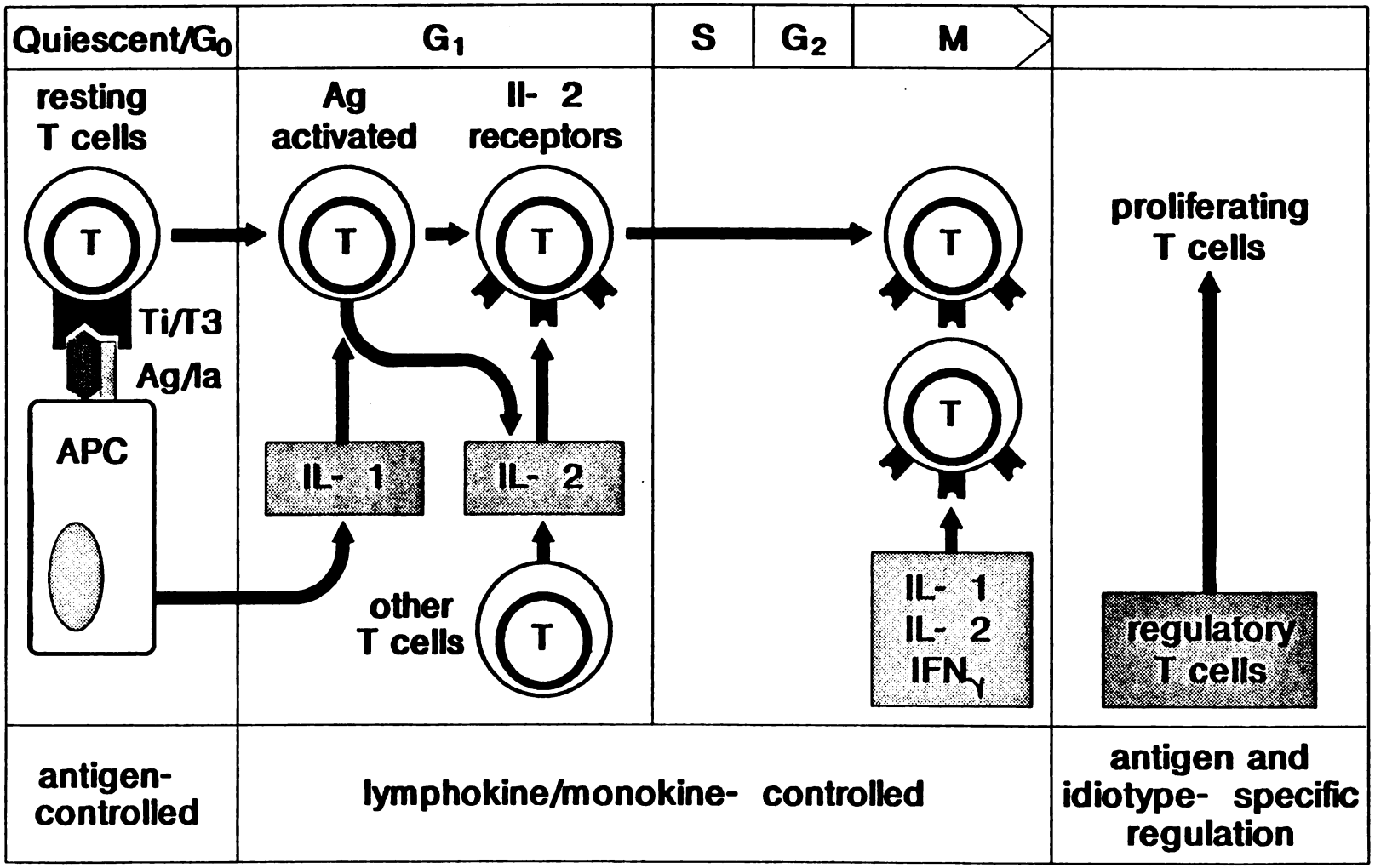

Fig. 4.4. Normal mechanism for activation of a T cell (revised from Male et al., 1987).

cells, ATL cells do not show a decline in IL 2 receptor numbers in culture (Waldmann et al., 1984). Thus, it was proposed that HTLV I proteins may play a role in the increased production of IL 2 and IL 2 receptors (Fig. 4.5).

Potential animal models for studying mechanisms of leukemogenesis induced by human retroviruses are few. The mechanisms in the existing animal models are thought to be oncogene-mediated (Hardy, 1985; Robinson et al.,1986). The similarities in the clinical pictures of the diseases caused by HTLV I and BLV, the presence of the pX regions in their genomes, and amino acid sequence homology between the two viruses suggest that BLV infection in animals may be a useful animal model for HTLV (Burny et al., 1984). BLV infects mainly B lymphocytes in cattle although recent evidence suggests that T lymphocytes may be infected also (Williams et al., 1989). In sheep, however, the primary target cells seems to be T lymphocytes (Horvath, 1982), the same as with the HTLV's. Both leukemia and lymphoma occur in BLV infected sheep after a latent period which is relatively short compared to that of cattle (Rogers et al., 1984).

Increased IL 2 levels in serum and plasma have been associated with several diseases of humans, and IL 2 levels in serum and plasma in humans usually exceed those found in vitro (Cornaby et al., 1988). Thus, serum and plasma may be more appropriate than lymphocyte supernatants as a medium for conducting biological assays of IL 2 activity.

