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The effects of bovine immunodeficiency-like virus on monocyte function

by

Anna Helen Rovid

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Microbiology, Immunology and Preventive Medicine
Major: Immunobiology

Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
For the Major

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For the Major Department

Signature was redacted for privacy.
For the Graduate College

Iowa State University
Ames, Iowa

1995

UMI Number: 9540936

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GENERAL INTRODUCTION

Bovine immunodeficiency-like virus (BIV) is a lentivirus of cattle which was first isolated and described over 20 years ago (1). BIV has been postulated to cause immunodeficiency, based on the clinical signs in the cow infected with the first (R29) isolate of BIV (1) and on the genetic relationship of BIV to the human immunodeficiency virus (HIV) (2). Until recently, however, little research has been done to examine this hypothesis. Recent studies on neutrophil function, T cell subsets, and lymphocyte blastogenesis in BIV infected cattle have found subtle alterations in neutrophil function (3), and decreased (4) or increased (3) lymphocyte blastogenesis. There is one report describing possible depletion of CD4+ T cells in infected animals (5). The primary target cell for most lentiviruses is, however, the macrophage (6). Although the cell tropism for BIV is not fully known, data presented in this dissertation and elsewhere (7) indicate that monocytes and macrophages are one of the cells infected by BIV. As described in the literature review in this dissertation, macrophage functional abnormalities have been found in HIV infection and in cats infected with the related feline immunodeficiency virus (FIV). Therefore, we examined macrophage function in cattle infected with BIV, as well as the direct effects of BIV virus on normal macrophages *in vitro*.

Dissertation Organization

The first chapter of this dissertation is an overview of known macrophage function defects in lentivirus infection. The second chapter is a manuscript on macrophage function and virus isolation in a group of cattle experimentally infected with the R29 isolate of BIV. This paper also describes the functional abnormalities found when normal monocytes are treated *in vitro* with supernatants from BIV infected cells. The third chapter is a manuscript describing the isolation of the active factor, the BIV Gag protein, from these cell supernatants. Finally, the fourth chapter is a manuscript describing an unusual lymphosarcoma in one of the BIV

infected cattle, which may have been associated with BIV infection. The dissertation concludes with a general summary. Also included are four appendices which contain more specific information on the macrophage function assays developed during this dissertation project, data describing how monocyte random migration, chemotaxis, and antibody-dependent cell-mediated cytotoxicity change during 5 day culture of monocytes *in vitro*, unpublished observations on *in vitro* infection of macrophages with BIV, and the development of a sandwich ELISA for the detection of BIV p26 antigens.

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LITERATURE REVIEW:
THE EFFECTS OF LENTIVIRUSES ON MACROPHAGE FUNCTION

The *lentivirus* genus of the *Retroviridae* (1) contains a group of complex viruses which tend to cause slow, progressive disease. Members of the genus include the human, feline, and simian immunodeficiency viruses (HIV, FIV and SIV, respectively), bovine immunodeficiency-like virus (BIV), caprine arthritis-encephalitis virus (CAEV), equine infectious anemia virus (EIAV), and ovine visna/maedi virus. The immune system plays a dual role in the pathogenesis of lentiviral disease; while the immune system can control and limit virus replication, it is also involved with the production of disease. For some lentiviruses, notably CAEV and visna, proliferation of immune cells and damage to tissues by chronic immune stimulation is an important mediator of disease (2). In contrast, the immunodeficiency-causing lentiviruses progressively destroy immune function.

Macrophages are target cells for all known lentiviruses (2); however, the relationship between macrophages and the immunodeficiency viruses is far more complex than simply that of virus and target cell. The macrophage is a cell with many roles. Macrophages are important in both innate and acquired immunity; their functions range from roles in tissue repair and basic "housekeeping" functions such as synthesis of complement components, to antigen presentation in a specific immune response. They phagocytose foreign invaders, secrete an array of cytokines, and may have a role in anti-tumor immunity. It should not, then, be surprising that macrophages have multiple roles in lentivirus infection. Macrophages harbor virus, secrete cytokines which influence virus replication, and may aid in controlling lentivirus infection by antigen presentation. In turn, however, lentiviruses may affect macrophage activities ranging from cytokine secretion to killing of bacteria.

This review will describe how the immunodeficiency viruses (HIV, FIV, SIV and BIV) affect macrophage functions, and what impact these effects might have on disease. Most research has focused on HIV infection; related lentiviruses such as

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SIV, FIV and BIV will be discussed where information is available. An initial overview will describe the role of macrophages in the pathogenesis of human immunodeficiency virus infection, and the evidence that macrophages are infected with lentiviruses. Subsequent sections will discuss the effects of virus on macrophage precursors; on cell surface molecules; on chemotaxis, antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, and oxidative metabolism; on antigen presentation and cytokine secretion; and on macrophage roles in tissue damage and repair. Molecular mechanisms have been discovered for some of these areas; for others, the science is still at a descriptive level. Finally, the hypothesis that defects in macrophage function are the result of chronic macrophage activation will be discussed.

Virus Structure and Replication

The lentiviruses are complex in both structure and genetic organization. These 100-130 nm enveloped viruses contain a central capsid surrounding a nucleoprotein complex of viral mRNA and nucleocapsid protein (1,2). A matrix protein is associated with the viral envelope (1,2). The lentivirus genome is a 9-10 kB single stranded RNA, flanked by 3' and 5' long terminal repeat (LTR) sequences in the proviral form (2). The structural genes for all lentiviruses include the *gag, pol* and *env* genes (1,2). The *gag* gene is transcribed and translated into a polyprotein precursor, Gag, which is proteolytically cleaved during the formation of mature virions into the virus capsid, nucleocapsid, and matrix proteins, as well as variably present small proteins of undetermined function (1,2,3). The processing of the Gag proteins appears to be quite complex: for BIV, a number of protein cleavage products ranging from 10 to 53 kDa have been shown or predicted to be present during processing of Gag (4).

The Pol precursor is cleaved into the reverse transcriptase, integrase, and protease (1). The *env* gene is transcribed, translated and modified into a glycosylated envelope protein which is cleaved into a large hydrophilic surface protein, and a

smaller hydrophobic transmembrane protein (1,2). In addition, lentiviruses contain gene segments coding for the positive regulatory proteins Tat and Rev (1,2). Other regulatory proteins are also present in lentiviruses; however, they may not be present in all members of the lentivirus genus. For example, the BIV genome does not appear to contain any region coding for the negative regulatory factor Nef, but does contain an open reading frame (ORF) for Vif and two unique ORFs known as W and Y (5).

Macrophage Infection by Lentiviruses

Numerous papers have documented that monocytes and macrophages can be infected by HIV, SIV, and FIV *in vitro* (1,6-15). Although there are no published reports which describe the full range of leukocytes infected by BIV, BIV does appear to infect macrophages (16,17). Factors influencing macrophage infection with HIV and SIV include the strain of virus, the donor source of monocytes/macrophages, and the state of macrophage activation (8,11,18,19). Macrophages appear to be the primary cell type infected with HIV and SIV in tissues (20,21). The percentage of HIV infected macrophages, as well as the amount of HIV RNA per cell, appears to be much higher in the tissues than in blood (2,20). In particular, productive infection of the macrophage appears to be higher in the tissues (2,20). In AIDS patients, while a very low percentage of blood monocytes appear to be infected (less than 0.001%), 1-10% of the brain microglia, 10% of lymph node dendritic cells, and 10-50% of alveolar macrophages may be infected with HIV (20). In children, even higher levels of macrophage infection may be seen, particularly in the lungs (22). Macrophages from tissues other than the lung have not been studied as intensively; however, Kupffer cells are infected in some patients (23), and infected macrophages have been detected in the brain (24,25), placenta (26) and bone marrow (24). Related cells such as dendritic and Langerhans cells also appear to be infected (20,27,28), although some have questioned these results (29). Tissue macrophages that have been infected with HIV *in vitro* include peritoneal macrophages (13) and Kupffer

cells (23).

In vitro studies have demonstrated that monocytes are easier to infect and produce greater amounts of HIV and SIV virus after differentiation into macrophages (8,11,18,19). Although most researchers agree that blood monocytes in some patients can be infected with HIV, it is still somewhat controversial whether monocytes are infected in all individuals. Some studies have found infected monocytes in most patients examined (30,31); however, others have found monocyte infection in few patients (32,33). A similar uncertainty exists for FIV infection (34). Some of this variability may be due to the technique used to detect virus. Many early experiments (32,33) used LTR and/or env gene primers in PCR detection of retroviral DNA. Recent reports (31,35) have, however, found that *gag* gene primers are most successful at amplification of HIV DNA from monocytes. Many of the earlier papers may have missed monocyte infection through the use of LTR primers, which are very poor at detecting HIV infection in monocytes. Furthermore, it seems unlikely that monocyte infection would be present in only a minority of patients. Recent studies demonstrate that macrophage tropism is necessary for infection of the brain and lungs by HIV or SIV (36,37), and macrophage infection in the lung and brain is common (20). Tissue macrophages are primarily replenished by the differentiation of blood monocytes; therefore, the most likely route for tissue invasion of macrophage-tropic viruses would be by infected monocytes.

Overview: the Role of Macrophages in the Pathogenesis of Disease

Of the immunodeficiency viruses, the pathogenesis of disease is most completely known for HIV. SIV infection in macaques appears to parallel HIV infection in humans and has been used as a model for AIDS (36,38); less is known about FIV. BIV has not yet been proven to cause any major immunodeficiency. In the initial studies of HIV infection, the CD4+ T cell appeared to be the only leukocyte important in disease. AIDS was seen as a simple progression: after a period of latent infection of T cells and no clinical signs, latent virus became

activated, and caused destruction of T cells, leading to clinical immunosuppression and death. More recent views of HIV infection are more complex, and the latent period is seen as a time when virus replication continues to occur at a high rate (39), damage to the immune system and decline in CD4+ cell numbers is progressive, and virus-immune system interactions may influence the length and possibly the outcome of disease (21). Macrophages appear to be involved, either directly or indirectly, in many aspects of pathogenesis.

Macrophage influence on virus variants

The initial interaction of HIV with the immune system occurs with an inoculum containing multiple virus variants. Some of these variants appear to be suppressed during or before transmission, or by the initial immune response (40-43). There is, in fact, intriguing evidence that macrophage tropic variants may be initially selected (40-43). HIV variants have been classified by several phenotypic characteristics, including cell tropism, the ability to induce syncytia in T cells, and rate of replication (40). While these characteristics may not be absolute (44), they have been useful in characterizing changes in virus populations over time. In general, the non-syncytium-inducing (NSI) variants share the characteristics of low rate of virus replication and the ability to replicate in macrophages as well as T cells. The syncytium inducing (SI) isolates, in contrast, have a higher replication rate, are more cytopathic, and have lost the ability to replicate well in primary macrophages (40,42,43). There is evidence that, although both SI and NSI variants are transmitted, SI (non-macrophage tropic) variants are generally eliminated early in the interaction with the immune system (40,43). As a result, in asymptomatic patients, NSI variants predominate (40,43). The important factor in the restriction of virus types during transmission is believed to be their macrophage tropism (40,42,43). In support of this theory, there is evidence from one patient that, when SI variants are monocytotropic, they do survive during the initial stages of disease (40).

The selection pressure causing this initial expansion of NSI viruses and

suppression of SI viruses is unknown. At least 2 theories have been proposed. One theory is that the early immune response clears the NSI variants, due to some phenotypic characteristic of the viruses (42). HIV is less cytopathic in macrophages than in T cells (45) and may, therefore, survive longer in macrophages while the immune system is still intact. In some culture systems, although HIV buds on the cell surface from T cells, it buds into intracytoplasmic vacuoles in macrophages (20). If this *in vitro* pattern reflects the pattern of virus budding *in vivo*, virus within macrophages might be hidden from the immune system. Others believe that, although this explanation may be true for a minority of cases, in most cases the virus tropism results from the initial cell type the virus encounters (43). According to this theory, except during intravenous transmission, the virus first encounters mucosal or placental macrophages and only the macrophage tropic isolates can replicate in these cells. Which theory, if either, is correct has yet to be determined.

Macrophages in early HIV infection

During the early period after infection, HIV and SIV viruses disseminate throughout the body (21), probably carried mainly by monocytes (36,37). Virus upregulation of macrophage adhesion molecules may occur, and could influence the invasion of tissues by infected cells (46). In the subclinical phase of infection, the immune system is apparently intact and both antibody and cell-mediated responses against HIV (or SIV) are seen (21,38). In cases where high neutralizing antibody titers exist, macrophage- T cell interactions or dendritic cell - T cell interactions may be an important means of virus transmission between cells (29,43). There is evidence that dendritic cells pulsed with HIV can infect T cells (29); whether the virus is spread by infection of the dendritic cell or simply uptake and sequestration of the virus (with consequent spread to T cells) remains to be determined. In this particular experiment, no budding of viruses from dendritic cells was seen (29).

During this early period, changes in macrophage-related cells in the lymph node occur. Follicular dendritic cells appear to be slowly destroyed or depleted

(20,21). Other, more subtle changes in macrophage lineage cells may also occur. Masutani et al (47) report a reduction in "adult T cell leukemia derived factor" (ADF), a cytokine secreted primarily by dendritic cells in the paracortex of the lymph node and macrophages in the sinuses (but not by follicular dendritic cells). In one AIDS-related complex (ARC) case, and several AIDS cases, lymph nodes were depleted of ADF-producing cells, particularly in the paracortical regions. This phenomenon did not appear to be due to cell death, as other proteins produced by paracortical dendritic cells did not appear to be affected.

Most overt defects in macrophage activity do not appear until late in disease, and macrophages may be activated during the early stages of infection. Cytokine derangements, particularly increased cytokine secretion, appear to be present in HIV (48-51) and SIV (52) infection. Recent evidence indicates that HIV replication may be occurring at high rates even early in disease (39). Virus replication at this time may be driven by increased levels of cytokines, particularly tumor necrosis factor (TNF) and interleukin-1 (IL-1), and again, the macrophage may be involved (21,53-59). HIV appears to induce release of some cytokines from macrophages (53-57), and these cytokines may feed back on the virus to further upregulate (or, in some cases, downregulate) replication (21,58,59).

Some authors speculate that, during this asymptomatic period, macrophages may also be involved in the selective loss of memory T cells and loss of proliferative responses to recall antigen, through inappropriate antigen presentation; antigen presentation without appropriate second signals may cause apoptosis in T cells responding to recall antigens (43).

Late stage disease

As disease progresses, viral variants emerge which have greater tropism for T cells than for macrophages (40,43). Although the macrophage tropic variants continue to be present at the previous levels, an increase in virus replication is seen. Most of the new viruses are SI variants, with high tropism for T cells and much less

for macrophages (40,43). A precipitous drop in CD4+ cell numbers eventually begins, and full-blown AIDS develops (21). During this time, macrophage infection may influence the clinical manifestations of disease. Encephalitis, myelopathy, pneumonitis, and lymphadenopathy are closely linked to virus replication in tissues, and tissue pathology is often correlated with HIV or SIV gene expression in macrophages (22,36,60). A high percentage of infected alveolar macrophages may contribute to the high incidence of lymphoid interstitial pneumonia in children with HIV (22). In SIV infection, giant cell pneumonia and granulomatous encephalitis have been associated with infection of tissue macrophages (36,35). Within the brain and lung, virus infection may influence the secretion and synthesis of a number of macrophage products which are able to cause tissue damage, including cytokines, eicosanoids, and reactive oxygen and nitrogen metabolites (61). Many of the macrophage defects discussed in this review appear at this late stage, although some, particularly cytokine derangements, may occur earlier. The following sections will examine the known effects of the immunodeficiency viruses on macrophage function in more detail.