Studies of the generation of colonies by T lymphocytes in vitro have provided valuable information on the normal mechanisms controlling T cell proliferation. The necessity of IL 2, IL 1, and accessory cells in the generation of colonies by human lymphocytes has been reported

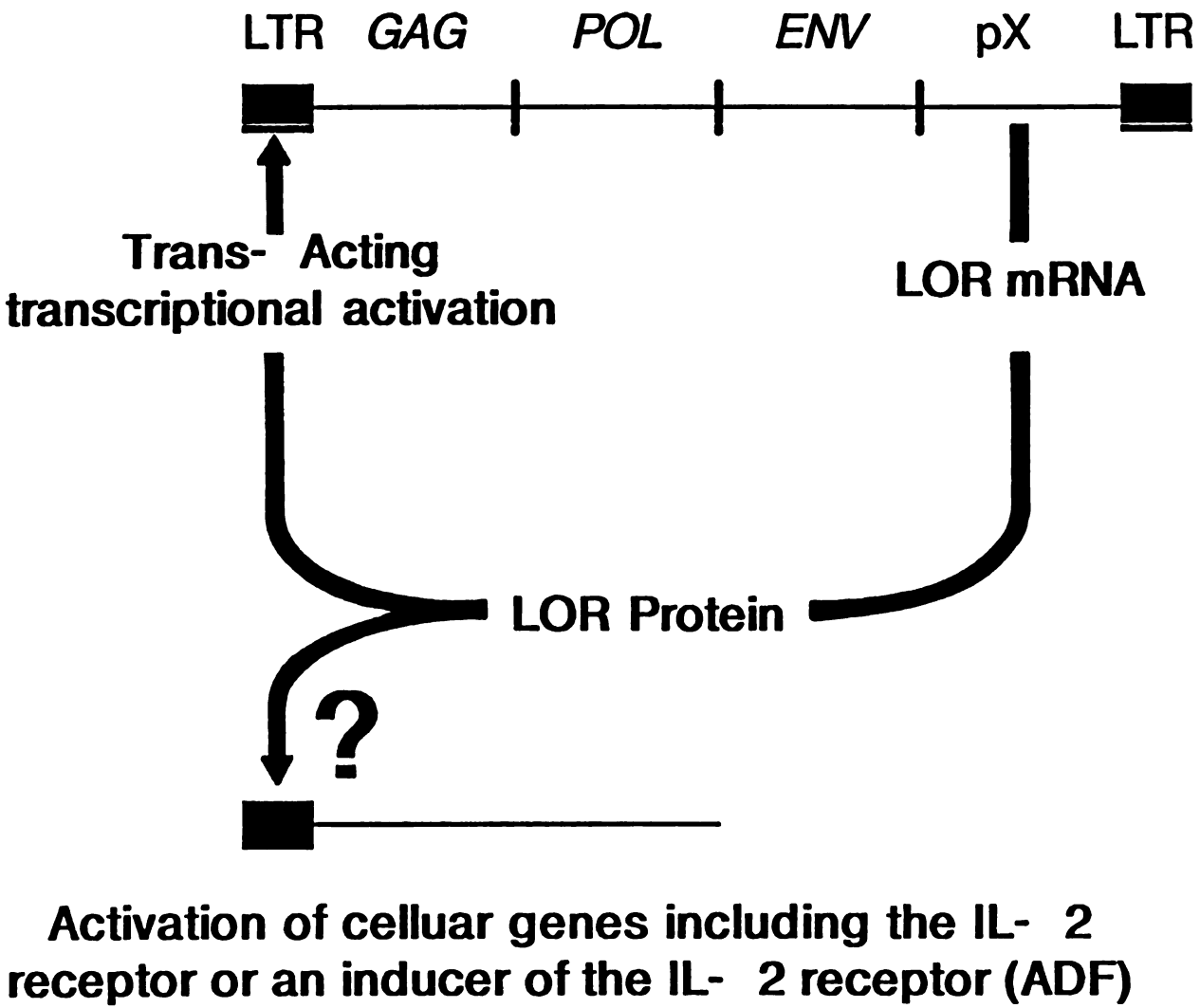

Fig. 4.5. Hypothetical mechanism for IL 2 receptor expression in HTLV I-infected cells.

(Winkelstein, 1983). Recombinant IL 2 (Jourdan et al. 1985) and lymphocyte derived IL 2 (Winkelstein, 1986) enhanced the clonal growth of lymphocytes, and a monoclonal antibody to the IL 2 receptor completely inhibited T colony formation (Jourdan et al., 1985). Thus, the T colony assay maybe a useful tool for studying cellular immune responses to BLV infection.

The major histocompatability complex (MHC) of humans and mice plays a vital role in directing and controlling the immune response. Studies of the MHC of sheep and cattle suggest that it may influence the susceptibility and resistance to some diseases (Cullen et al., 1984; Outteridge et al.,1985). Leucocyte antigens of cattle have been shown to affect the susceptibility of cattle to persistent lymphocytosis (Lewin et al., 1986). The MHC of sheep infected with BLV may also influence their apparent susceptibility to the development of tumors.

The discovery of an ever-increasing number of human and animal retroviruses (Gurgo and Gallo, 1987) lends support to the need for developing strategies to protect against diseases caused by these agents. The absence of suitable animal models for some of the viruses, the similarities between BLV and HTLV I and between ATL and bovine leukemia, the susceptibility of sheep to BLV infection, the abbreviated period for the induction of tumors in sheep, and the fact that T lymphocytes are a target cell of BLV in sheep suggest that sheep may be a useful animal model for understanding the biological behavior of retroviruses. We used these facts as a basis for testing the following hypotheses: (1) that BLV infection in sheep would result in increased colony formation by lymphocytes in vitro, due possibly to BLV-induced abnormalities in the IL 2/IL 2R system. (2) that serum from BLV infected sheep would cause

increased colony formation by lymphocytes from BLV infected sheep, when compared to serum from noninfected sheep. Evidence to support this potential abnormality in cells from sheep infected with BLV could provide an incentive for further studies of the usefulness of BLV in sheep as a model for studying diseases caused by human retroviuses.

MATERIALS AND METHODS

Serum collection and storage

For the serologicl tests (see below), ten ml of whole blood was collected into coagulant tubes from an aseptically prepared jugular vein of each sheep. The blood was allowed to clot overnight at 26 C. The clots were removed, the remaining erythrocytes were pelleted by centrifugation at 400 g for 10 min, and the serum from each sheep was stored at -30 C until further use. To obtain autologous sheep serum (ASS) and pooled sheep serum (PSS), a volume of whole blood equal to twice the volume of ASS desired was collected from each sheep into 10.0 ml coagulant tubes from an aseptically prepared jugular vein. The blood was allowed to clot overnight at 26.0 C, the clots were removed using aseptic technique, and the remaining erythrocytes were pelleted by centrifugation at 400 g for 10.0 min. The serum was aspirated, filtered through a 0.20 micron syringe filter (Millpore Prod., Bedford, MA), heat-inactivated at 56 C for 30.0 min and frozen immediately in 1.5 to 4.0 ml aliquots at -30.0 C. Pooled sheep serum (PSS) was prepared similarly by mixing equal volumes of serum from the infected sheep (IPSS) or from the control sheep (CPSS). The ASS sera had been stored 6 to 18 days, and the PSS had been stored 5 to 18 days prior to being used in the cell cultures.

Storage of phytomitogens

Concanavalin A (CON A) (Sigma Chemical, St. Louis, MO) was dissolved in sterile complete Dulbecco's modified Eagle's medium (CDMEM, pH 7.4, Gibco, Grand Island, NY) containing 200 IU per ml of penicillin g, 0.2 ug per ml of streptomycin and 0.6 ug per ml of amphotericin B (antibiotic/antimycotic, Sigma Chemical). The concentration of this stock CON A was 0.385 ug per ul. It was aliquoted and stored at -18 C.

Serological test for BLV and BSV antibodies

The agar gel immunodiffusion (AGID) test was used to examine sera from both cattle and sheep for antibodies to BLV and BSV. The details of this test were given in chapter 2 of this thesis.

Cattle used to infect sheep with BLV

The procedure for selecting the cattle which were used to infect the sheep with BLV were given in chapter 2 of this thesis.

Selection of sheep to be infected with BLV

The procedure for selection of sheep which were infected with BLV were given in chapter 2 of this thesis.

Infection of the sheep with BLV

The procedure for infection of the sheep with BLV were given in chapter 2 of this thesis.

Collection of blood for cell cultures

Twenty to fifty milliliters of blood was collected from each sheep into sterile, 10-milliliter, heparinized (14.3 IU per ml), prewarmed (37 C +/- 3 C) evacuated tubes (Becton Dickinson, Rutherford, NJ) after

aseptic preparation of a jugular vein. The blood was transported in a 37 C portable incubator.

Collection of peripheral blood mononuclear cells

The procedure for collection of the mononuclear cells which were used in the cell cultures were given in chapter 3 of this thesis.

Lymphocyte colony assay for sheep lymphocytes

A lymphocyte colony assay was used to culture the cells from the sheep. The details for the lymphocyte colony assay for sheep lymphocytes were given in chapter 3 of this thesis. The assay had been standardized during a previous experiment in our laboratory to determine the optimal T lymphoctye mitogens, mitogen doses, cell number, distribution of the cells in the culture vessel and duration of the culture.

Addition of ASS, IPSS, CPSS and CON A

The CDMEM was aspirated from each well after the 4.5 hour incubation period. One hundred ul of either ASS, IPSS or CPSS was added to the cells in each well, followed by the addition of 50 ul of CON A. Control wells received 50 ul of CDMEM. After addition of the ASS, IPSS, or CPSS and CON A, the contents of the plates were gently mixed manually for 4.0 min. The final serum concentration was 18.0 percent, and the final CON A concentration was 35 ug per ml of the final culture volume for all experiments.

Addition of CDMEM-agar

The details of how the agar was added were given in chapter 3 of this thesis. However, there was one minor change. The final cell concentration was 1.81×10^6 per ml for all cultures. The agar-overlays

were allowed to harden by incubating the plates at 26 C for at least 30 to 45 min. The plates were then gently shaken by hand to confirm solidification of the agar. They were incubated at 37 C, 5% CO_2, 90% humidity for 5 to 6 days.

Colony counts

The details of how the colonies were counted were given in chapter 3 of this thesis.

Isolation of sheep leucocyte DNA

The buffy coat from 14 ml of anticoagulated (EDTA) whole blood was collected by centrifuging the blood at 750 g for 20 minutes. The buffy coat was washed twice to lyse the erythrocytes. It was then incubated overnight at 55 C in lysis buffer at a ratio of 1 ml of buffer to 4 ml of the original volume of whole blood from which the buffy coat was obtained and in proteinase K (400 ug per ml of lysis buffer). The digested leukocytes were incubated in Rnase (100 ug per ml of lysis buffer) at 37 C for 60 min. The protein was initially extracted from the mixture with an equal volume of a solution consisting of phenol/chloroform/isoamyl alcohol at a ratio of 5:4.8:2. The extraction process was repeated on the aqueous phase which was recovered from the first extraction. The aqueous phase was then extracted with an equal volume of chloroform/isoamyl alcohol (9.6:0.4). The final aqueous phase of the above extractions was mixed with a 3M solution of sodium acetate at a ratio of 9:1. An equal volume of absolute ethanol at 20C was added and mixed by inversion. The precipitated DNA was pelleted, washed twice with 70% ethanol, and allowed to air dry for 30 to 60 min.