Effects on Macrophage Precursors

HIV,SIV,FIV

AIDS patients, SIV infected macaques, and FIV infected symptomatic cats all have hematologic abnormalities including lymphopenia, anemia, neutropenia and thrombocytopenia (62-68). Depressed monocyte numbers have not been reported (69); however, selective decreases in the monocyte and granulocyte bone marrow precursor CFU-GM (as well as in the erythrocyte precursor BFU-E) have been reported following infection with either SIV or HIV (62,63,65,66). One study also found a selective suppression of some precursor populations when bone marrow cultures from HIV-infected individuals were grown *in vitro* (65). While some hematopoietic precursors were produced in normal numbers, the differentiation of granulocyte/macrophage precursors and erythrocyte precursors was selectively

suppressed in cultures from HIV infected individuals (65). In HIV infection, the numbers of circulating monocyte precursors also appear to be depressed during the asymptomatic stage of infection (62). In contrast, abnormalities in bone marrow culture have not been found in asymptomatic FIV infected cats (64) or in one symptomatic cat (68), even though hematologic abnormalities have been seen in infected cats. Additional studies, particularly in symptomatic cats, are needed to confirm the finding that bone marrow culture is normal in FIV-infected cats.

The mechanisms for virus suppression of hematopoietic precursors have not been fully determined. Possible mechanisms for suppression include altered cytokine production, cellular or viral inhibitory factors, the killing of infected precursors by leukocytes, or infection of precursors resulting in death or metabolic alterations (63). There is little evidence that altered levels of cytokines are responsible for the bone marrow abnormalities seen in HIV and SIV infection. There is one report that the HIV transactivating protein, Tat, induces bone marrow macrophages to secrete the inhibitory cytokine transforming growth factor β (TGF-β) (70). The relevance of this finding to the *in vivo* situation remains to be determined. Decreases in IL-3 and granulocyte-monocyte colony stimulating factor (GM-CSF) have also been postulated to be involved in the bone marrow abnormalities: IL-3 and GM-CSF stimulate the production of macrophages and neutrophils, and IL-3 and GM-CSF levels have been reported to be depressed in AIDS patients (62,71). Nevertheless, physiologic doses of IL-3 and GM-CSF do not appear to be able to restore normal bone marrow function (62,63). In some cases, high *in vitro* doses of GM-CSF and IL-3 appear to increase CFU-GM colony formation from SIV and HIV infected individuals (62,63); however, the relevance of such high doses of cytokines to *in vivo* hematopoiesis is unknown.

Infection of hematopoietic precursors does not appear to be responsible for bone marrow suppression by HIV or SIV (62,63,67). Although there have been reports of infection of precursors by large doses of HIV (72), these cells do not appear to be a major reservoir of virus *in vivo* (62). SIV virus also appears to be harbored in bone marrow stromal macrophages and not in hematopoietic precursors

(67). In SIV (66) and HIV infections (62), increased virus burden in macrophages and peripheral blood mononuclear cells (PBMC) has been correlated with suppression of bone marrow precursor colony formation. *In vitro* studies have, however, also demonstrated virus-mediated depression in CFU-GM without evidence of latent or productive infection (62,63). Lymphocytotropic HIV IIIB (which is unable to infect macrophage precursors) can also suppress colony formation of CFU-BM (73). It appears, therefore, that a virus product, or a virus-mediated change in cellular products, may be responsible for the selective suppression of bone marrow precursors in HIV and SIV infection. A good candidate is the envelope glycoprotein, gp120. In one study, pre-incubation of virus with antibodies to gp120 neutralized the effect of HIV virus on macrophage precursors (62). Recombinant gp120/gp160 also appears to suppress colony formation (74). One hypothesis is that the effect of gp120/gp160 is to induce apoptosis in some hematopoietic precursors (62). No evidence has yet been published to support this hypothesis.

Very little is known about the cause of bone marrow suppression in cats infected with FIV. There is one report of serum from a symptomatic FIV positive cat suppressing autologous CFU-GM but not CFU-GM from healthy FIV negative cats, suggesting that some serum substance was acting only on CFU-GM infected or altered by FIV (68). This serum factor was not identified further.

Macrophage Cell Surface Molecules

HIV

Macrophage surface molecules have mainly been examined in HIV infection. Cell surface expression and/or function of MHC class I and II molecules, as well as macrophage Fc, complement and mannose receptors, may be altered by infection with HIV. These molecules will be discussed in conjunction with defects in antigen presentation (MHC molecules), chemotaxis (complement receptors), and phagocytic function (Fc and mannose receptors). The expression of CD4 on macrophages also appear to be decreased after infection (75-77), although not all groups could confirm

this finding (78). CD4 is of interest primarily due to its role in infection of macrophages by HIV. One study compared neutral glycolipids (which are involved in cell interactions, differentiation, and binding microorganisms) in promonocytic U937 cells infected by HIV or the human T lymphotropic virus (HTLV I) (79). Although infection by the oncovirus HTLV I affected ganglioside concentrations in the cell membrane, there was no difference in any glycolipids examined after HIV infection.

Integrins

Integrins play roles in both macrophage migration and cell interactions such as cell cytotoxicity and antigen presentation. They also appear to be involved in macrophage recognition of opsonins leading to phagocytosis (80): increased expression of integrins is associated with increased phagocytic ability for *Staphylococcus aureus* and *Escherichia coli* (46). Increased expression of integrins has also been postulated to be involved in the transport of HIV into the brain by infected macrophages (46).

Integrins are cell surface molecules composed of an alpha and a beta chain. The 3 integrins Mac-1 (CR3), LFA-1, and p150,95 (CR4) share a common β chain (CD18); however, each has a unique alpha chain. Mac-1, LFA-1, and p150,95 are all found on monocytes and macrophages, and levels of LFA-1 and p150,95 appear to be influenced by infection with HIV. Expression of the alpha subunit of p150,95 (CD11c) is increased by HIV infection (80,81). Levels of the LFA-1 α subunit, CD11a, have also been found to be increased on monocytes or macrophages from HIV-infected patients by some (81,82) but not all (80,83) groups. In contrast, although increased (81) or decreased (83) expression of the Mac-1 α subunit CD11b has been found, most evidence indicates that CD11b is not altered by HIV infection (78,80,84). The effect of HIV on the common β subunit, CD18, is also still controversial, as both unchanged (80) and increased (82) levels have been reported.

The effect of HIV infection on integrin levels on promonocytes, but not promyelomonocytes, may be similar to the effect on mature monocytes and

macrophages. In one study, promonocytic lines (U937 and THP-1) chronically infected with HIV had increased CD11c expression (as well as CD15, HLA-DR, and HLA-DQ), when compared to uninfected cells (85). Promyelomonocytic lines, in contrast, had decreased levels of CD11c and HLA-DR (but increased CD15 and HLA-DQ) (85). Petit et al (86) have confirmed that chronically infected U937 cells upregulated p150,95, as well as CD11a and CD18.

It appears, therefore, that the integrin molecules p150,95 and LFA-1 but not Mac-1 are upregulated by HIV infection of monocytes, macrophages, and promonocytic cell lines. The mechanisms for integrin upregulation or suppression by HIV have not been determined. Integrins are upregulated during monocyte activation; therefore, one explanation for the increased expression of integrins is that HIV infection is causing monocyte activation. Some evidence exists in support of this hypothesis, including possible changes in FcIII receptors (87) and the increased secretion of some cytokines after HIV infection (48-51,54,88).

Chemotaxis

HIV

A decrease in monocyte chemotaxis has been well documented in HIV-infected individuals (69,89-91). In AIDS patients, a significant decrease in chemotactic migration, with preservation of normal random migration, was first reported by Smith et al (69). A marked reduction of migration in response to all stimuli examined - C5a, the bacterial peptide fMLP (N-formyl-methionyl-leucyl-phenylalanine), *Giardia lamblia*, and "lymphocyte-derived chemotactic factor" - was seen. In this study, patients with persistent lymphadenopathy also had depressed chemotactic function, intermediate between normal controls and AIDS patients, although this was not significant at the $P < 0.05$ level. Poli et al (90) also observed a selective depression of chemotaxis with no other monocyte abnormalities in a group of 17 AIDS patients. These results have been confirmed in other studies (89,91). Martin et al (92), however, found that different defects in chemotaxis and random

migration may be found, depending on the method used to study migration (blind well chamber or migration under agarose), and the stage of disease. The results of Martin et al (92) are plausible, as migration under agarose depends more on cell adhesiveness than does migration in blind well chambers and, may, therefore, be affected differently by HIV infection (92). Unfortunately, this study used unfractionated mononuclear cells rather than monocytes, and differences in monocyte percentages between patients may have affected the migration results. Therefore, although the study is intriguing, it remains to be confirmed using purified monocytes.

The suppression of migration in blind well chambers appears to be a result of interaction with viral proteins and is not necessarily a result of infection of the macrophage. Decreased migration appears to be mediated through an interaction with the envelope and/or transmembrane proteins of HIV (89,91). Chemotaxis can be broken down into stages of ligand binding, monocyte polarization toward a stimulus, and movement. The defect in chemotaxis appears to be mediated at the step of ligand binding and/or monocyte polarization. In one study, *in vitro* infection of monocytes with HIV suppressed monocyte polarization to a stimulus, with significant suppression first seen 24 to 36 hours after infection (89). This effect could be duplicated by the addition of gp120 to monocytes, with similar kinetics observed (89). From these results, it appears that the initial binding of virus to monocytes during infection was able to mediate a decrease in migration. A concurrent decrease in fMLP receptor and C5a receptor staining was seen, suggesting that decreased ligand binding had resulted in the suppression of chemotaxis (89). These researchers also found an upregulation of class II MHC molecules with the same kinetics as the depression in chemotaxis, indicating that the effect on migration might be part of a general activation of the cell.

The HIV capsid protein, p24, does not appear to have any effect on monocyte migration (91); however, the transmembrane glycoprotein (gp41) appears to mediate a decrease in monocyte polarization (91). Recombinant gp41, as well as serum

fractions of 25-30 kDa from the serum of asymptomatic HIV infected individuals and AIDS patients, was able to suppress migration. The effect of 25-30 kDa serum fractions could be abolished by the addition of an antibody to gp41; it appears that a breakdown product of gp41 was responsible for the suppression observed.

Retroviral transmembrane peptides related to the murine leukemia virus p15E protein have also been found in the serum of oncovirus infected and cancer patients (93) and may mediate immunosuppression (94,95). The p15E peptide is derived from a highly conserved portion of the retroviral transmembrane protein, and related sequences are present in HIV (96). Some researchers have suggested that these p15E-related peptides may mediate the decreased chemotaxis seen in AIDS (94,97). The breakdown product of gp41 found in the above experiment does not, however, appear to be the p15E related portion of the protein (91). Some serum samples in this study also contained small (less than 25 kDa) factors which were serologically related to p15E and which could inhibit monocyte polarization. These small factors were, however, detectable only in the serum of HIV seronegative homosexual individuals and did not appear to be related to gp41 (91). In addition, a study using synthetic transmembrane peptides from HIV and other retroviruses has shown that, although many retroviruses contain p15E-related peptides able to suppress monocyte polarization, the HIV peptide has a relatively weak effect (94). It therefore appears that, although p15E related peptides may contribute slightly to suppression of chemotaxis, they are unlikely to be the major factor responsible for the effect. Other gp41 breakdown products and/or gp120 appear to be more likely candidates for the role.

SIV,FIV,BIV

Macrophage chemotaxis does not appear to have been examined in SIV or FIV infections. Onuma et al (16), however, found suppressed chemotaxis in 3 BIV infected calves, early in infection. We were not able to confirm this result. In our studies, BIV infection status had no effect on random or chemotactic migration of

monocytes isolated from infected animals (17). There was, however, a significant enhancement of both random and chemotactic migration when monocytes were treated *in vitro* with BIV-containing cell supernatants or recombinant p26 Gag protein (98).

Antibody-Dependent Cell-Mediated Cytotoxicity

HIV

There are few reports on antibody-dependent cell-mediated cytotoxicity (ADCC) of monocytes in HIV infected individuals. In an early report on 17 ARC and 9 AIDS patients, ADCC was normal, although monocyte chemotaxis was suppressed in the same patients (90). Two, more recent, reports have found depressed monocyte ADCC either in AIDS patients or in macrophages infected with HIV *in vitro* (84,99). Bender et al (84) found depressed ADCC in a group of AIDS patients. In addition, relatively high concentrations of serum (25 or 40%) from these individuals were able to suppress ADCC of normal mononuclear cells. A criticism of this study was that it used unfractionated mononuclear cells rather than isolated monocytes. ADCC is also a function of NK cells, and it is unclear from the methods whether NK cell or monocyte ADCC was being measured. In another study, however, monocyte derived macrophages (MDM) infected *in vitro* also had reduced ADCC against 5 leukemic target cell lines (99). This study is, perhaps, the most convincing evidence that monocyte ADCC mediated killing may be suppressed by HIV. Molecular mechanisms for the suppression of ADCC have not been determined.

SIV,FIV,BIV

ADCC function does not appear to have been examined in SIV or FIV infected macrophages. Monocyte ADCC does not appear to be affected in BIV infected animals; however, treatment of monocytes *in vitro* with BIV resulted in a dose-dependent decrease in ADCC function, consistent with the effects seen with

HIV (17). Infection of the monocyte was not necessary for this suppression.

Killing of Bacteria and Fungi
HIV

Killing of bacteria and fungi by macrophages is a complex process. Most extracellular organisms are killed by phagocytosis, in conjunction with reactive oxygen metabolites, reactive nitrogen intermediates, and/or lysosomal enzymes. Cytokine upregulation of macrophage activity is necessary for effective killing of some bacteria, particularly intracellular bacteria which can multiply within un-activated macrophages. A number of opportunistic infections, as well as disseminated infections by pathogens, are common in AIDS; therefore, a wide variety of organisms have been used in studies on bacterial and fungal killing by macrophages. Although it has not been found that HIV-infected macrophages have a generalized defect in killing of all microorganisms, defects have been found in the killing of some specific microorganisms.

The most general studies have examined the final result of the macrophage interaction with a microorganism: the reduction in colony counts when bacteria or fungi are mixed with HIV-infected macrophages. Defective killing of *Aspergillus fumigatus* by monocyte-derived macrophages (MDM) from HIV infected children (100), and of *Candida* by macrophages from HIV infected adults (99,101) have been observed. Killing of *Leishmania donovani* does not appear to be affected by HIV infection; however, *Leishmania* is a more difficult organism to kill than *Candida* (101). An early study reported that the killing of *Toxoplasma gondii* by macrophages from infected patients was also unchanged (101). In contrast, Murray et al found that killing of *T. gondii* and *Chlamydia psittaci* by macrophages from AIDS patients was defective when using unstimulated macrophages; however, these macrophages were able to kill bacteria normally after they were activated with interferon γ (102,103). Murray et al (103) suggest that the defects found in macrophage killing of bacteria are simply a reflection of decreased stimulation by interferon γ *in vivo*; however,

other studies using macrophages infected *in vitro* or treated with purified HIV proteins do not support this hypothesis (99,104-106).

Another study also found that HIV is able to affect macrophage-mediated killing of *T. gondii* by a different mechanism involving interferon (107). This particular study also illustrates the complications which may occur in killing assays. Killing of *T. gondii* by interferon stimulated macrophages was found to be depressed in HIV infected, asymptomatic individuals and patients with lymphadenopathy. No defects in killing were, however, seen in unstimulated macrophages. On closer examination, the "defect" in interferon-stimulated macrophages appeared to be due to an enhancement in growth of *Toxoplasma* when the organisms were stimulated with interferon (107). A similar phenomenon has been demonstrated for other microorganisms; the growth of mycobacteria may also be stimulated by interferon (108). In the *Toxoplasma* study, it appeared that increased interferon was reaching intracellular organisms in the HIV-infected macrophages. Any interferon-mediated enhancement in macrophage activity could not, apparently, overcome the increase in the replication rate of the toxoplasma organisms. The authors speculate that an increase in the number of interferon receptors on macrophages from HIV infected patients was responsible for allowing additional interferon to reach the organisms (107). They did not, however, stain directly for interferon receptors.