Concentration and purity of the DNA

The purity and concentration of the dissolved DNA was evaluated by spectrophotometry. The samples were evaluated at wavelengths of 280 nanometers (nm), 260 nm, 250 nm, and 230 nm. Ratios between the absorbances at 260 and 280, 260 and 250, and 230 and 260 wavelengths were determined. These ratios represent the quantity of protein and phenol contamination, carbohydrate contamination, and protein contamination, respectively. Acceptable ranges for the ratios are 1.8 to 2.0, 1.0 to 1.5, and 0.4 to 0.5. DNA samples outside of these ranges were reextracted with phenol and chloroform, or a new DNA sample was collected.

DNA Digestion

Twenty ugs of DNA from each sheep were digested overnight at 37 C with the restriction endonuclease (RE) Taq I (4 units of RE per 1 ug of DNA) . The digestion was terminated by cooling the reactants, and an appropriate amount of 5X gel loading buffer was added to each sample.

Electrophoresis

Agarose was dissolved in Tris*HCL/acetic acid/EDTA (TAE) electrophoresis buffer to attain a concentration of 0.75%. One hundred ml of molten agarose was poured into a 10 cm by 15 cm frame for the class II gels. The poured mixtures were allowed to harden and cure for 1 hr prior to running the gels. The digested DNA was loaded into the wells formed by combs, and the current was applied. Two standards of known base-pair size were electrophoresed with each gel. Biotinylated lambda DNA which was digested with BstE II was used for class II gels.

Class II gels were electrophoresed for 4 hours with 50 mA. The gels were run in a submerged mode with recirculation of the electrode buffers. Electrophoresis was terminated when the two dye components of the gel-loading buffer had trisected the gel. The gels were then removed from the frames of the electrophoresis chamber and placed into distilled water with ethidium bromide (0.5 ug/ml). They were stained in this mixture for 30 min, while being rotated, and subsequently washed twice with distilled water for 15 to 30 min while being rotated. Transillumination with ultraviolet light and a Polaroid MP-4 camera with type 667 film were used to photograph the gels. A wratten filter was used to block stray UV light. A scale was placed next to the gel. The photography documented the migratory distance of the standards in order to generate a size and migration scale to which the unknown fragments could be compared.

Transfer of electrophoresed DNA to membranes

The Southern method was used to transfer the electrophoresed DNA from the agarose gel to nylon membranes. Briefly, the gels were soaked in a solution of 1.5 M sodium chloride 0.5 M sodium hydroxide for 1 hr. They were then transferred to a solution consisting of 1.0 M tris and 1.5 M Nacl at pH 8.0, rinsed briefly in a 10X SSPE, and inverted onto a solid support covered with filter paper containing tails that served as wicks. A nylon membrane prewetted with with 10X SSPE was placed atop the gel. Two wet filter papers were then place atop the membrane and the papers were covered with several inches of paper towels. This stack was covered with a support and a 9 cm by 20 cm by 6 cm, 264 gm weight was set atop. The wicks allowed the passage of 10X SSPE from the reservoir through the gel and into the paper towels by capillary action. DNA in the liquid was

carried from the gel to the membrane. After a 16 to 20 hr transfer, the origins of the lanes were marked with a pencil on the membrane. The membrane was rinsed with 6X SSPE for 1 to 2 min and air-dried for 1 hr. The DNA was fixed to the membrane by baking at 80 C under vacuum pressure for 2 hr.

Prehybridization

The membranes were pre-wetted with water and transferred to a hybridization bag. Hybridization buffer, determined by the size of the membrane, was added to the bag, the bag was heat-sealed, and rocked for 16 to 20 hr at 42 C.

Hybridization

A biotinylated probe was added to the hybridization buffer, and the mixture was raised to 42 C and rocked. Class II membranes were hybridized for 5 days, and 1 ug of biotinylated HLA-DRA, -DRB, -DQA, -DQB, -DPA, or -DPB probe was added to the class II membranes.

Stringency washes

All membranes were washed 3 times with a solution of 2X SSPE and 0.5% tween 80 at 26 C for 15 min. Class II membranes were washed at 55 C and air dried.

Nonradioactive development

Class II membranes were rinsed with a solution of PBS and 5% triton X-100 for 5 min prior to a 1 hr incubation in streptavidin horse radish peroxidase conjugate which was diluted in this same solution to a concentration of 0.29 ug HRP per ml. The membranes underwent 5, five-min washes in a solution of PBS and 5% triton X-100 buffer that included 1M

urea and 1% dextran sulfate. They were developed in a 10mM EDTA solution at pH 5.0 that included 0.4mM tetramethylbenzidine and 0.0014% hydrogen peroxide. The colors were developed for 60 to 90 min, rinsed with distilled water, and photographed using Kodak Technical Pan film. The film was developed using Tech Ethol developer and Rapid Fix, and printed on medium contrast film.

Calculation of fragment length of the unknowns

The lengths of the fragments of the RFLPs were calculated using the method of least squares. The length of the unknown fragments was determined by comparing the migration distance of these fragments with those of the standards which were run in parallel with each gel.

Statistical analysis

A two-factor nested analysis of variance (ANOVA) model was used to test the hypothesis that the number of colonies formed by lymphocytes from BLV infected sheep would be different from the number formed by cells from the control sheep (Neter et al.,1985). The statistical model was:

$Y_{ijk} = u + a_i + b_{j(i)} + e_{k(ij)}$, where

Y_{ijk} = the expected number of colonies formed,

u = the mean number of colonies for all observations,

a_i = the effect of the treatment (i.e., BLV infection),

$b_{j(i)}$ = the effect of a sheep that is nested either within the treatment or the control group,

$e_{k(ij)}$ = random error.

An average of 10 measurements per sheep per group was made.

A two-factor crossed ANOVA model was used to test the hypothesis that the number of colonies formed from lymphocytes of infected sheep, whose cells were cultured in PSS from infected sheep, would be different from the number formed by these same cells if PSS from the control sheep was used. The statistical model was:

$Y_{ij} = u + a_i + b_j + e_{ij}$, where

Y_{ij} = the expected number of colonies formed,

u = the mean number of colonies for all observations,

a_i = the effect of the serum (i.e. treatment)

b_j = the effect of the sheep whose cells received both of the treatments,

e_{ij} = random error.

An average of 5 measurements were made on the cells of each sheep treated with each type of serum. These data were analyzed using the computer program SAS (SAS, 1985).

RESULTS

Heterogeneity of the sheep leucocyte DNA

Electrophoretic studies of the sheep leucocyte DNA suggested that the sheep originated from a heterogeneous population with respect to these antigens. The leucocyte DNA of the one sheep which developed lymphosarcoma did not have a DNA migratory pattern which differed appreciably from the protein migratory pattern of the other infected sheep or of the control sheep (Fig. 4.6).

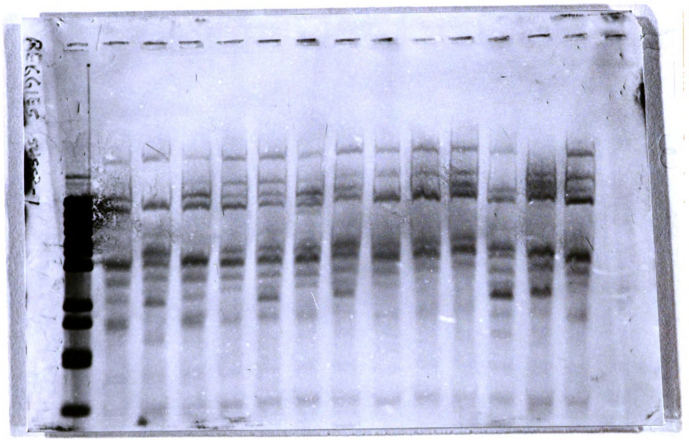

Fig. 4.6. Southern blot of sheep leucocyte DNA using the HLA MHC probe DQB.

Colony formation by BLV infected sheep

As a group, there was no statistically significant difference ($p=0.51$) between the number of colonies formed by lymphocytes from the infected sheep and the control sheep (Fig. 4.7). There were significant differences in the number of colonies formed when individual sheep were compared to each other ($p=0.0001$). The lymphocytes from one sheep in the infected group failed to produce any colonies, although the cells were cultured on several occasions. Nonmitogen-stimulated lymphocytes also failed to produce colonies.

Effects of IPSS and CPSS

As a group, there was no statistically significant difference ($p=0.052$) between the number of colonies formed by lymphocytes from the BLV infected sheep when these lymphocytes were cultured in pooled serum either from the infected sheep only or from the control sheep only (Fig. 4.8). There were also significant differences in the number of colonies formed when responses from individual sheep used in this experiment were compared.