Studies of phagocytosis do not suffer from such complications, as they measure a single aspect of bacterial killing, and often use killed organisms or inert particles. As a result, however, they present a more limited picture of the effects of HIV on bacterial killing. A number of studies have examined phagocytic function of HIV infected macrophages directly. The simplest studies have examined the ingestion of latex beads or red blood cells, to determine if there are any general defects in macrophage internalization of particles. Most of these studies have found no defects in phagocytosis of inert, non-opsonized particles (84,90,101) even when antibody-mediated phagocytosis was impaired in the same study (84). A more sophisticated approach is to examine the internalization of pathogenic or

nonpathogenic organisms, with or without opsonization by antibody or complement. Such studies have found defective phagocytosis of a variety of organisms, particularly fungi (100,101-106). Crowe et al demonstrated defective phagocytosis of opsonized *Candida albicans* or *T. gondii* by monocyte derived macrophages infected with the Ba-L or DV strains of HIV, or a clinical monocyte-tropic isolate (104). Using monocyte-derived macrophages from HIV-infected children, Roilides et al (100) also found defective phagocytosis of *Aspergillus fumigatus*. Alveolar macrophages from HIV-infected adults appear to have phagocytic defects against *Pneumocystis carinii* (105) and *Cryptococcus neoformans* (106).

No common mechanism has been found for these defects in phagocytosis. One common factor, however, may be that only adherent macrophages have their phagocytic activity altered by HIV infection. All of the studies which found phagocytic defects used macrophages grown in adherent systems; in a single study using monocyte derived macrophages cultured in Teflon bags and infected with a monocyte tropic isolate of HIV, no phagocytic (or killing) defects of macrophages against a variety of bacteria could be found (78).

A variety of cell surface receptors appear to be involved in the phagocytic defects found, with different receptors involved for different organisms. Changes in antibody Fc receptor expression do not, however, appear to be involved in phagocytic defects. Fc receptors on macrophages do not appear to be downregulated in HIV infection (78,84,87,109). These results do not, however, exclude the possibility of impaired receptor function with normal surface expression.

The decreased phagocytosis of *Pneumocystis carinii* appears to be due to impaired mannose receptor function (105). The apparent mechanism is competition of HIV proteins with *Pneumocystis* for binding to the mannose receptor. The HIV gp120 envelope protein (but not the p24 Gag protein) was able to suppress binding of macrophages to either mannose-rich proteins or to *Pneumocystis* (105). Interestingly, gp120 was also able to inhibit phagocytosis of *Cryptococcus neoformans*, but not by competition for a receptor (106). Gp120 depressed macrophage

internalization, but not binding, of *C. neoformans* by an unknown mechanism (106). The gp41 transmembrane protein has not been tested for effects on phagocytosis. Retroviral p15E-related transmembrane peptides have been tested only against opsonized chicken erythrocytes; in this system, no effect on monocyte phagocytosis was seen (97).

In the *Candida albicans* system, the mannose receptor does not appear to be involved in phagocytosis; instead, the defect in phagocytosis of this organism appears to result from the downregulation of the Mac-1 integrin (complement receptor CR3) by HIV (104). Most other studies have found no differences in CR3 levels in HIV infection (78,80,84); however, these studies used different virus isolates, or macrophages from infected patients, and do not necessarily invalidate these results. Bender et al (91) found defective clearance of antibody or complement opsonized inert particles by the reticuloendothelial system in AIDS patients, although no decrease in the number of Mac-1/CR3 receptors was seen (84). The clearance of unopsonized particles was not, however, affected. It is possible that a direct effect of HIV infection of macrophages *in vitro* is to depress Mac-1/CR3 expression, but that cytokine or other interactions *in vivo* result in normal expression, with impaired function. HIV proteins were not tested in the *C. albicans* system.

As different receptors are used for the phagocytosis of different organisms, it is difficult to determine whether a generalized phagocytic defect exists for HIV infected macrophages. Most evidence, to date, indicates that it does not. In addition, there are some organisms for which killing defects have been found, but phagocytic function does not appear to be impaired. One study found normal phagocytosis, in spite of a defect in killing *Candida pseudotropicalis*, when monocyte-derived macrophages were infected *in vitro* with the monocyte-tropic HIV-JRFL isolate (99). Another study using macrophages from AIDS and LAS (lymphadenopathy syndrome) patients also found normal phagocytosis with concurrent defects in killing of bacteria (49). As described above, the growth of *Toxoplasma gondii* may be stimulated in HIV infected macrophages, after interferon stimulation (107). It is not known whether

phagocytosis is also affected for *T. gondii*. Furthermore, mycobacteria may also grow better in HIV-infected macrophages than in uninfected macrophages. Newman et al found that macrophages infected *in vitro* with the Ba-L isolate of HIV, then superinfected with *Mycobacterium avium* or *Mycobacterium tuberculosis* had increased intracellular growth of the bacteria in HIV infected macrophages, as well as cytokine derangements (54). It is not known whether interferon receptors were altered in this study. Unfortunately, a nearly identical study was unable to confirm these results (110). Currently, the best evidence indicates that phagocytic defects exist for internalization of some organisms, and that killing of other organisms may be impaired by mechanisms other than a defect in phagocytosis.

SIV,FIV,BIV

There have been relatively few studies examining macrophage phagocytosis or microbial killing by macrophages infected with lentiviruses other than HIV. These studies are, however, particularly interesting as most of them have been done at very early stages of infection. This is generally not possible with HIV. Phagocytic function of macrophages from SIV infected monkeys does not appear to have been measured; however, peritoneal macrophages of cats in an early stage of infection with FIV were found to have increased antimicrobial activity against *Toxoplasma gondii* (111). Macrophages from FIV infected cats, therefore, appear to be activated for microbial killing during early infection. The equivalent studies from very early stages of infection are not available for HIV; however, results from macrophages infected with HIV *in vitro* show suppressed rather than increased antimicrobial activity.

Unlike FIV, BIV does not appear to activate macrophages for killing of microorganisms *in vivo*. In one study, BIV infection suppressed the ingestion of latex beads by monocytes from 2 of 3 infected calves (16). In BIV infected calves followed for 2 years PI, we also found a tendency for a decrease in phagocytosis of opsonized *Staphylococcus aureus* early during infection. This was not, however, statistically significant, and did not persist after the first 8 months PI. In contrast, monocytes

treated *in vitro* with supernatants from BIV-infected cells (17) or with recombinant BIV Gag (98) had a significant dose-dependent increase in *S. aureus* ingestion. The discrepancies between these *in vivo* and *in vitro* results remain to be resolved. It is likely, however, that the enhancement of phagocytosis was not seen *in vivo* because monocytes were not exposed to high levels of the BIV Gag proteins. In addition, monocyte exposure to immunosuppressive cytokines, or other viral interactions, may have masked any effect of low levels of BIV Gag proteins *in vivo*.

Therefore, it appears that FIV and BIV do not affect antimicrobial activity as reported for HIV. While HIV appears to decrease antimicrobial activity *in vivo* and *in vitro*, FIV infection *in vivo* and BIV *in vitro* both appear to enhance antimicrobial activity (17,98,111). Few studies have examined macrophage function in lentiviruses other than HIV, and it may be dangerous to generalize from a small number of studies. Currently, however, suppression of antimicrobial activity does not appear to be a general effect of lentiviruses.

Reactive Oxygen Metabolites

HIV

Reactive oxygen intermediates are involved in the killing of some microorganisms. Increases in oxidative metabolism may, however, also produce tissue damage, particularly lung damage (112). Currently, there does not appear to be a consensus on whether oxidative metabolism is affected in AIDS. Suppression of the oxidative burst in HIV-infected individuals (101), no effect (49,101,113), or an increase in the oxidative burst with the progression to AIDS (112,114) have all been reported. Some of the variability in results may result from differences in patient selection. Some investigators have found that the effect on oxidative metabolism varies with the stage of infection. In one study, increased macrophage chemiluminescence was found in a group of asymptomatic, HIV antibody positive, antigen negative patients, with depressed chemiluminescence in the antigen and antibody positive group (114). Some studies, particularly earlier studies (49,101), may

also have been influenced by the presence of occult bacterial or fungal infection in HIV-infected patients. In one group of asymptomatic, HIV positive individuals which were carefully screened to rule out the presence of occult lung infections, alveolar macrophages from HIV-infected individuals were found to have an enhanced spontaneous release of superoxide anion (112). This upregulation was transient, and suggestive of macrophage activation (and possibly exposure to interferon γ) *in vivo*.

Interestingly, the oxidative response of monocytes to interferon may be altered in some HIV infected individuals. Pennington et al (113) found normal O_2-production in the monocytes of AIDS patients; however, treatment of these patients with recombinant interferon γ suppressed rather than enhanced the respiratory burst of isolated monocytes. Furthermore, treatment of monocytes from infected patients with interferon *in vitro* resulted in a heterogeneous set of responses (unlike that of monocytes from normal controls). Interferon treatment consistently enhanced the respiratory burst of monocytes from normal controls; however, monocytes from 3 AIDS patients responded to interferon with an increased respiratory burst, and 3 with a decreased respiratory burst. This result is consistent with a study demonstrating that the HIV p24 protein may alter the pattern of gene activation after interferon treatment (115). No obvious patient factors explained the variability between individuals.

It appears that *in vitro* studies may be necessary to clarify the results obtained from monocytes infected *in vivo*; however, few *in vitro* studies on monocyte oxidative metabolism have been done. One study found normal chemiluminescence in infected macrophages; however, the authors infected macrophages with a monocyte tropic isolate (Ba-L) which had been grown in mitogen-stimulated peripheral blood lymphocytes (PBLs) (78). Growth of virus in mitogen-stimulated PBLs is known to restrict virus tropism in favor of exclusively lymphocyte-tropic strains, and may, therefore, have resulted in alterations in virus characteristics. Other investigators, in contrast, have demonstrated a suppression of the monocyte respiratory burst (101). While it is unknown whether gp120 or gp41 affect the monocyte oxidative burst, the

p15E retrovirus consensus sequence, CKS-17, is able to suppress the respiratory burst in monocytes (97). Further research is necessary to resolve the discrepancy between the *in vivo* results, demonstrating a possible upregulation in oxidative metabolism, and the *in vitro* results demonstrating no change or a suppression in the respiratory burst. It is possible that the direct effect of HIV virus is to suppress oxidative metabolism, but that *in vivo* interactions modulate this response.

SIV,FIV,BIV

Oxidative metabolism does not appear to have been measured in FIV or SIV infection. Monocyte chemiluminescence was measured in 3 calves infected with BIV and found to be suppressed; unfortunately, the statistics attempt to compare variability between samples in 1 calf to variability in a group of 5 control calves (16). Therefore, this study must be repeated before conclusions can be drawn.

Reactive Nitrogen Intermediates

Data on the reactive nitrogen intermediate (RNI) system is currently sparse; however, envelope gp120 appears to increase the production of nitric oxide in 4 day old (but not 0 day old) monocyte-derived macrophages (116). This effect could not be suppressed by the addition of soluble CD4, indicating that it was not mediated by binding of gp120 to the usual cellular receptor CD4. The sugar mannan and a yeast chelate did, however, abolish the effect. The effect of gp120 on the RNI system, therefore, appears to be mediated through the macrophage mannose receptor.

Antigen Presentation

HIV

Antigen presentation by HIV infected macrophages is difficult to measure well. Tests are complex, and generally involve the depletion of monocytes from T cell cultures, the isolation and infection of purified monocytes, the presentation of an antigen by monocytes, and the measurement of lymphocyte proliferation in the

reconstituted cultures. While a number of studies have examined aspects of antigen presentation, such as the levels of MHC class II molecules or accessory cell function (cytokine secretion and levels of co-stimulatory molecules), fewer papers have examined the antigen presenting ability of macrophages more directly. One early paper examined the proliferative response to *Candida*, in 2 sisters with identical MHC class II types. One sister was infected with HIV, and did not have a blastogenic response to *Candida*. When macrophages from this patient were mixed with lymphocytes from her sister, blastogenesis to *Candida* was suppressed (117). Other papers have reported antigen presentation or accessory cell function defects in dendritic cells and macrophages (43), or monocytes (118,119) from asymptomatic, HIV infected individuals, patients with LAS, and AIDS. HIV may also have an effect on actual numbers of antigen presenting cells. Numbers of antigen presenting (but not phagocytic) macrophages in the duodenum appear to be reduced, as determined by staining for specific markers (120).

Some experiments have determined the effect of HIV infection *in vitro* on macrophage accessory cell function. Many of these have measured mitogen-induced blastogenesis. Although mitogen-induced blastogenesis does not require antigen presentation by macrophages, macrophages are necessary in this system for their costimulator function. Lacroix et al (121) tested the accessory cell function of monocyte derived macrophages infected with HIV (IIIB strain) at 2 or 7 days PI. They measured the proliferation of autologous T cells depleted of monocytes, and reconstituted with either uninfected or infected monocyte derived macrophages. Both phytohemagglutinin (PHA) and anti-CD3 antibody stimulated blastogenesis were suppressed when HIV-infected macrophages were tested; the addition of uninfected monocytes restored normal proliferative responses. Live virus was necessary for this effect. IL-1 and interleukin-6 (IL-6) were found to be low in the culture supernatants; addition of IL-1 and IL-6 to the cultures partially restored blastogenesis in response to PHA stimulation. Although unchanged or elevated, rather than depressed, levels of IL-1 have been found in infected macrophages

(50,51,56,88,122-125), these studies were all performed in isolated macrophages and not stimulated mononuclear cell cultures. It is possible that interactions between T cells and HIV-infected macrophages result in lower than normal levels of IL-1 than direct stimulation of these macrophages by cytokines or lipopolysaccharide. Other experiments have confirmed that antigen presentation ability and IL-1 synthesis declined concurrently in HIV infected macrophage hybridomas (126) and U937 promonocytic cells (118). In U937 cells, the suppression of antigen presentation coincided with the period of virus proliferation; however, simple addition of virus to T cells (at the level present in the cultures) did not suppress blastogenesis (118).

Although decreased levels of cytokines may be partially responsible for the suppression of PHA blastogenesis (118,121), addition of cytokines does not appear to restore blastogenesis in anti-CD3 stimulated cultures (121). The authors of one study speculate that HIV infection also inhibits function of macrophage Fc receptors (121). Macrophage Fc receptors are necessary in the anti-CD3 system, to cross-link the antibody which stimulates CD3 receptors. Other studies have not demonstrated a decrease in Fc receptors I and II (78,84,87,109); however, function of the Fc receptor might be affected without an effect on expression (121). It is unclear whether such inhibition of Fc receptors has any relevance to antigen presentation *in vivo*.

In addition to cytokine production, antigen presentation ability of macrophages depends on a number of factors, including expression of class II molecules and other accessory molecules. A number of studies have stained infected macrophages for class II MHC antigens. Decreased (83,86,114,126,127), unchanged (77) and increased (46,82,85,101,112) class II expression have been reported. Sperber et al (126) report a concurrent decline in class II MHC expression, IL-1 production and antigen presentation ability of HIV infected macrophages. Some of the discrepancy in the results of different studies may result from measuring class II expression at different stages of infection. Alterations in cytokine release by HIV and subsequent effects on class II expression might also be seen in some systems. Even with isolated promonocytic cells, the effects of HIV on class II expression are still

controversial. Decreases in class II molecules, with no change in class I expression, were seen in U937 cells infected with HIV in one system (86); however, increases in class II molecules on these cells (HLA-DR, DQ), under similar conditions, were found by others (85).

Other accessory molecules involved in antigen presentation have rarely been studied; one study found decreased expression of accessory molecules (B7/BB-1) after infection with HIV (43). Additional research will be needed to resolve the discrepancies, and determine if changes in class II or B7 expression are involved in antigen presentation defects in AIDS. It is quite likely that a combination of changes in cytokine secretion, class II MHC, and other macrophage molecules are responsible for defective antigen presentation by HIV infected macrophages.

Finally, one caveat must be observed in determining the effects of HIV on antigen presentation: in many experiments, it is difficult to determine whether antigen presentation by macrophages is defective, or whether antigen presentation is normal and macrophages are actively inhibiting T cell proliferation by some other mechanism. In at least one case, adherent cells seemed to actively inhibit T cell proliferation; depletion of adherent cells from cultures enhanced blastogenesis (119). This inhibitory activity was not due to virus production, and appeared to be due to a heat-labile protein. Other experiments have implicated prostaglandin E_2 (PGE$_2$) in the inhibition of lymphocyte proliferation by HIV-infected macrophages. Prostaglandin E_2 downregulates the immune system, and there are reports of possible prostaglandin dysregulation in AIDS (49,101,114). Although plasma levels of PGE$_2$ appear to be normal, their synthesis and release by unstimulated monocytes may be altered (49,101). One paper describes a prostaglandin-mediated suppression of lymphocyte blastogenesis and lymphokine activated killer (LAK) cell induction in asymptomatic, HIV positive individuals (114). In this experiment, monocytes from AIDS patients actively suppressed blastogenesis and LAK cell induction with lymphocytes from normal individuals (114). Addition of indomethacin to block prostaglandin synthesis, and IL-2, restored levels to normal.