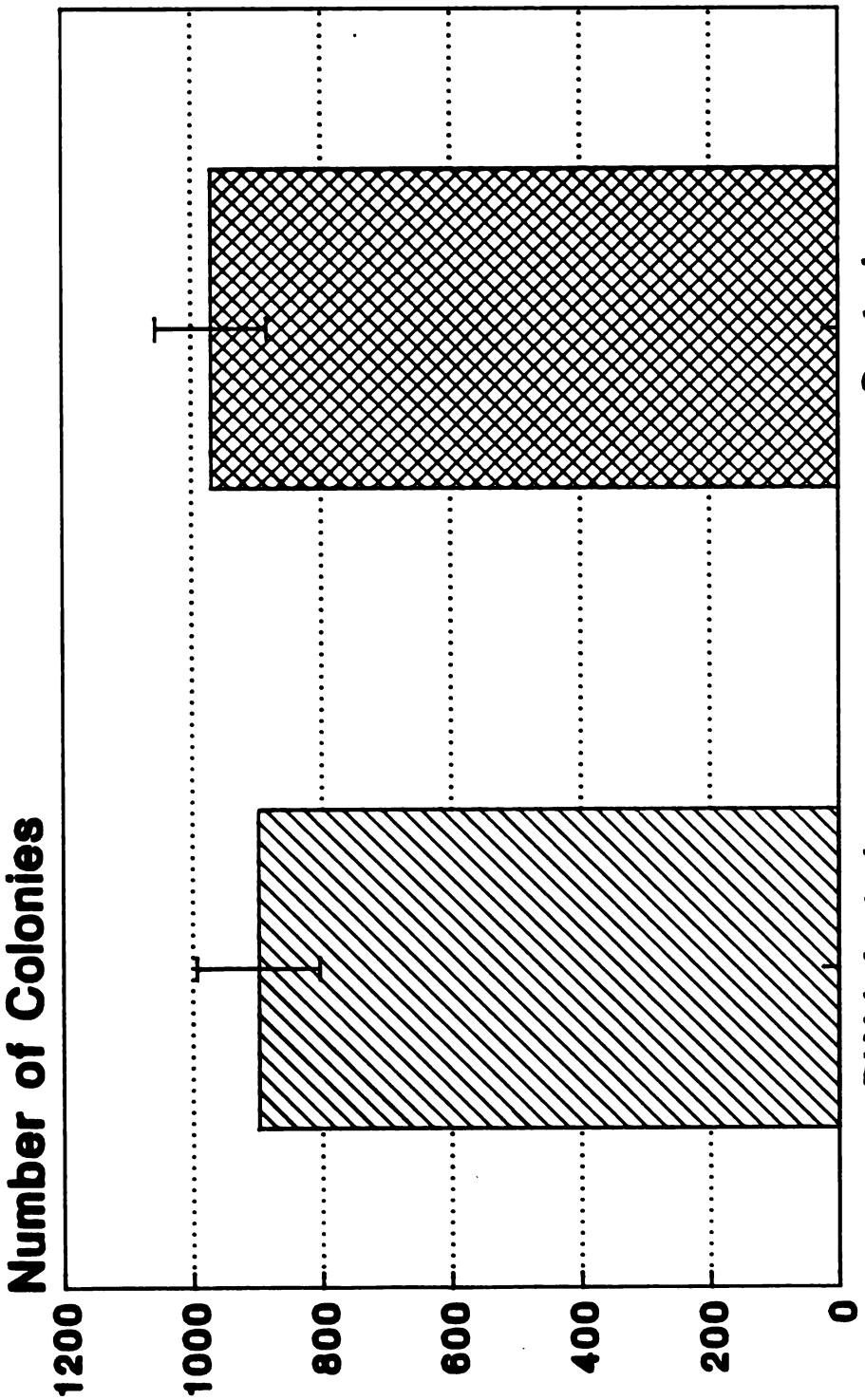

Fig. 4.7. Comparison of lymphocyte colony formation by lymphocytes from BLV infected and control sheep.

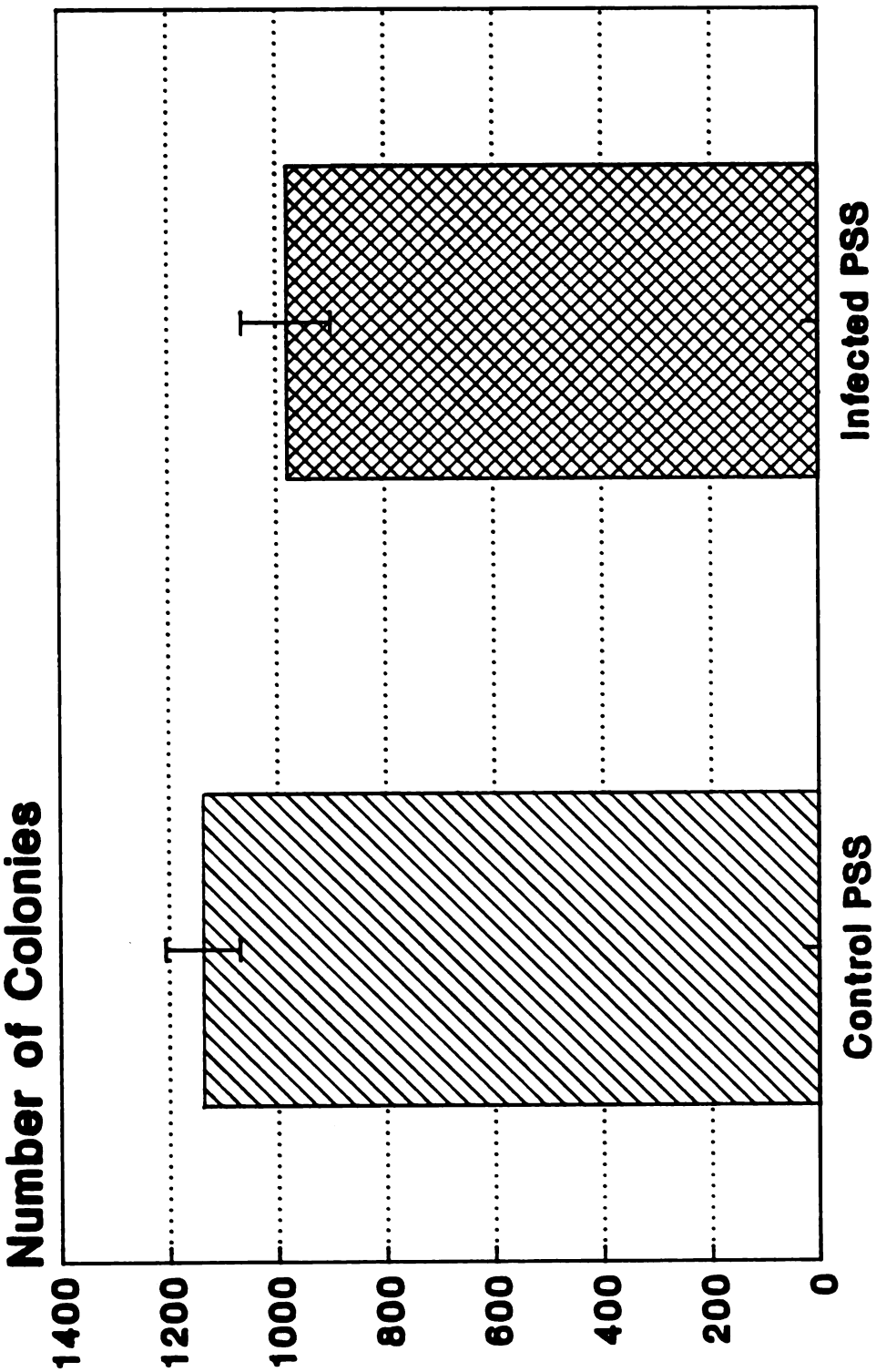

Fig. 4.8. Comparison of lymphocyte colony formation by lymphocytes which were cultured with IPSS or with CPSS. The lymphocytes were taken from BLV infected sheep.

DISCUSSION

It is possible that the MHC proteins may determine the susceptibility of sheep to the development of tumors. Therefore, we thought that it was necessary to provide some assurance before proceeding with our other immunologic studies that the sheep were from a heterogenous population with respect to their leucocyte antigens. The observed differences in the protein migratory patterns indicate that the antigens were in fact heterogeneous. Consequently, any differences between treatment groups and between individual sheep, with respect to responses to infection with BLV, could be more likely attributed to the virus alone, and not to extremes in the susceptibility of the sheep to BLV.

The exact mechanism of leukemogenesis and lymphomagenesis by bovine leukemia virus is unknown. The current virus-induced leukemogenic models cannot explain the transformation of cells after infection by BLV for several reasons: (1) A virus-encoded oncogene has not been implicated because the proviral sequences in tumor cells of BLV infected, lymphosarcomatous cattle have no homology with normal cellular sequences (Deschamps et al. 1981). No evidence of oncogenes or oncogene-bearing viral proteins have been detected by immunoprecipitation (Mamoun et al. 1983). (2) The downstream promotion of a cellular oncogene in the tumors does not seem to be a likely mechanism, since BLV does not integrate at a specific chromosomal site (Kettmann et al., 1983; Gregoire et al., 1984). (3) Products of the LOR region which participate in trans activation of transcription of the BLV long terminal redundancies (LTR) are not expressed in all tumor clones (Rosen and Sodroski, 1985). Thus, this

promoter is not universally expressed among the cells susceptible to BLV infection.

Likewise, cells from leukemic patients infected with HTLV I contain only one or a few copies of integrated provirus, and the site of integration is different in cells from different patients (Seiki et al., 1985; Wong-Staal and Gallo, Greene et al., 1986). Thus, the possibility of insertional mutagenesis directly activating a cellular oncogene is unlikely, and nucleotide sequence studies of HTLV I suggest that the acquisition of a viral oncogene from a cellular proto-oncogene is also unlikely. No viral transcription has been detected in leukemic cells, indicating that the consistent expression of HTLV I is not needed for maintainance of the neoplastic state (Franchini et al.,1984).
Although the exact mechanism by which HTLV I and BLV induce neoplastic proliferation of cells in not completely understood, it appears that a mode of leukemogenesis different from that of previously known retroviruses may be responsible. Cells from healthy HTLV I seropositive people and healthy BLV seropositive cattle harbor the virus in their lymphocytes, and virus particles are released upon cultivation of these cells in vitro, if they are stimulated with a mitogen. The human cells acquire a transformed phenotype (Miyoshi et al., 1981; Popovic et al., 1983, Yamamato et al., 82), progress from a state of polyclonality to one of monoclonality (DeRossi et al., 1985), and gradually become independent of IL 2 (Arya et al., 1984). Although the human leukemic cells also loose the ability to produce IL 2, increased levels of the IL 2 receptor persist on the surface of both cultured cells as well as primary malignant cells (Depper et al., 1984; Yodoi et al., 1983), concomitant with the expression of HTLV I viral proteins. Induction of the expression

of the genes for IL 2 and the IL 2 receptor by the pX gene product has been reported (Greene et al., 1986; Greene et al., 1987), and expression of the IL 2 genes is an early response of the cells specifically to the expression of $p40^{X}$ (Inoue et al., 1986). IL 2, IL 2 receptors and the mRNA for these proteins reached maximum levels 20 hours after transient expression of $p40^{X}$. Thus, $p40^{X}$ is a prime candidate for induction of leukemogenesis by HTLV I. The BLV protein p38 is functionally analogous to the p40 of HTLV I (Derse et al., 1987, Rice et al.,1987), but no studies of IL 2 production by lymphocytes in cattle infected with BLV have been reported.

Monoclonal antibodies to the IL 2 receptor on human lymphocytes and assays for human IL 2 are commercially available, and these have been used to study lymphocytes from patients with adult T cell leukemia. Similar markers and assays for sheep lymphocytes would enable efficient testing of hypotheses concerning derangements in the IL 2-IL 2 receptor system of lymphocytes from sheep infected with BLV, but these are not currently available. Several laboratories have reported the isolation from sheep lymphocytes of factors with T cell growth factor activity (English and Whitehurst, 1984; Knisley and Pearson, 1987; Ellis and DeMartini, 1985). However, there have been no reports of the isolation of sheep IL 2 in a relatively pure form, nor of the sheep IL 2 receptor. Thus, monoclonal antibodies which could be used in immunoassays of these proteins are unavailable. Neither are there cell lines bearing IL 2 receptors which could be used to assay for sheep IL 2.

Given the absence of such immunoassays, only indirect measures of the IL 2-IL 2R system in sheep lymphocytes are possible. Under the assumption that normal function of both the IL 2-IL 2R systems are a

necessary prerequisite for colony formation by sheep lymphocytes, we decided to use this assay to investigate colony formation by lymphocytes from BLV infected sheep. We were not able to demonstrate increased lymphocyte colony formation in these sheep which had been infected with BLV for 112 weeks when the colony assays were done. This suggests that there are no abnormalities in IL 2 production by lymphocytes from aleukemic sheep infected with BLV. One sheep died of lymphosarcoma 96 weeks after infection. Unfortunately, lymphocytes from that sheep were not cultured by using the colony assay, because it had not been completely standardized for sheep lymphocytes at that time.

Supernatants from cultured lymphocytes have frequently been used in in vitro assays of IL 2 activity. The availability of commercial IL 2 ELISA tests have made it possible to measure Il 2 levels in vivo by using serum and plasma. The assays have been used to study human patients undergoing rejection episodes following kidney and liver transplantation (Cornaby et al.,1988), and patients with systemic lupus erythematosus (Huang et al., 1988). The IL 2 levels in serum and plasma in humans were much higher than those found in culture supernatants. Therefore, we decided to investigate the effects of serum from the infected sheep on colony formation, rather than to attempt to assay lymphocyte supernatants for IL 2 activity. We were not able to demonstrate an enhanced lymphocyte colony response due to the serum from the infected sheep.