SIV,FIV,BIV

There appear to be no studies which have examined the effect of lentiviruses other than HIV on macrophage antigen presentation ability.

Effects on Cytokine Release

HIV,SIV,FIV

In addition to their roles in antigen presentation, cytokines are involved in regulating monocyte chemotaxis, phagocytic function, and ADCC. Cytokines may also act on infected cells to inhibit or activate virus replication (21,58,59,128,129). Some cytokines which may be altered by HIV infection include TNFα, IL-1, IL-6, and GM-CSF.

Tumor necrosis factor α

TNFα, IL-1, IL-6, and GM-CSF are cytokines which have been documented to activate HIV. TNF appears to be particularly important in AIDS, as it both activates HIV and mediates some of the physiologic effects of infection (21,58). A number of studies have measured the release of TNFα, as well as other cytokines, from HIV infected macrophages. Some early research determined that the spontaneous or LPS-stimulated production of TNFα from monocytes or macrophages of AIDS patients was increased (25,130,131). A problem with many of these studies was the inclusion of patients with secondary infections or tumors (25,130). In a study of AIDS patients without active secondary infections (132), there were no differences in serum TNF, or TNFα from cultured PBMC, when mean values were compared to normal controls. The AIDS patients did, however, have increased variability within the group; 10 of 26 patients had TNF levels that were either higher or lower than the control mean by greater than 2 standard deviations. In a study using alveolar macrophages from asymptomatic, HIV positive individuals, there was a trend toward lower TNF production than controls when macrophages were stimulated with lipopolysaccharide (LPS) (133). It is somewhat difficult to directly compare this study

to the others, as a different macrophage type, as well as a different stage of disease, were studied. In fact, Lathey et al (48) have recently found that TNF produced from blood monocytes may vary with the stage of disease. They report that TNFα (as well as IL-1β) levels appear to be upregulated in asymptomatic HIV infected individuals, with a loss of this upregulation as the patients progress to AIDS. In asymptomatic individuals, TNF mRNA was present in unstimulated monocytes, although TNF production after stimulation with LPS was not different from controls. From this data, it appeared that monocytes were activated in asymptomatic individuals. TNF mRNA was, however, no longer found in unstimulated monocytes from AIDS patients. The monocytes from AIDS patients still appeared to be receiving activation signals (i.e., neopterin levels were increased); however, they no longer appeared to be reacting to the signals for increased TNF production. Unfortunately, this study also includes patients with secondary diseases, as well as some individuals on AZT.

A more direct approach, which eliminates the problem of concurrent infections, has been to examine TNF levels in monocytes infected *in vitro*. Conflicting results have also been found, with this approach. While some studies (53,54,56,88) have found that infection with HIV is able to induce spontaneous and/or elevated production of TNFα *in vitro* from peripheral blood monocytes, others (123-125,134) find no elevation of TNF production after infection. Some studies which find no effect of infection have used monocyte tropic strains of HIV such as Ba-L; however, the specifics of virus challenge, including virus strain and multiplicity of infection, as well as the length of macrophage culture and method of cytokine stimulation may account for the conflicting results. It is also possible that the timing of some of these studies may have missed the burst of TNF production, 2 to 6 hours after infection, found by Merrill et al (56). Merrill et al (56) found that binding of HIV to CD4 mediated an increase in TNFα production with no requirement for productive infection of the macrophage. More recently, Clouse et al (57) have confirmed that recombinant gp120 is able to induce TNF production by binding to macrophages. Studies using HIV infected promonocyte cell lines have also found increases in TNF

after HIV infection (122,135). There is, therefore, evidence that the initial perturbation of the cell membrane through gp120 binding to CD4 results in a transient burst of TNFα production. Nevertheless, a consensus has not been reached on whether HIV infection changes the levels of TNF production from monocytes *in vivo*. Increased TNF *in vivo* may also result from macrophage activation from concurrent infections, or influences of other cytokines.

Tumor necrosis factor production from macrophages has also been studied in both FIV infection and SIV infection, but not BIV. In rhesus macaques, TNF levels have been found to be significantly increased in asymptomatic animals, then depressed after the progression to AIDS (52). This correlates well with the situation found in HIV infected humans by Lathey et al (48). In asymptomatic FIV infected cats, although no abnormalities in serum TNFα were found for the first 6 months post-infection, increased levels of TNFα production were found after vaccination or challenge with FeLV, compared to cats not infected with FIV (136).

Interleukin 1

IL-1 production appears to be increased by infection with HIV, although, again, this may vary with the stage of disease. Although not all reports agree (90,124), most studies have found increases in IL-1 production, in either the asymptomatic stage only (48), or in patients with persistent lymphadenopathy (49) or AIDS (49-51). Furthermore, Berman et al (50) have found that AIDS patients have both increased IL-1 activity and an increase in IL-1 inhibitors. This study may explain why some studies using thymocyte proliferation assays to test for IL-1 have not found increased levels of IL-1. In the thymocyte proliferation assays, the biological response would appear to be a response to normal levels of IL-1, due to the masking of high IL-1 levels by the IL-1 inhibitors. Infection of monocyte derived macrophages (54,56,88) or promonocyte lines (122) has also been found to increase IL-1 production and/or IL-1 specific mRNA. The studies in immature monocyte lines may, however, simply reflect a slight maturation of promonocytes induced by

infection with HIV, which might increase the cell capacity to make IL-1 (122).

Interleukin 6

Although fewer studies have examined IL-6 than IL-1 or TNF, IL-6 levels have usually been found to be normal (48,55) in HIV infected patients. IL-6 levels from macrophages infected *in vitro* have, however, been found to be elevated in some studies (54,88). Inactivated, noninfectious HIV (55) has also been found to induce IL-6 from monocytes, although plasma IL-6 levels were found to be normal in asymptomatic, ARC, or AIDS patients in the same study (55). It is possible that local levels of IL-6 are high in tissues with active HIV infection, but that this is not reflected in serum IL-6 levels. A recent study found that the HIV transactivating protein Tat was able to induce IL-6 (but not GM-CSF or TNFα) from peripheral blood monocytes (137). Tat has also been reported to induce TGFβ by bone marrow macrophages (70).

Other cytokines

A few studies have examined the production of other cytokines from infected macrophages. Alveolar macrophages from HIV infected patients were found to spontaneously release small amounts of GM-CSF, and to produce greater than normal levels of GM-CSF after LPS stimulation (138). This phenomenon appears to support the theory that macrophages from HIV infected patients are activated *in vivo*. Others have found that monocytes infected *in vitro* with HIV then stimulated with LPS produced elevated levels of IL-8 (139) but normal M-CSF (139). In a nonadherent system, depressed levels of M-CSF were found (88). Relatively few studies have investigated interferon production from macrophages; however, one intriguing study found that cultured macrophages infected with HIV and then treated with a double stranded RNA or with 2 different RNA viruses were able to produce normal levels of TNFα, IL-1, IL-6, or interferon β (IFNβ), but did not produce IFNα (124). The block appeared to be at the transcriptional level. There have been reports

that the interferons may have antiretroviral activity against HIV (128,129,140); suppressed secretion of interferon might interfere with this effect. Others have also found that monocytes and PBMC from infected patients have a decreased ability to secrete IFNα or to produce IFNα mRNA (130,141,142).

Finally, there is one report that treatment of monocytes with the HIV p24 core protein alters the response of monocytes to interferon γ (115). Recombinant p24 treatment of the THP-1 monocyte line inhibited the normal interferon induced increase in HLA-DR (class II MHC) and cytochrome b mRNA.

Alterations in the cytokine response to pathogens

Increased or decreased cytokine levels may be in themselves interesting, particularly when the cytokine is one like IL-1 or TNF which can cause side effects such as fever or muscle wasting, or reveal macrophage activation present *in vivo*; however, the primary importance of cytokines is their regulation of the immune response to pathogens. One particularly interesting study, rather than examining cytokine levels in HIV, examined the cytokine response to *Pneumocystis carinii* by HIV infected macrophages (143). Monocyte derived macrophages were infected with HIV Ba-L, then exposed 10-14 days later to *P. carinii*. Uninfected macrophages had higher levels of IL-1 and TNFα than HIV infected macrophages when treated with *P. carinii*. IL-6 levels were comparable in infected and uninfected macrophages. Similar results were seen in response to a *Pneumocystis* 95-115 kDa gpA protein (143). This cytokine response may be organism specific; in contrast to the results with *P. carinii*, HIV infected macrophages appear to secrete increased IL-1β and TNFα when coinfected with *Mycobacterium avium* (143).

Macrophage Roles in Tissue Repair

Most studies of macrophages infected with HIV have examined immune functions and not basic macrophage "housekeeping" functions. A few interesting studies have, however, examined the role of infected macrophages in tissue repair.

Some researchers have found that HIV infected macrophages adhere to and spread on extracellular matrix proteins more efficiently than control cells (144). While this effect may be partly due to increased expression of integrins, other mechanisms also appear to be involved. Dhawan et al (144) found that when equal numbers of HIV infected and control monocytes were cultured on a matrix of basement membrane proteins, the HIV infected macrophages not only spread faster, but also degraded the matrix around them. These macrophages expressed p24 and were productively infected. Culture fluids from HIV infected macrophages contained higher levels of a 92 kDa metallogelatinase than supernatants from control macrophages. Neutral proteases such as metallogelatinase are secreted by tissue macrophages and are involved in remodeling, wound healing, and inflammation; this report (144) raises the possibility that healing functions may be affected by HIV infection of macrophages.

Another report has implicated macrophages in the glomerulosclerosis observed in some AIDS patients (145). Increased mesangial cell proliferation and matrix synthesis may be responsible for this phenomenon, and Mattana et al report that supernatants from HIV-serum treated macrophage cell line (J774.16) significantly increased mesangial cell synthesis of collagen (145). This experiment used a macrophage cell line, and is rather vague on the patient profiles; this study should be confirmed using tissue or monocyte-derived macrophages.

Finally, macrophages in AIDS patients may be less able to participate in normal hemostasis. Tissue factor is a macrophage cytokine that initiates the coagulation protease pathway, and may, therefore, be involved in wound healing. Tissue factor production from LPS stimulated monocytes was found to decrease with the progression to AIDS (48).

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The Activation Hypothesis

Do HIV effects on macrophage activation eventually result in a state of generalized macrophage dysfunction?

In an early review on monocyte function in AIDS patients, Estevez and Sen (101) suggested that monocytes and macrophages might become chronically activated by HIV, which would eventually lead to generalized defects in macrophage function. In this theory, infected macrophages are activated and produce proinflammatory cytokines (e.g. IL-1, TNF, and IL-6). These cytokines lead to increases in virus concentrations, and increased stimulation of macrophages, T and B cells. Eventually, negative feedback mechanisms on the macrophage would result in a concurrent increase in PGE_2. The permanently activated state, they hypothesized, would lead to a state of macrophage dysfunction. This hypothesis is consistent with some but not all of the *in vivo* data presented above. Some evidence suggests that macrophages are activated by HIV infection: this evidence includes increases in integrin (and possibly MHC class II) expression, increased IL-1 and possibly TNF synthesis, and increased reactive nitrogen metabolism. Interestingly, Allen et al (87) have shown an increased expression of FcγRIII on freshly isolated monocytes from AIDS patients. Expression of this receptor is correlated with macrophage activation or differentiation. The theory cannot, however, account for some changes in macrophage function in cells infected by HIV *in vitro*, including suppression of antigen presentation, phagocytosis, and ADCC. The chronic activation hypothesis predicts an initial increase in these parameters, followed by suppression. Activation for these functions is not, however, seen in macrophages infected *in vitro*. Therefore, although a unifying theory for the multiple effects of HIV on macrophage function is attractive, the activation hypothesis cannot explain all of the defects in monocyte function seen following HIV infection. This does not preclude the possibility that HIV causes macrophage activation; however, individual virus proteins or fragments (particularly gp120 and gp41, but also Tat) also appear to have direct suppressive effects on macrophage functions.

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Summary/Conclusions

It is apparent that HIV is able to affect macrophage function, mainly to the detriment of the immune system. The formation of monocyte precursors in the bone marrow, as well as function of mature cells (including chemotaxis, microbial killing mechanisms, and, possibly, ADCC function) appear to be suppressed by interactions with HIV. Upregulation of surface molecules such as integrins may be seen, while the function and/or expression of other cell surface molecules (i.e. complement receptors) may be downregulated. While some of these effects are controversial and not widely accepted, others appear to be emerging as real phenomena. It is plain that HIV infection can no longer be viewed exclusively as an infection of T cells, with the macrophage as an unimportant secondary target. While the primary effect of HIV on T cells appears to be cell death (21), the effects on macrophages are subtle and varied. The overall result, however, appears to be a downregulation of monocyte functions and a resultant suppression of immune defenses against pathogens and tumors. The effects of SIV, FIV and BIV have yet to be fully described and their effect on monocyte function remains to be determined. With the possible exception of SIV, however, these lentiviruses do not appear to affect monocytes identically to HIV.

Some of the effects of lentiviruses on macrophage function remain to be more fully characterized and described. Much of the current focus, however, is on determining mechanisms for the immune function changes caused by HIV. Retroviral proteins, particularly the envelope and transmembrane protein, appear to be good candidates to mediate many of the alterations in macrophage function. In addition, the p24 core protein has been recently implicated in changes in immune function (115,146,147). The effects on macrophage molecules are just beginning to be determined, and signal transduction mechanisms have not been described for any of these phenomena. This molecular emphasis will, most likely, define the direction of future research on lentiviral effects on macrophage function.

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MONOCYTE FUNCTION IN CATTLE EXPERIMENTALLY INFECTED WITH BOVINE IMMUNODEFICIENCY-LIKE VIRUS

A paper published in Veterinary Immunology and Immunopathology[1]

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Abstract

The effects of bovine immunodeficiency-like virus (BIV) on the function of monocytes recovered from experimentally infected cattle or upon monocytes infected *in vitro* were examined. Infection with the R29 isolate of BIV appeared to have relatively little effect on monocyte function in cattle during the first 2 years post-inoculation (PI). For the first 4 to 8 months PI, monocyte phagocytosis of opsonized *Staphylococcus aureus* tended to be lower (P = 0.06) in BIV infected calves than in control animals. After 8 months PI, however, phagocytosis became equal between the two groups. Random and chemotactic migration and antibody-dependent cell-mediated cytotoxicity (ADCC) did not appear to be affected by BIV infection. Monocytes from BIV infected cattle were able to respond to *in vitro* treatment with interferon γ similarly to monocytes from control cattle. Although experimental infection with BIV R29 resulted in minimal effects on monocyte function, this result could have been due either to a low virus burden *in vivo* or because BIV is intrinsically unable to affect monocyte function. To distinguish between these possibilities, monocytes from control, uninfected cattle were treated with BIV virus *in vitro*. Treatment of normal monocytes with supernatants from BIV-infected fetal bovine lung cells significantly (P < 0.05) increased phagocytosis

and random and chemotactic migration and decreased ADCC, in a dose-dependent manner. It appears, therefore, that the normal function of peripheral blood monocytes in the BIV R29 infected animals may be due to a low virus burden rather than to the inability of BIV to affect monocyte function. The *in vitro* infection results also raise the possibility that the function of monocyte derived cells at local sites of BIV replication may be altered.