A model for the mechanism of leukemogenesis by these retroviruses has been proposed. The tat-induced coexpression of both IL 2 and its cellular receptor suggests that infection by HTLV I or II results in an autocrine mechanism of cell proliferation (Yoshida et al.,1987). Initially,

infection of the cells by the virus is thought to lead to the expression of $p40^x$ at low levels. The $p40^x$ could stimulate the viral promoter and increase the expression of viral antigens, including $p40^x$ itself. The accumulated $p40^x$ could then activate the transcription of the IL 2 and the IL 2R in T cells which have differentiated to this particular stage of maturation. These T cells could eventually undergo uncontrolled proliferation subsequent to several proposed intermediate steps.

It is also possible that BLV infection has no effect on lymphocyte colony formation. However, it was recently reported that HTLV I infected cells and HTLV I virions were able to induce the proliferation of T colony-forming cells in the absence of exogenous IL 2 and accessory cells (Duc Dodon and Gazzolo, 1987). HTLV I particles were treated with HTLV I antisera against the envelope and core proteins of the virus in order to inhibit binding of the virus to lymphocytes. Since colony formation was subsequently decreased, it was concluded that virus particles were directly involved in colony formation, and that this involvement may have an accessory cell function which subsequently triggers the autocrine secretion of IL 2. Direct activation of resting T lymphocytes, induction of IL 2 secretion and IL 2 receptor expression by HTLV I was later shown by the same investigators (Gazzolo and Duc Dodon, 1987). Colony formation was directly attributed to these events.

Given the preexisting similarities between BLV and HTLV I, it should not be unreasonable to speculate that BLV may also adversely affect T colony formation. There are several possible explanations for our failure to demonstrate this potential adverse effects of BLV. Unfortunately, the hypotheses which were proposed by us could only be tested in an indirect manner. This underscores the need for the

development of tools such as IL 2 assays and monoclonal antibodies to the IL 2 receptor for cells of domestic species. These tools could only enhance our ability to test similar hypotheses. It will be of interest to test the responses of lymphocytes from BLV infected sheep to B and T cell human recombinant interleukins. These interleukins may provide a more direct measure of IL 2 activity than the lymphocyte colony assay. It is also possible that BLV induces proliferation of cells by a different mechanism, but the transactivation mechanism is still thought to play a major role in the induction of tumors by BLV (Burny et al., 1988). Our inability to demonstrate the hypothesized abnormalities also may simply be a matter of having conducted our studies using aleukemic sheep. Cell proliferation in aleukemic sheep may not have reached the stage in which it has been " switched on " irreversibly. Consequently, cellular and subcellular indicators of this stage of the infection may not be expressing themselves in a detectable fashion. Experiments using lymphocytes from sheep with leukemia or lymphoma are now in order.

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CONCLUSIONS

The objective of this research was to develop a sheep model for studying the mechanisms of leukemogenesis induced by a new class of retroviruses which has long been known to infect cattle and was recently shown to infect humans. Bovine leukemia virus (BLV) causes neoplastic proliferation of B lymphocytes in cattle, and human T cell leukemia virus type I and type II (HTLV I and II) cause a proliferation of T lymphocytes in humans. The current mechanisms of leukemogenesis of retroviruses, e.g. oncogene-mediated mechanisms, cannot explain the induction of neoplasia by this new class of retroviruses. Thus, the existing animal models are of limited value for studying mechanisms of leukemogenesis by these viruses, because most existing models induce neoplasia via oncogenes.

It has been proposed that BLV infected cattle may serve as an animal model for the HTLVs due to their various similarities. However, the period for the induction of neoplasia in cattle requires many years, and most infected cattle never develop tumors. Experimental studies of BLV infection in sheep suggest that sheep are actually more susceptible to the induction of leukemogenesis by BLV than are cattle. There is evidence that BLV infection in sheep causes a T lymphocyte proliferation, as do the HTLVs in humans. BLV infection in sheep may be a more useful animal model than BLV infection in cattle for studying these viruses.

Thus, we attempted to develop a model by using BLV infection in the sheep. This model could be used by individuals in a variety of disciplines to study the biological behavior of animal and human retroviruses from the subcellular level to the level of the population.

These viruses are different from most retroviruses in that they have a region referred to as the tat region in their genome. The tat region is presumed to encode for proteins that play a key role in the induction of tumors induced by these viruses. The hypothesized mechanism in our studies was that the tat gene encodes a protein(s) that induces the secretion of interleukin 2 (IL 2) and the IL 2 receptor (IL 2R) via an autocrine mechanism which eventually results in the uncontrolled proliferation of tumor cells.

We investigated a similar hypothesis by using lymphocytes from aleukemic sheep infected with BLV. A lymphocyte colony assay, which itself has been shown to depend on IL 2 for the colonies to form, was standardized for culturing sheep lymphocytes. We were not able to demonstrate increased colony formation by lymphocytes from aleukemic sheep infected with BLV. We also investigated the effects of serum from BLV infected sheep on lymphocyte colony formation, based upon previous reports of increased serum IL 2 and soluble IL 2R in humans with leukemia. Neither were we able to demonstrate increased colony formation by serum from BLV infected sheep. Antibodies to BLV did persist throughout the experiment, and one sheep did develop tumors, however.

The usefullness of this animal model will be dictated by the nature of the questions which an investigator wishes to explore. Although sheep develop the tumors more rapidly than cattle do, the period of induction is still rather prolonged. Unfortunately, this long period of induction

may compromise the usefullness of this model for studies which would be conducted during the tumor phase of infection with BLV. However, the model may still be used for studies which would be conducted in the early phases of BLV infection, and this should be taken into consideration in the design of future studies of BLV infection.

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