Introduction

Bovine immunodeficiency-like virus (BIV) is a lentivirus of cattle that shares genetic, antigenic, and structural similarity with the human immunodeficiency virus type 1 (HIV-1) (Gonda et al., 1987). BIV was first isolated in 1969 from an 8-year old cow with persistent lymphocytosis, generalized lymph node hyperplasia, and progressive weakness and emaciation (Van der Maaten et al., 1972). More recently, the AIDS epidemic and interest in animal models for AIDS has renewed interest in BIV. Although serologic surveys demonstrate BIV antibodies in 4% to 11% of cattle in some areas of the U.S. (Amborski et al., 1989; Black, 1990), relatively little is known about the effects of BIV infection on the bovine immune system. It is not yet known whether BIV causes significant immunosuppression; however, changes in immune function have been reported relatively early after BIV infection (Stott et al., 1989; Martin et al., 1991; Flaming et al., 1993). In initial observations, inoculation of calves with the original R29 isolate produced a mild nonpersistent lymphocytosis and enlargement of subcutaneous lymph nodes (Van der Maaten et al., 1972). More recently, we (Carpenter et al., 1992) have confirmed that calves inoculated with BIV have early histopathological changes similar to those seen in early cases of HIV-1 infection, including follicular hyperplasia in lymph nodes and spleen. Other recent reports describe a selective depletion of CD4$^+$ T cells 3 to 4 months after inoculation with the R29 isolate (Stott et al., 1989), both decreased (Martin et al., 1991) and increased (Flaming et al., 1993) lymphocyte blastogenesis, and depressed neutrophil function (Flaming et al., 1993). Recently, Onuma et al.

have observed a depression of monocyte functions on a single occasion in three animals (Onuma et al., 1992). Long-term studies on the influence of BIV infection on monocyte function have not, however, been published.

In this study, we have measured phagocytosis, ADCC, and random and chemotactic migration in monocytes isolated from a group of cattle experimentally infected with the R29 isolate of BIV. We also determined whether monocytes from BIV infected cattle were capable of responding to activating agents such as interferon. In addition, we treated normal monocytes *in vitro* with BIV, to determine whether the virus, in sufficient concentration, is able to affect monocyte function.

Materials and Methods

Experimental animals

Experimental infection of the calves with BIV and the virologic aspects of infection have been previously described (Carpenter et al., 1992; Flaming et al., 1993). Briefly, 2-4 month old male Holstein calves were determined to be serologically negative for BIV and bovine leukemia virus and randomly assigned to BIV (n = 4) or control groups (n = 5). All but one BIV infected animal were also free of antibodies to bovine syncytial virus. Stocks containing the only available isolate of BIV were known to contain a noncytopathic strain of bovine virus diarrhea virus; therefore, all calves were first immunized 2 and 4 weeks prior to BIV inoculation with a commercial killed bovine virus diarrhea (BVD) vaccine. The BIV animals each received an intravenous dose of 1.8×10^4 syncytium forming units of the R29 isolate of BIV in fetal bovine lung (FBL) cells. The control animals were simultaneously inoculated with a non-cytopathic strain of BVD (field isolate of a noncytopathic BVD virus from persistently infected cattle obtained from S. Bolin, NADC, Ames, IA) and FBL cells. No immune functions were examined for the first 3 months of infection to allow the animals time to eliminate the BVD virus and to prevent interference of BVD with immune function. Starting 4 months after inoculation with BIV, blood monocyte function was assayed every 2 to 4 weeks for 2

years.

Monocyte isolation for function assays

Whole blood was collected into acid citrate dextrose anticoagulant and mononuclear cells were isolated by centrifugation at 1500 x g, collection of the buffy coat, and hypotonic lysis of erythrocytes as previously described (Roth et al., 1981b). Monocytes for functional assays were isolated by adherence to tissue culture flasks. Flasks were prepared by coating for 15-20 minutes with autologous plasma before adding RPMI 1640 (GIBCO Laboratories, Chagrin Falls, OH) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and fetal bovine serum (FBS) for a final concentration of 10% FBS and 10% autologous plasma in RPMI. An average of 3 x 10^8 mononuclear cells were added to each flask and, after a 1 to 2 hour incubation period (37°C in 5% CO_2) to allow the monocytes to adhere, the nonadherent cells were removed by washing 5 times with warm (37°C) RPMI without antibiotics or FBS. The adherent monocytes were then detached by incubation in warm (37°C) phosphate buffered saline for 45 minutes (Bendixen, 1981). Monocytes were resuspended for assays at 3.5 x 10^6 cells/ml in medium M199 (GIBCO Laboratories, Chagrin Falls, OH) with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. Monocyte purity (93% ± 4% by nonspecific esterase staining) and viability (94% ± 5% by trypan blue stain) were generally high.

Virus recovery

BIV virus was isolated by co-cultivation of monocytes or unfractionated mononuclear cells with fetal bovine lung (FBL) cells, an indicator cell line which forms syncytia when infected with bovine retroviruses. Mononuclear cells were isolated as described above and 10^7 cells were added to cultures of FBL cells (except at 6 months, when 2 x 10^7 cells were used). Virus replication was detected by syncytium formation in the FBL cells and indirect immunofluorescence as previously described (Carpenter et al., 1992). Co-cultures of FBL cells and mononuclear cells were followed for 5 passages before they were considered to be

negative for virus.

To detect BIV in monocytes, the adherent monocyte population was isolated from mononuclear cells. An excess of mononuclear cells was added to tissue culture flasks (0.5×10^8 or 1×10^8 mononuclear cells/ T25 flask). The adherent cells were washed repeatedly on day 0 and day 1 before adding FBL cells to each flask of monocytes. Monocytes purified in this way generally result in approximately 0.2 to 1 $\times 10^6$ monocytes per 25 cm^2 tissue culture flask, as well as improved purity over monocytes prepared for monocyte function assays. After purification of monocytes, equal numbers of FBL cells were added to each flask of monocytes. Co-cultures were then monitored daily for syncytium formation. Cultures were followed for at least one month (3-4 passages) before determining cultures to be negative for virus.

In all cases, virus identity was serologically confirmed by indirect fluorescent antibody staining of infected FBL cells with bovine anti-BIV antibodies as previously described (Carpenter et al., 1992). Parallel cultures were stained with antibodies to bovine leukemia virus to confirm the absence of co-infection with bovine leukemia virus (BLV).

Monocyte function assays

Parameters for bovine monocyte function assays were established in initial experiments (data not shown) by varying incubation times and reagent concentrations.

Monocyte phagocytosis was measured by the ingestion of heat-killed (^{125}I) iododeoxyuridine-labelled *Staphylococcus aureus* (Roth et al., 1981a) during a 12-14 hour incubation (overnight) beginning immediately after monocyte isolation. Monocytes were added to 12 x 75 mm polystyrene tubes (Becton-Dickinson Labware, Lincoln Park, NJ), at a concentration of 5.25×10^5 cells in 450 μl M199 (supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS) per sample. Monocytes were allowed to re-adhere for 30 minutes before adding labelled *S. aureus* (at a bacteria to monocyte ratio of 140:1) and bovine anti-*S. aureus*

antibody. Samples were placed in a 37°C humidified 5% CO_2 incubator for 12 to 14 hours. The extracellular bacteria were then lysed with lysostaphin (Sigma Chemical Co., St. Louis, MO), and cells were washed free of debris by centrifugation 2 times at 1250 x g for 10 minutes in cold (4°C) phosphate buffered saline. Radioactivity associated with internalized bacteria was then counted in a gamma counter. All samples were tested in duplicate. Results are reported as the percent specific ingestion of bacteria for the average of the two replicates per sample.

Antibody-dependent cell-mediated cytotoxicity, random migration and chemotactic migration were measured the day after monocyte isolation. Monocytes for ADCC were aliquoted to wells of a 96 well plate (Becton-Dickinson Labware, Lincoln Park, NJ) at 3.5 x 10^5 monocytes per well and allowed to adhere overnight. Target cells used were ^{51}Cr labelled chicken red blood cells (cRBC), in a monocyte to target cell ratio of 3:1. Antibody-dependent cell-mediated cytotoxicity was measured over 6 hours as the percent specific lysis of target cells coated with a subagglutinating dose of bovine antibodies to cRBC (Roth et al., 1981a). All samples were tested in duplicate.

Monocytes for migration assays were held overnight in siliconized glass tubes in a 37°C humidified 5% CO_2 incubator. Monocytes from control and BIV infected calves were assayed for random and chemotactic migration at 1.6 x 10^5 monocytes/chamber in a 48 well blind well chamber (Neuroprobe, Rockville, MD) with 5.0 μm pore polycarbonate filters (Nucleopore, Pleasanton, CA). Cell culture medium (M199) without fetal bovine serum, or 10% zymosan activated bovine serum (serum treated with zymosan to activate complement, Roth et al, 1981a) in culture medium were used as attractants for random or chemotactic migration, respectively. Filters were stained with Diff-Quik (American Scientific Products, Columbia, MD) and the number of monocytes that had migrated through the filter was quantified by counting 10 oil immersion fields on a light microscope. Each sample was tested in duplicate. Results are reported as the average number of monocytes migrated per oil immersion field (1000x) over 2 hours.

Non-specific esterase staining was performed as previously described for bovine macrophages (Osbaldiston et al., 1978; Dhingra et al., 1982).

Interferon treatment

On 5 days during months 7 and 8 post-inoculation (PI), monocytes from each animal were divided into two aliquots. One was treated overnight (12-14 hours, in a 37°C humidified 5% CO_2 incubator) with 200 U/ml bovine recombinant interferon γ (lot AE62, gift of Ciba-Geigy, Basel, Switzerland); the other aliquot served as an untreated control. Antibody-dependent cell-mediated cytotoxicity, random migration and chemotactic migration were assayed the following day as above. Not all assays were done on all animals on all days, as insufficient numbers of monocytes were isolated from some animals on some days.

Monocytes treated in vitro with BIV

BIV virus isolated from monocytes of animal #346 was grown in FBL cell culture and 1.3×10^4 syncytium forming units of virus in FBL cells were inoculated intravenously into a 5 month old, BVD and retrovirus negative calf. BIV was isolated by co-culturing monocytes from this calf with FBL cells every 2 to 4 weeks for 2 years, starting 2 weeks post-inoculation. Virus isolated at 6 months PI at a low passage number (P1) was used to produce stocks of cell-free BIV. Virus cultures were fed daily with uninfected FBL cells, and cell supernatants were removed daily, pooled, and clarified by centrifugation at 850 x g for 30 minutes. Virus supernatants contained 800 syncytium forming units/ml. Control supernatants were simultaneously collected from uninfected FBL cells.

Eleven age-matched juvenile Holstein cattle were determined to be negative for BIV, BLV and BSV by virus isolation from mononuclear cells, as well as by Western blot and agar gel immunodiffusion for antibodies to BIV, or BLV and BSV, respectively. Monocytes were isolated from each animal and divided into 4 aliquots. Each aliquot of monocytes was then treated overnight (12-14 hours in a

37°C humidified 5% CO_2 incubator) with dilutions of either cell-free virus or control supernatants. The multiplicity of infection was 10^{-4} syncytium forming units (SFU) of virus per monocyte for migration and ADCC assays, and 10^{-3} SFU for *Staphylococcus aureus* ingestion. Monocyte random and chemotactic migration, ADCC, and *Staphylococcus aureus* ingestion were then tested.

Statistical analysis

Data comparing BIV to control animals were analyzed using the general linear models procedure of SAS (SAS Institute Inc. Cary, NC). A repeated measures analysis of variance was used to account for the repeated sampling over time. Type I sums of squares were calculated for treatment (BIV or control), animal within treatment, date, treatment by date interaction, and animal by date within treatment. The animal within treatment term was used as the error term for testing treatment effects.

The effects of treatment with interferon γ were tested using a split, split plot analysis of variance. BIV infection status was the whole plot; interferon treatment was used as a subplot, with date treated as a sub-sub plot. Type I sums of squares were used for testing, with animal within treatment as the whole plot error term. Animal by interferon within treatment group was used as the error term for testing interferon effects and the treatment by interferon interaction. Interferon effects were evaluated using only data from days where interferon was used.

The effects of *in vitro* treatment with BIV were analyzed by linear regression, testing for lack of fit, non-zero slope and adequacy of linear model. Samples were blocked by animal.

Results

Virus isolation and clinical signs

BIV was consistently recovered from mononuclear cells except on two occasions (table 1). At 12 and 18 months PI, BIV could not be recovered from

Table 1: BIV isolation from infected animals

Animal	4 - 8 Months PI		9 - 17 Months PI		18 - 24 Months PI	
	Monocyte[a]	MNL[b]	Monocyte	MNL	Monocyte	MNL
340	0/2[c]	3/3	0/4	0/1	2/6	1/2
341	0/2	3/3	0/4	0/1	3/6	1/2
342	0/2	3/3	0/4	1/1	6/6	2/2
346	1/3	3/3				

Virus isolation is summarized as the ratio of positive isolations from mononuclear cells or monocytes divided by the number of times virus isolation was attempted during the time period. BIV could not be isolated from any of the control animals at any time.

[a]Monocytes: BIV isolation from monocytes.
[b]MNL: BIV isolation from unfractionated mononuclear cells.
[c] Number of times BIV was recovered/number of attempts to isolate virus.

mononuclear cells of animals #340 and #341. At these times, BIV was isolated only with difficulty from animal #342, requiring more passages *in vitro* before first detection of virus. At 24 months PI, virus was again isolated from mononuclear cells of all infected animals.

Interestingly, although virus could be recovered from unfractionated mononuclear cells early in infection, virus was not recovered from purified monocytes of most animals until 18 months PI (table 1). After 18 months PI, BIV was recovered consistently from monocytes from animal #342. In animal #340 and #341, virus was isolated from monocytes first sporadically, then later more consistently. With increasing time after infection, syncytia were detected at earlier passages of the FBL cells during co-culture. BIV was not isolated at any time from the control animals.

Calf #346 died during the course of the experiment. This animal developed lymphosarcoma 5 months after BIV inoculation and died at 7 months PI. It is uncertain whether the death of this animal was related to the BIV infection. During the period before its death, this animal had a persistent monocytosis (manuscript in preparation). Virus could be isolated from purified monocytes of this animal much earlier than from the healthy BIV-infected animals. Virus isolation from monocytes from #346 had been previously unsuccessful at 4 months PI; however, monocyte cultures established from this animal during month 7 PI produced infectious BIV. Monocyte functions for animal #346 were not significantly different between this animal and others in his group. Other data from this animal will be described further in a separate paper (4th chapter of dissertation).

All animals except #346 remained clinically normal throughout the observation period. Bovine leukemia virus could not be isolated from any of the BIV infected cattle, including animal #346. In addition, all BIV infected cattle remained seronegative by agar gel immunodiffusion for bovine leukemia virus antibodies.

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Monocyte function

Data for monocyte function were analyzed separately for three time periods. (table 2). The early period includes months 4 to 8 PI, when virus could not be isolated from monocytes but could be isolated from mononuclear cells. During this time, the greatest differences in monocyte function were observed between the BIV infected and control groups (table 2). The second period includes months 9 to 17 PI, when virus isolation was consistently unsuccessful from monocytes and isolated in only 1 of 3 animals from mononuclear cells. Monocyte functions during this time were very similar between the two groups. The final period includes months 18 to 20 PI. At this time, BIV could be isolated from both monocytes and mononuclear cells.

Monocyte functions measured were not' significantly different between the BIV infected and control animals at the $P < 0.05$ level during any of the three time periods. Phagocytic activity (table 2) tended to be reduced ($P = 0.06$) in the BIV group only in the 4 to 8 month period. Chemotactic migration, random migration and ADCC were not significantly different between the two groups during any of the three time periods (table 2).

Interferon treatment effects

Monocytes from the control and BIV infected cattle were also treated with 200 U/ml of recombinant bovine interferon gamma during months 7 and 8 PI. Interferon γ almost completely inhibited random migration and significantly ($P < 0.05$) decreased chemotactic migration (table 3). Interferon γ also significantly ($P < 0.05$) increased ADCC (table 3). Interferon effects on phagocytosis of *S. aureus* by control monocytes were highly variable; therefore, these results are not presented. Treatment with interferon affected monocyte chemotaxis, random migration, and ADCC similarly in both groups, when data were evaluated by a split plot analysis of variance.

Table 2: Monocyte function in BIV infected and control cattle

Assay	4 - 8 Months PI			9 - 17 Months PI			18 - 20 Months PI		
	Control n = 5	BIV n = 4*	Pr > F	Control n = 5	BIV n = 3	Pr > F	Control n = 5	BIV n = 3	Pr > F
S. aureus Ingestion (%)	29	22	.06	30	29	.23	28	28	.74
ADCC (% specific lysis)	34	35	.82	30	32	.51	16	21	.24
Random Migration (monocytes/hpf)	10.3	14.3	.30	5.1	3.4	.35	9.6	6.3	.23
Chemotaxis (monocytes/hpf)	58.6	76.4	.13	29.1	24.2	.26	51.4	42.7	.50

Pr > F: The probability of having a greater F value by chance for the comparison of BIV to control groups.
Abbreviations used: hpf = high power field. ADCC = antibody-dependent cell cytotoxicity. PI = post-inoculation.
n = number of animals in each group
(* n = 4 for months 4 to 7 PI. n = 3 for month 8 PI).

Table 3: The effect of interferon γ on monocyte function assays in control and BIV infected animals

Assay	Controls (n = 5)		BIV (n = 4)	
	No interferon	Interferon γ 200 U/ml	No interferon	Interferon γ 200 U/ml
ADCC (% specific lysis)	$35.9^a \pm 4.2$	$50.6^b \pm 6.0$	$39.3^a \pm 4.6$	$58.2^b \pm 5.2$
Random Migration (monocytes/hpf)	8.3 ± 3.7	0.2 ± 0.1	15.1 ± 7.1	0.6 ± 0.5
Chemotaxis (monocytes/hpf)	$38.3^c \pm 10.5$	$1.8^d \pm 0.4$	$63.2^c \pm 14.1$	$1.4^d \pm 0.6$

[a] Mean ± standard error of the mean.
Values with different superscripts are significantly different at $P < 0.05$.

In vitro treatment of monocytes with BIV

Monocytes treated *in vitro* with supernatants from BIV infected FBL cells ("cell free virus") were significantly different from monocytes treated with uninfected FBL cell supernatants in all functions examined. Cell-free virus significantly ($P < 0.05$) increased random and chemotactic migration and *S. aureus* ingestion, and significantly ($P < 0.05$) decreased ADCC (figure 1). These effects were all dose-dependent and repeatable. Monocyte viability was not, however, affected by treatment with BIV (data not shown). Monocyte supernatants were also able to affect monocyte function after pelleting out virus by centrifugation at 75,000 x g for 16 hours. The absence of infectious virus in this centrifuged supernatant was confirmed by co-cultivation with FBL cells.

BIV infection of FBL cells does result in the formation of syncytia and eventual lysis of some cells. As a control for the effects of FBL cell lysis by BIV,
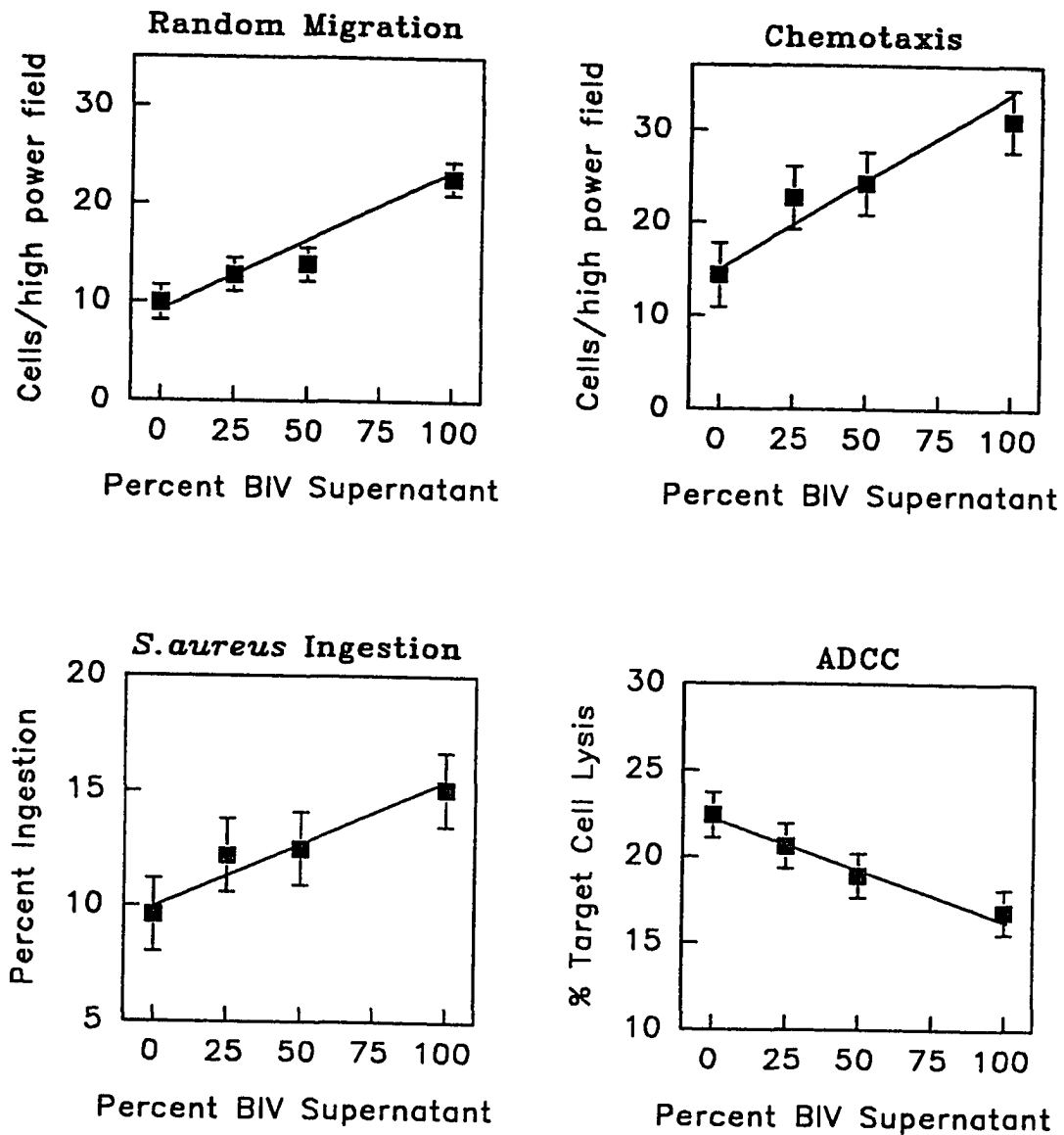
Figure 1: The effects of treatment of monocytes *in vitro* with dilutions of supernatants from BIV-infected FBL cells (percent BIV supernatant). Monocyte migration, *Staphylococcus aureus* ingestion, and ADCC function were measured. Data points for all assays do not show a significant deviation from a straight line, and all lines have a significant non-zero slope (P < 0.01 for ADCC, random and chemotactic migration. P < 0.04 for *Staphylococcus aureus* ingestion.)

uninfected FBL cells were lysed by repeated freeze-thawing. Supernatants collected from these lysed FBL cells were then compared to supernatants from unlysed, uninfected FBL cells. Both random and chemotactic migration were nearly identical between monocytes treated with lysed or unlysed FBL supernatants (data not shown). The effects of cell-free virus did not, therefore, appear to be simply due to FBL cell lysis by virus.

Discussion

In cattle experimentally infected with the R29 isolate, we found no significant effect of infection on monocyte function during the first 2 years PI. Chemotactic and random migration and ADCC were not significantly different between the BIV and control groups at any time (table 2). Phagocytosis tended to be reduced (P = 0.06) in the BIV group early in infection, but became normal after 8 months PI. In addition, treatment with interferon significantly (P < 0.05) decreased migration and increased ADCC of monocytes from both groups of cattle, indicating that the cells from the R29 infected cattle were capable of responding normally to activating cytokines. Nevertheless, when directly treated with virus-containing supernatants *in vitro*, all monocyte functions examined were significantly affected.

Macrophages are important target cells for most lentiviruses. Our experiments support the findings of Onuma et al. (1992) that BIV, like other lentiviruses, infects macrophages. In our experiments, BIV could not be recovered from monocytes until 18 months post-inoculation. The virus isolated from monocytes of animal #346 was inoculated intravenously into another calf. BIV could be consistently isolated from monocytes in this animal starting at 2 weeks PI, for at least the first year after infection. The virus recovered from monocytes did, therefore, appear to retain its ability to productively infect monocytes. These data are suggestive of an expansion of cell tropism in the group of R29 virus infected cattle. Virus recovery by cell type is not, however, available for the earliest periods

of infection. We cannot, therefore, conclusively determine whether the R29 virus has expanded or shifted its tropism during the course of infection. We also cannot exclude the possibility that monocytes were infected either with latent virus or at a low level that could not be detected. Technical difficulties are, however, unlikely to account for the inability to recover virus during this time, as BIV was recovered from monocytes of other BIV-infected animals at the same time (data not shown).

Infection with the R29 isolate of BIV did not appear to produce dramatic impairments in monocyte function during the first 2 years PI. In fact, during the last period monocyte function data were analyzed for the R29 BIV infected cattle, monocytes were producing infectious BIV in culture. Nevertheless, monocyte migration, phagocytosis and ADCC continued to be normal. Monocyte functions have also been found to be normal in the calf inoculated with monocyte-derived virus from animal #346 (data not shown). One possibility is that virus replication might not be high enough in $vivo$ during the first 2 years PI to significantly affect monocyte function. Alternatively, BIV might simply be in too low concentration in the environment of peripheral blood monocytes to affect function, although virus concentrations might be higher in other tissues. In BIV infected cattle, a very low percent of blood leukocytes appears to be infected with the virus (Carpenter et al., 1992). Our experiments treating monocytes in $vitro$ with BIV do support the hypothesis that BIV can affect monocyte function, if virus is in sufficient concentration. In this experiment, supernatants from BIV-infected FBL cells were known to contain 10^{-4} or 10^{-3} infectious virions per monocyte; however, these supernatants are also expected to contain substantial amounts of inactivated or infectious virus. We cannot at this time determine whether the apparent effects of BIV in $vitro$ are due to a direct effect of the virus or possibly to a cytokine or other factor induced by BIV infection of FBL cells.

Onuma et al. (1992) found depressed monocyte phagocytosis on a single occasion in 3 calves infected with BIV. Phagocytosis was depressed in 2 calves

measured at either 15 or 27 weeks PI but not in one calf measured at 45 weeks PI. This is consistent with the tendency that we found for depressed monocyte phagocytosis at 4-8 months PI. Interestingly, this occurred at a time when virus was readily recovered from mononuclear cells. Monocyte phagocytosis did return to normal after this time, concurrent with decreasing ease of virus recovery. The physiologic significance of the transient decrease in phagocytosis found in both experiments is, however, unclear. In addition, further experiments are necessary to resolve the discrepancy between the *in vivo* and *in vitro* effects of virus on phagocytic function.

Onuma et al. (1992) also found significant decreases in monocyte oxidative metabolism and monocyte chemotaxis on one occasion in each infected animal. Although we did not measure the oxidative burst, our data generally show no significant effect of BIV on monocyte chemotaxis in infected animals.

In HIV-1 infection, most monocyte abnormalities appear late in the course of disease. Although decreases in monocyte chemotaxis (Smith et al.,1984; Poli et al., 1985) and ADCC (Bender et al., 1988) have been reported in AIDS patients, these abnormalities do not appear to be present in asymptomatic, HIV-1 infected individuals (Poli et al., 1985; Estevez et al., 1989). Therefore, the absence of functional impairment of monocytes in clinically normal cattle is consistent with studies of monocyte function in HIV-1 infected, asymptomatic individuals. In addition, our studies with *in vitro* treatment of monocytes with BIV support the likelihood that monocyte function might be affected in cattle at times when the virus burden is high, or possibly in environments where virus is actively replicating, possibly lymph node or lung.

A tendency toward transient suppression of monocyte phagocytosis (P = 0.06), therefore, appeared to be the only abnormality in monocyte function seen during the first 2 years after inoculation of cattle with the R29 virus. Nevertheless, it appears that BIV can affect monocyte function when monocytes are inoculated with

virus *in vitro*. The BIV infected cattle are part of a long-term study on immune function and BIV; more dramatic impairments in monocyte function may appear over the course of time, if virus replication increases late in the course of infection.

Acknowledgements

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ENHANCEMENT OF MONOCYTE MIGRATION AND PHAGOCYTOSIS BY THE BOVINE IMMUNODEFICIENCY-LIKE VIRUS GAG PROTEINS

A paper submitted to Cellular Immunology

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ABSTRACT

Supernatants from bovine immunodeficiency-like virus (BIV) infected fetal bovine lung cells have been previously shown to affect monocyte random migration, chemotaxis, phagocytosis, and antibody-dependent cell-mediated cytotoxicity (ADCC) *in vitro* (1). The experiments in this report demonstrate that the BIV Gag (core) proteins can enhance monocyte random migration, chemotaxis and phagocytosis. Supernatants from BIV-infected cells contained 10-30 kDa and 30-50 kDa proteins which significantly ($P < 0.05$) increased monocyte chemotaxis. The 30-50 kDa protein(s) could be cleaved by limited proteolysis into 10-30 kDa active components. Affinity purification with monoclonal anti-p26 (capsid) antibodies yielded preparations which were active in the random migration, chemotaxis, and phagocytosis assays, but did not affect ADCC. Furthermore, the activity of the affinity purified preparation could be specifically neutralized by hyperimmune rabbit serum against the BIV Gag proteins. A recombinant Gag protein, consisting primarily of BIV p26, also enhanced monocyte random and chemotactic migration. It appears, therefore, that direct treatment with affinity purified BIV Gag proteins, or with a recombinant Gag protein, is able to significantly affect the function of normal monocytes *in vitro*. The factors affecting monocyte migration and phagocytosis appear to be one or more breakdown products of the BIV Gag polypeptide precursor, particularly those containing the p26 (capsid) protein.

INTRODUCTION

Bovine immunodeficiency-like virus (BIV) is a lentivirus of cattle that has been postulated to affect immune function. BIV infection, to date, has not been associated with clinical immunodeficiency (1-6); however, experimental infection has been associated with follicular hyperplasia in lymph nodes and spleen (7,8) and subtle changes in numbers and function of neutrophils and T cells (2,3,5). Although the complete cell tropism of BIV has not been reported, there is evidence that monocytes are one of the target cells for BIV (4 and unpublished data).

The related (9) lentiviruses HIV and FIV cause alterations in monocyte function (10-15); such changes may be present in BIV infection, as well. Onuma et al (4) reported decreases in monocyte phagocytosis, migration, and chemiluminescence in a single sample of 3 BIV infected calves. We were able to confirm only a tendency ($P = 0.06$) for decreased phagocytic function in experimentally infected calves followed for 2.5 years post-inoculation (1). *In vitro* experiments, however, raised the possibility that changes in monocyte function were not seen in these animals because titers of circulating virus were not sufficiently high to affect monocyte function. *In vitro* treatment of normal bovine monocytes with crude BIV-containing cell supernatants did result in significant ($P < 0.05$) dose-dependent increases in monocyte random migration, chemotaxis and phagocytosis, and decreases in antibody-dependent cell-mediated cytotoxicity (1).

Further experiments were necessary to determine whether the factor(s) affecting monocyte function in these crude preparations were viral components or virus-induced cellular molecules. We have previously reported that infection of monocytes was not required for changes in cell function (1). This report further characterizes the active factor in the BIV-containing cell supernatants, and the evidence that this factor is one of the BIV Gag (core) proteins.

MATERIALS AND METHODS

Experimental Animals

Eleven age-matched young adult (1-2 year old) male Holstein cattle were tested and found to be negative for BIV, bovine leukemia virus (BLV) and bovine syncytial virus (BSV) by virus isolation from mononuclear cells, and by Western blot or agar gel immunodiffusion for antibodies to BIV, BLV and BSV (1,5,7). Monocytes from these animals were isolated in single cell suspension by adherence to tissue culture flasks and recovery with phosphate buffered saline (PBS, pH 7.2), as previously described (1). Monocytes isolated from each animal were divided into several aliquots. Each aliquot was treated overnight (12-14 hours) with various BIV or control preparations diluted in tissue culture medium. Tissue culture medium used for all experiments was medium M199 (GIBCO Laboratories, Chagrin Falls, OH) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Monocyte random migration, chemotactic migration, antibody-dependent cell-mediated cytotoxicity (ADCC) or *Staphylococcus aureus* ingestion were then tested.

Monocyte Function Assays

Random and chemotactic migration, antibody-dependent cell-mediated cytotoxicity (ADCC), and *Staphylococcus aureus* ingestion were performed as previously described (1), with minor modifications in cell numbers or incubation times.

Briefly, random and chemotactic migration were performed in blind well chambers, with 5.0×10^4 monocytes/chamber. Cell culture medium or 10% zymosan activated serum (bovine serum treated with zymosan to activate complement) in culture medium were used as attractants for random or chemotactic migration, respectively. Filters were stained with Diff-Quik (American Scientific Products, Columbia, MD) and the number of monocytes that had migrated through the filter was quantified by counting 2 oil immersion fields on a light microscope. Each

sample was tested in duplicate. Results are reported as the average number of monocytes migrated per oil immersion field (1000x) during 90 minutes.

Monocyte phagocytosis was measured as the percent specific ingestion of heat-killed (^{125}I) iododeoxyuridine-labelled *Staphylococcus aureus* (16) during a 6 hour incubation at 37°C (1). Each sample contained 7.0 x 10^5 monocytes in 550 μl M199, *S. aureus* (at a bacteria to monocyte ratio of 100:1) and bovine anti-*S. aureus* antibodies. After incubation, extracellular bacteria were lysed with lysostaphin (Sigma Chemical Co., St. Louis, MO), and cells were washed free of debris by centrifugation in cold phosphate buffered saline. Results are reported as the percent specific ingestion of bacteria for the average of the two replicates per sample.

Antibody-dependent cell-mediated cytotoxicity was measured over 6 hours as the percent specific lysis of ^{51}Cr-labelled chicken red blood cells (cRBC) coated with a subagglutinating dose of bovine antibodies to cRBC (1,16). All samples were tested in duplicate.

Virus Source

The BIV virus used in all experiments was obtained from monocytes of a healthy, persistently infected animal (#808), 2.5 years after inoculation with the R29 isolate of BIV (1,17). Fetal bovine lung (FBL) cells were infected by co-cultivation with monocytes isolated from the infected animal, as previously described (1). These FBL/virus cultures were then used to produce stocks of cell-free BIV. Virus cultures were fed daily with uninfected FBL cells, and cell supernatants were removed daily, pooled, and clarified by centrifugation at 850 x *g* for 30 minutes. Virus supernatants contained 1-6 x 10^3 syncytium forming units/ml of BIV. Control supernatants were simultaneously collected from uninfected FBL cells.

Virus identity was confirmed by indirect fluorescent antibody staining of infected FBL cells with bovine anti-BIV antibodies as previously described (7). Parallel cultures were stained with antibodies to bovine leukemia virus, bovine syncytial virus and bovine virus diarrhea virus to confirm the absence of co-infection

with these viruses.

Recombinant Gag

Recombinant Gag3 and control (trpE) protein preparations were a gift of Dr. Jeffrey Isaacson. The 65 kDa Gag3 protein (corresponding to nucleotides 631-1442 of the R29 isolate of BIV) consists of the p26 capsid protein and small flanking regions, expressed as a trpE fusion protein (18,20,21). The Gag3 protein preparation is partially purified from *Escherichia coli* cells by salt precipitation (21). The trpE control is a similar protein preparation from *E. coli* cells (transfected with the pATH vector) which express the 37 kDa trpE protein (21).

Anti-Gag Antibodies

The production of a monoclonal antibody (#104) specific for the BIV p26 (capsid) protein has been previously described (18). To isolate large quantities of antibody, hybridoma cells were grown in cell culture in roller bottles (19), and proteins in the culture medium were concentrated 32x with a 10 kDa filter (Amicon Inc., Beverly, MA). Concentrated monoclonal antibodies were bound to protein G-coated agarose beads (Sigma Chemical Co., St. Louis, MO) by incubation for 2 hours at 37°C with rocking, then washed 6 times with PBS (pH 7.2), to remove non-adherent proteins. The protein G beads contained recombinant protein G which does not bind albumin, cell membranes, or Fab fragments (Sigma #P7700).

Rabbits were also immunized with the recombinant Gag fusion protein (Gag3), as previously described (20). High titered serum (reactive to Gag at 1:500 on Western blot) was heat inactivated at 56°C for 30 minutes to inactivate complement. Control sera were obtained from rabbits immunized with the control (trpE) antigen preparation and heat inactivated. For some studies, rabbit antibody was bound to protein G beads, similarly to monoclonal antibodies.

Purification of Supernatants

BIV-containing and control supernatants were size fractionated using Centriprep filter concentrators (Amicon Inc., Beverly, MA). These concentrators retain molecules of approximately 10 kDa, 30 kDa, 50 kDa and 100 kDa. The 10-30 kDa and 30-50 kDa fractions were further purified with monoclonal anti-p26 antibodies or polyclonal anti-Gag serum. Fractionated supernatants were incubated with antibody-coated protein G beads for 2 hours at 37°C, then overnight at 4°C. The beads were washed 6 times with PBS (pH 7.2), then incubated in PBS (pH 2) to separate antigens from antibodies. After 1 hour at room temperature, antibodies and large proteins were separated from the solution with Centriprep filter concentrators and the pH was readjusted to 7. Proteins greater than 50 kDa in molecular mass were discarded; proteins of approximately 10-30 kDa or 30-50 kDa were retained. The exact protein concentration of all preparations was not tested; however, it was estimated that most antibody purified proteins were used at approximately 0.1 μg/ ml (approximately 0.05 μg per 10^6 monocytes).

Neutralization by Rabbit Serum

In neutralization studies, monoclonal antibody purified 30-50 Kda BIV or control supernatants (each purified from equal volumes of supernatant, approximately 4 - 6 ml) were incubated with anti-Gag or control rabbit serum for 45 minutes at 37°C. PBS (pH 7.2) washed protein G-agarose beads were then added (19 mg per treatment) and the preparations were incubated for an additional hour at 37°C. The beads, with associated antibodies and bound antigen, were removed from the supernatants by centrifugation at 400 x g for 5 minutes. The supernatants were filter sterilized with a 0.2 μm syringe filter (Gelman Sciences, Ann Arbor, MI) and diluted with tissue culture medium to 4 ml.

Heat Treatment

BIV or control supernatants were incubated at 4°C, 20°C, 37°C, 60°C or 100°C for 30 minutes. To prevent denaturation of essential proteins in the tissue culture medium, concentrated BIV or control preparations were first incubated at each temperature, then diluted with fresh tissue culture medium.

Protease and RNase Treatment

The 30-50 kDa fractions from BIV-containing or control supernatants were incubated for varying lengths of time at 37°C with protease coated agarose beads (Sigma Chemical Co., St. Louis, MO). Protease coated bead preparations consisted of 6 units of trypsin, 6 units of chymotrypsin, and 2-6 units of a nonspecific bacterial protease (*Streptomyces griseus*) for most experiments. Some preparations were treated with 15 units of bacterial protease to confirm the results obtained with a combination of proteases. BIV or control supernatants were treated in parallel with RNase coated agarose beads (Sigma Chemical Co., St. Louis, MO), as a control for exposure to agarose. After incubation, the beads were removed from the preparations by centrifugation at 400 x g for 5 minutes. The supernatants were then filter sterilized with a 0.2 µm syringe filter, and diluted with tissue culture medium. Coomassie blue staining of denaturing SDS-PAGE gels demonstrated a number of additional smaller molecular mass bands in the protease treated but not RNase treated preparations.

Statistical Analysis

Data comparing BIV to control animals were analyzed using the general linear models procedure of SAS (SAS Institute Inc. Cary, NC). Data were blocked by animal, and Type I sums of squares were calculated for treatment (BIV or control), block and error. Heat treatment data were analyzed by linear regression, testing for lack of fit, non-zero slope and adequacy of a linear model. Samples were blocked by animal.

RESULTS

Physical Characterization - Size, Heat Resistance, and Protease Susceptibility.

For the initial characterization of the active factor(s), bioactivity was measured as the ability to significantly (P < 0.05) enhance monocyte chemotaxis. BIV and control supernatants were fractionated into approximate size ranges of 10-30 kDa, 30-50 kDa, 50-100 kDa, and greater than 100 kDa. Both the 10-30 kDa and 30-50 kDa fractions of the BIV supernatants significantly (P<0.05) increased monocyte chemotaxis (Table 1). Fractions greater than 50 kDa did not increase chemotaxis. The 30-50 kDa chemotaxis enhancing factor was determined to be a protein: complete protease digestion (6 hours at 37°C) destroyed the activity (data not shown). The activity was heat stable to 60°C for 30 minutes, but destroyed by boiling (means: 63-65 cells/hpf for control supernatant at 37°C or 100°C and BIV supernatant at 100°C; 80-92 cells/hpf for BIV supernatant at 4°C, 20°C, 37°C, and

Table 1: Chemotaxis of monocytes after treatment with size-fractionated control and BIV supernatants.

Size	Mean (monocytes/hpf)			
	Control	BIV	SEM	Pr > F
10-30 kDa	56	65	1.6	0.005
30-50 kDa	23	32	1.8	0.02
50-100 kDa	44	44	6.2	0.98
Greater than 100 kDa	64	55	5.3	0.28

Monocytes were treated with size fractionated BIV and control supernatants, and the effect on chemotaxis was measured. Migration data from different cell fractions cannot be directly compared, as not all fractions were measured on the same date and this assay has high variability between different dates (n = 7-9 animals). Values are representative of multiple experiments. Abbreviations used: hpf = high power field (1000X). SEM = standard error of the mean. Pr > F = the probability of having a greater F value by chance for the comparison of each BIV treatment to its control.

60°C; means for all BIV supernatants at temperatures except 100°C are significantly different from the control values at P < 0.05 and fit a straight line with a slope of zero).

Limited proteolysis (37°C for 30 minutes), did not destroy activity; however, it altered the size of the active component. BIV and control 30-50 kDa preparations were treated with either protease or RNase beads, then separated into 10-30 kDa and 30-50 kDa fractions. Before size separation, both RNase and protease treated supernatants retained activity (Table 2). The enhancement of chemotaxis, with either preparation, was similar to increases mediated by non-enzyme treated BIV

Table 2: Limited proteolysis, but not treatment with RNase, reduces the size of the active factor in BIV supernatants.

	Whole 30-50 kDa supernatants	Supernatants after separation by size	
		10-30 kDa	30-50 kDa
	Mean ± SEM	Mean ± SEM	Mean ± SEM
RNase treated supernatants			
Control (cells/hpf)	43 ± 2.0	38 ± 1.8	48 ± 2.1
BIV (cells/hpf)	52 ± 2.0*	34 ± 1.8	59 ± 2.1*
Protease treated supernatants			
Control (cells/hpf)	49 ± 2.7	34 ± 4.2	51 ± 1.5
BIV (cells/hpf)	59 ± 2.7*	50 ± 4.2*	52 ± 1.5

30-50 kDa BIV and control supernatants were treated with protease or RNase for 30 minutes at 37°C; after removal of enzyme-coated beads, some preparations were separated into 10-30 kDa and 30-50 kDa fractions. Monocyte chemotaxis was tested on both the RNase or protease treated, whole supernatants and on these supernatants after separation into 10-30 kDa and 30-50 kDa fractions. Values with asterisks (*) are significantly different from their control at P < 0.05 (n = 5-9 animals). Values cannot be compared across columns, as some preparations were tested on different dates. Abbreviations used: hpf = high power field (1000X). SEM = standard error of the mean.

supernatants. After size fractionation, however, the active component was found to be 10-30 kDa, and not 30-50 kDa, in the protease treated BIV preparation. The activity was retained in the 30-50 kDa fraction in the RNase treated preparation.

Immunologic Characterization

Preliminary data indicated that the factor affecting chemotaxis could be neutralized by serum from a steer immunized with killed BIV virus, sera from some naturally infected animals, or polyclonal rabbit serum against the BIV Gag (core) proteins (data not shown). It, therefore, appeared to be a viral and not a cellular component. In particular, it appeared to be one of the BIV Gag proteins. The BIV Gag proteins are synthesized as a 53 kDa polypeptide precursor (22). One potential candidate, found in FBL cell supernatants, is the p26 (capsid) protein. Other putative cleavage products of the 53 kDa Gag precursor protein include numerous 10-49 kDa proteins (22); all of the Gag proteins except p53 fall into the size range containing the component active on chemotaxis.

To determine whether the active factor might, in fact, be one of the Gag proteins, antigens were isolated from 30-50 kDa BIV and control supernatants by binding to anti-Gag antibodies on protein G beads. Serum was obtained from rabbits immunized with a recombinant Gag-trpE fusion protein (mainly containing the p26 protein) and from control rabbits immunized with the control (trpE) preparation. Protein G beads were coated with either control (anti-trpE) or anti-Gag antibodies; 30-50 kDa BIV and control supernatants were then incubated with the antibody coated beads. Bound antigens were isolated from the beads by a pH change and separated from whole antibodies by size fractionation. Both rabbit polyclonal preparations (anti-trpE and anti-Gag) were incubated with control and BIV supernatants. Only antigens isolated from the BIV preparations with anti-Gag serum were able to enhance monocyte chemotaxis (mean ± SEM: 26-30 ± 1.9 cells/hpf, for control antigens isolated with control or anti-Gag antibodies; 57 ± 3.7 cells/hpf, for BIV antigens isolated with anti-Gag antibodies; $Pr > F = 0.005$).

Antigens from control supernatants, or from BIV preparations incubated with anti-trpE serum, did not increase monocyte chemotaxis. Similarly, affinity purification of either 10-30 kDa or 30-50 kDa BIV fractions with anti-p26 monoclonal antibody coated beads resulted in preparations which also significantly (P<0.05) increased monocyte chemotaxis (data not shown).

The effect of these antigens could be neutralized with Gag-specific serum. Antigens were first affinity purified from the 30-50 kDa BIV fractions with monoclonal anti-p26 antibody coated beads. These antigens were then neutralized by treatment with rabbit anti-Gag serum. In initial studies, heat inactivated serum from the rabbits immunized with Gag, but not the control rabbits, was able to neutralize the effect of the affinity purified antigens on monocyte chemotaxis (data not shown). To control for the possibility of immune complexes acting upon the monocyte to suppress migration, this technique was then modified slightly: affinity purified antigens were incubated with rabbit serum, then allowed to react with protein G coated agarose beads. After the beads, associated immunoglobulins, and any bound antigen were removed, the remaining preparations were tested on monocyte chemotaxis. Affinity purified antigens were neutralized by treatment with rabbit anti-Gag serum (mean $\pm$ SEM: 60 $\pm$ 2.3 cells/hpf for controls; 59 $\pm$ 2.3 cells/hpf for BIV antigens; Pr > F = 0.83). BIV antigens were not, however, affected by treatment with control serum (mean $\pm$ SEM: 60 $\pm$ 1.4 cells/hpf for controls; 69 $\pm$ 1.4 cells/hpf for BIV antigens; Pr > F = 0.001).

Finally, monocytes were treated with a recombinant Gag fusion protein. This protein contains the BIV p26 protein and small flanking regions on both ends of this protein, expressed as a trpE fusion protein. Control preparations were treated in parallel with the trpE protein. Treatment of monocytes with a minimum of 0.5 μg/ml recombinant Gag resulted in a significant (P < 0.05) increase in monocyte chemotaxis, as well as random migration (Table 3). Viability of cells treated with the recombinant Gag fusion protein or the control (trpE) preparation were not significantly different from each other.

Table 3: The effect of 1 μg/ml recombinant Gag on random migration and chemotaxis.

	Control Mean[a] $\pm$ SEM	Recombinant Gag Mean $\pm$ SEM	Pr>F
Chemotaxis	33 $\pm$ 1.6	44 $\pm$ 1.6	0.002
Random migration	14 $\pm$ 1.4	20 $\pm$ 1.4	0.03

Monocyte migration assays were tested after treatment with 0.1 μg/ml Gag3 (approximately 0.5 μg Gag3 per 10^6 monocytes). Mean[a] = monocytes/ high power field (n = 10 animals). Abbreviations used: SEM = standard error of the mean. Pr > F = The probability of having a greater F value by chance for the comparison of BIV to control groups.

Other Assays

Monocyte random migration, *Staphylococcus aureus* ingestion, and ADCC assays were also tested. Monocytes were treated overnight (12-14 hours) with affinity purified antigens isolated with the monoclonal anti-p26 antibody (Table 4). Random migration and *Staphylococcus aureus* ingestion were significantly (P < 0.05) enhanced by treatment with the affinity purified antigens. ADCC activity was not, however, affected, by either the affinity purified antigens (Table 4) or by recombinant Gag protein at 1 μg/ml (data not shown).

DISCUSSION

From these experiments, it appears that proteolytic cleavage products of the BIV Gag precursor are able to affect monocyte function *in vitro*. The Gag proteins appear to be at least one of the active factors, previously identified in BIV-infected cell supernatants, which enhance monocyte migration and phagocytosis (1). Antigens isolated by binding to either a monoclonal antibody specific for p26, or to rabbit polyclonal anti-Gag serum, significantly (P < 0.05) enhanced monocyte chemotaxis,

Table 4: The effect of core proteins isolated with monoclonal anti-p26 antibody on monocyte ADCC, *Staphylococcus aureus* ingestion, and random migration.

Assay	Control Mean ± SEM	BIV Mean ± SEM	Pr>F
Random migration (monocytes/hpf)	24 ± 0.9	31 ± 0.9	0.001
Staphylococcus aureus ingestion (percent ingestion)	41% ± 1.1	44% ± 1.1	0.02
ADCC (percent target cell lysis)	17% ± 1.0	19% ± 1.0	0.21

Monocytes were treated with approximately 0.1 μg/ml monoclonal antibody purified Gag (approximately 0.05 μg per 10^6 monocytes for random migration and ADCC, and 0.005 μg per 10^6 monocytes for *S. aureus* ingestion. Abbreviations used: ADCC = antibody-dependent cell-mediated cytotoxicity. hpf = high power field (1000X). SEM = standard error of the mean. Pr > F = the probability of having a greater F value by chance for the comparison of BIV to control groups (n = 10 animals).

random migration and *Staphylococcus aureus* ingestion. The antigens isolated from the monoclonal antibody preparations could be specifically neutralized by rabbit anti-Gag serum. Finally, a recombinant Gag fusion protein, containing the p26 protein, enhanced monocyte chemotaxis and random migration similarly to the molecule(s) isolated from infected cell supernatants. From the latter data, it appears that the active component is contained either within or near the p26 protein. A second, as yet uncharacterized, factor also appears to be present within the BIV supernatants, as monocyte ADCC was suppressed by supernatants from BIV infected cells (1), but was not affected by the BIV Gag proteins.

The 53 kDa Gag precursor is cleaved into p26 capsid, p14 nucleocapsid, and p17 matrix proteins (9,22,23). Proteolytic processing of these components is poorly understood; it appears, however, that multiple breakdown products, including

products of sizes ranging from 10 kDa to 49 kDa are produced (22). In our experiments, whole virus (in the >100 kDa fraction) did not appear to affect monocyte function. This is not surprising, as the Gag proteins are contained within the virion and would not be expected to contact monocytes. The Gag proteins can, however, be detected in FBL cells (data not shown), and Gag proteins may be released into tissues or cell cultures during cell lysis. They may also be present after degradation of virus particles. The active factor in the 10-30 kDa fraction is most likely to be the p26 protein; however, this fraction could also contain a number of other proteolytic cleavage products of Gag, some of which contain portions of p26. The 30-50 kDa fraction may contain active p32, p37, p39, p42 and/or p49 proteins, which are known or predicted breakdown products of p53 (22). It is likely that, during limited proteolysis, some of these proteins are broken down into smaller fragments similar to those found in the 10-30 kDa cell supernatant fraction. It is also possible that the 10-30 kDa and 30-50 kDa fractions contain some of the same (approximately 30 kD) proteins, as size fractionation by Centriprep concentrators is not exact.

While it may at first appear surprising that the Gag protein is able to affect monocyte function, it is not unprecedented. Recently, the related (9) HIV-1 capsid protein has been shown to affect monocyte function and lymphocyte blastogenesis (24-26). Treatment with HIV p24 appears to inhibit the induction of lymphocyte responses to antigens (24,25) and alter the monocyte response to interferon gamma (26). The blastogenesis inhibiting activity has been traced to a fragment of the HIV p24 protein containing the conserved 7 amino acid sequence RGSDIAG (25). BIV-R29 does not contain this particular amino acid sequence within its p26 protein (27); however, a relatively conserved amino acid sequence, KTDEIIG, is present at a similar position in the BIV p26 protein (amino acids 239-246 of BIV) (27). It is not yet known whether this or other BIV peptides are able to affect monocyte function.

The mechanism for the effect of the BIV Gag proteins on migration and

phagocytosis is not known. It is likely to be a receptor mediated event, as low concentrations of Gag are able to affect monocyte function, and the lentiviral Gag proteins are not hydrophobic. One possibility is that Gag mimics a normal cell ligand. Potential mechanisms for the enhancement of migration and phagocytosis include cytokine induction from monocytes by Gag, or a direct alteration in signal transduction leading to a change in the monocyte intracellular environment.

In previous experiments, it had been found that monocyte function in infected animals was relatively normal (1); however, this may have been a result of low levels of virus proteins in the blood. The few reported abnormalities seemed to occur during the early stages of infection (1,4), when virus production appeared to be at its highest levels (1). These *in vivo* abnormalities may, however, be a result of virus induced alterations in cytokine levels, or other *in vivo* interactions; the alterations in monocyte function reported *in vivo* (a suppression in migration and phagocytosis (4) or tendency for decreased phagocytosis (1)) are opposite to those found when monocytes are treated directly with BIV *in vitro*.

Although circulating monocytes from infected animals do not appear to be greatly affected (1), and virus levels in the blood appear to be low (7), Gag protein production at local sites may be high enough to affect the function of tissue macrophages. *In vitro* treatment with recombinant Gag at concentrations of at least 0.5 μg/ml results in monocyte functional alterations; such levels might be reached in areas of high virus production. The BIV p26 protein has, in fact, been found in lymph node follicles by immunohistochemical staining (28). In localized areas, therefore, BIV proteins may result in alterations in monocyte function. Although there are no published reports of clinical immunodeficiency proven to be caused by BIV infection, it is possible that alterations in macrophage function are present within the tissues but result in only subtle changes in immunity. It is also not certain that the relatively minor changes in monocyte function caused by Gag would cause significant clinical abnormalities. They may, however, enhance immunodeficiency caused by stress or other microorganisms. At present, it is not clear whether BIV

causes immunodeficiency, as is suggested by the previous reports of BIV suppression of ADCC *in vitro* (1) and several monocyte functions *in vivo* (1,4); or whether BIV enhances monocyte function, as suggested by the results reported in this paper. It appears that BIV has (or induces from infected cells) at least two components that affect monocyte function. One appears to enhance functions such as phagocytosis and migration; the other appears to suppress ADCC.

Finally, the finding that BIV Gag is able to affect monocyte functions, coupled with the presence of BIV p26 protein in lymph nodes (28) and the inhibition of lymphocyte blastogenesis by HIV p24, raises the possibility that BIV p26 might affect antigen presentation by macrophages. In both BIV and HIV-1 infections, reactivity to the Gag protein is lost during the course of infection (20). In contrast to HIV, BIV virus production is not likely to be high enough to induce the formation of immune complexes; therefore, other mechanisms are more likely to result in the loss of anti-Gag antibodies (20). One intriguing hypothesis is that the BIV p26 protein, like HIV p24, might inhibit the induction of antigen specific responses, including the response to p26 itself. As BIV p26 is able to affect other monocyte functions, it is interesting to speculate that this could take place by inhibiting the antigen presentation ability of macrophages in the lymph nodes.

Until recently, it appeared that the lentiviral core proteins merely served a structural function. Now, however, it seems that BIV p26, like HIV p24 (24-26), may be involved in viral modulation of immune function. In addition to its effects on monocyte migration, ADCC, and phagocytosis, there is the potential for its involvement in antigen presentation defects and the loss of reactivity to p26 in infected animals. These latter effects are speculative, yet they provide intriguing ground for further research. The Gag proteins are one of the major proteins in infected cells and lentiviral virions (23,29-31); it is likely that they play a greater role in lentivirus infection than previously suspected.

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ACKNOWLEDGEMENTS

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AN ATYPICAL T CELL LYMPHOSARCOMA IN A CALF WITH BOVINE IMMUNODEFICIENCY-LIKE VIRUS INFECTION

A paper submitted to Veterinary Pathology

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Abstract

This paper describes a case of T cell lymphosarcoma in an 11 month old Holstein calf experimentally infected with bovine immunodeficiency-like virus (BIV). Lymphosarcoma developed at 5 months post-inoculation, concurrent with progressive monocytosis. Tumors were found in the thymus, multiple lymph nodes, and brain. Tumor cells were $CD2^+$ $CD4^-$ $CD8^-$ T cells. Infectious BIV could be recovered from splenic tissue and blood mononuclear cells. Bovine leukemia virus was not present. Because this calf was part of an ongoing experiment on the pathogenesis of BIV infection, immune function data were also available both before and after lymphosarcoma developed. Neutrophil and monocyte function were normal, but lymphocyte blastogenesis was enhanced before the development of lymphosarcoma. Follicular hyperplasia in lymphoid tissues was also seen. This case raises the possibility that BIV infection may cause or be associated with some cases of atypical T cell lymphosarcoma, without evidence of immune suppression at the time of tumor onset.

Brief Communication

Bovine immunodeficiency-like virus (BIV) is a lentivirus of cattle that shares some characteristics with the immunodeficiency-causing lentiviruses: human immunodeficiency virus-1 (HIV-1), feline immunodeficiency virus (FIV) and simian

immunodeficiency virus (SIV).[7] Lentivirus-associated lymphomas have been recognized in humans, non-human primates, and cats.[1,4] In cattle, there is a clear association of bovine leukemia virus (BLV), an oncogenic retrovirus, with multicentric lymphosarcoma in adult cattle; however, no tumors have been described in conjunction with the lentivirus BIV. An increased incidence of lymphosarcoma has been observed in some herds with BIV; however, these herds were also reported to be infected with BLV.[10,12] In this report, we describe a case of multicentric T cell lymphosarcoma in an 11 month old calf experimentally infected with BIV, but known to be negative for bovine leukemia virus.

A male Holstein calf (animal #346), 6 months of age, was inoculated intravenously with 1.8×10^4 syncytium forming units of the R29 isolate of BIV. This animal was part of a long term study on the pathogenesis of BIV in experimentally infected cattle. The details of experimental inoculation, control animals, and methods for clinical evaluation have been previously described.[2,6] Briefly, this animal was evaluated twice each month, starting at 4 months postinoculation (PI), for total and differential white blood cell counts, neutrophil function, monocyte function, mononuclear cell subset analysis (CD4, CD8, CD6, CD2, and markers for B cells, monocytes, and $\gamma\delta$ T cells) and lymphocyte blastogenesis to mitogens. These assays have been previously described.[3,6,8] Lymphocyte blastogenesis data was available only to day 115 PI. The presence of infectious BIV in cells and tissue samples was detected by co-cultivation with fetal bovine lung (FBL) cells. [2,8] Virus isolation from peripheral blood mononuclear cells (PBMC) was attempted at 2, 6, 8, 16, 18, 27 and 29 weeks PI, and virus isolation from monocytes was attempted three times, at 17, 18 and 29 weeks PI.

At 23 weeks (160 days) following inoculation with BIV, systemic enlargement of lymph nodes was observed in animal #346. A diagnosis of lymphosarcoma was made by lymph node biopsy. Prior to the detection of the tumors, an absolute monocytosis was detected by flow cytometry, and confirmed by non-specific esterase staining. Monocyte numbers progressively increased from day 112 to day 190, at

which date 55% of mononuclear cells were monocytes. The calf's condition deteriorated over the next month, and the calf died at 29 weeks PI (day 203). Interestingly, BIV was detected in purified monocytes isolated from whole blood collected on the day of death, but not on the 2 previous attempts. BIV had been, however, consistently isolated from peripheral blood mononuclear cells (PBMC) at each attempt following infection. PBMC were negative for BLV and bovine syncytial virus (BSV) at all times prior to and following experimental infection with BIV.

At post mortem examination, a gray-white subcutaneous mass approximately 15 x 15 x 20 cm was present in the caudoventral cervical region. The mass extended through the thoracic inlet into the thoracic cavity for a distance of 4 cm. Upon incision, bands of white fibrous connective tissue divided the mass into irregular lobules. Multiple foci of caseous necrosis, or necrosis with hemorrhage, up to 3 cm in diameter were randomly distributed within the tumor. Dorsally the mass was concave and caused compression of the trachea and esophagus. Carotid arteries in the caudal cervical region were embedded within the mass, and jugular veins were compressed laterally. This mass was presumed to be a thymic tumor.

The thorax contained approximately 5 liters of clear amber fluid and a few fine strands of fibrin. Paired lymph nodes, including parotid, mandibular, retropharyngeal, superficial cervical, and superficial inguinal nodes, were symmetrically enlarged to 5 - 10 times normal size. Thoracic and abdominal nodes ranged from normal size and appearance to 20 times normal size for some mesenteric nodes. Variable enlargement and pallor of hemal nodes was also found in the subcutis, thorax and abdomen. A cluster of enlarged hemal nodes occurred in the subiliac region. Plaques of gray-white tissue were found in the frontal sinuses and on the ventrorostral dura mater extending caudally to the hypothalamic area.

Small areas of atelectasis were present in anteroventral lung lobes. Liver, spleen, and bone marrow were unremarkable.

Microscopically, the thymic mass was composed of sheets of lymphoid cells with slight to moderate amounts of lightly eosinophilic cytoplasm (Fig. 1). Cytoplasmic borders were usually indistinct in compact areas, and the cells were round to oval with distinct cytoplasmic borders at the periphery where invasion of adipose tissue occurred. The majority of cells were 8 to 10 micrometers in diameter and had round to oval, darkly staining nuclei with clumped chromatin. Mitoses were variable and ranged from 0 to 6 per high power field. Fibrous connective tissue stroma varied from fine trabeculae to thick bands. Foci of necrosis and hemorrhage were irregularly distributed in the mass. Tumorous lymph nodes (Fig. 2) and hemal nodes were composed of diffuse sheets of large lymphocytes comparable to those in the thymic mass. Similar neoplastic cells were in the mucosa of the frontal sinus and cerebral meninges. Residual pituitary acidophils were identifiable among the neoplastic lymphoid cells in part of the meningeal mass. Follicular hyperplasia was common in lymph nodes and hemal nodes not effaced by tumor and in other lymphoid tissues including spleen, bronchus-associated lymphoid tissue, and gut-associated lymphoid tissue. Subepithelial lymphoid follicular hyperplasia causing obstruction of bronchioles was found in areas of pulmonary atelectasis.

Tumor samples collected at necropsy were analyzed by flow cytometry and the predominant cell type present in lymph node tumors was phenotypically identified as a $CD2^+$ $CD4^-$ $CD8^-$ lymphocyte. Very few cells with this phenotype were found in the blood, indicating the absence of leukemia with this lymphosarcoma. Circulating atypical lymphocytes were not detected by microscopic examination.

Various tissue samples collected at necropsy were analyzed for infectious BIV and BLV by co-cultivation of tissue homogenates with FBL cells. BIV was recovered from the spleen; all other tissues were negative for both BIV and BLV.

Bovine lymphosarcomas are generally classified into 4 groups: multicentric

Figure 1.   Thymic mass. Sheets of neoplastic lymphocytes subdivided
by fine to moderate bands of dense fibrous connective
tissue. Harris' hematoxylin and eosin stain. Bar = 240 μm.
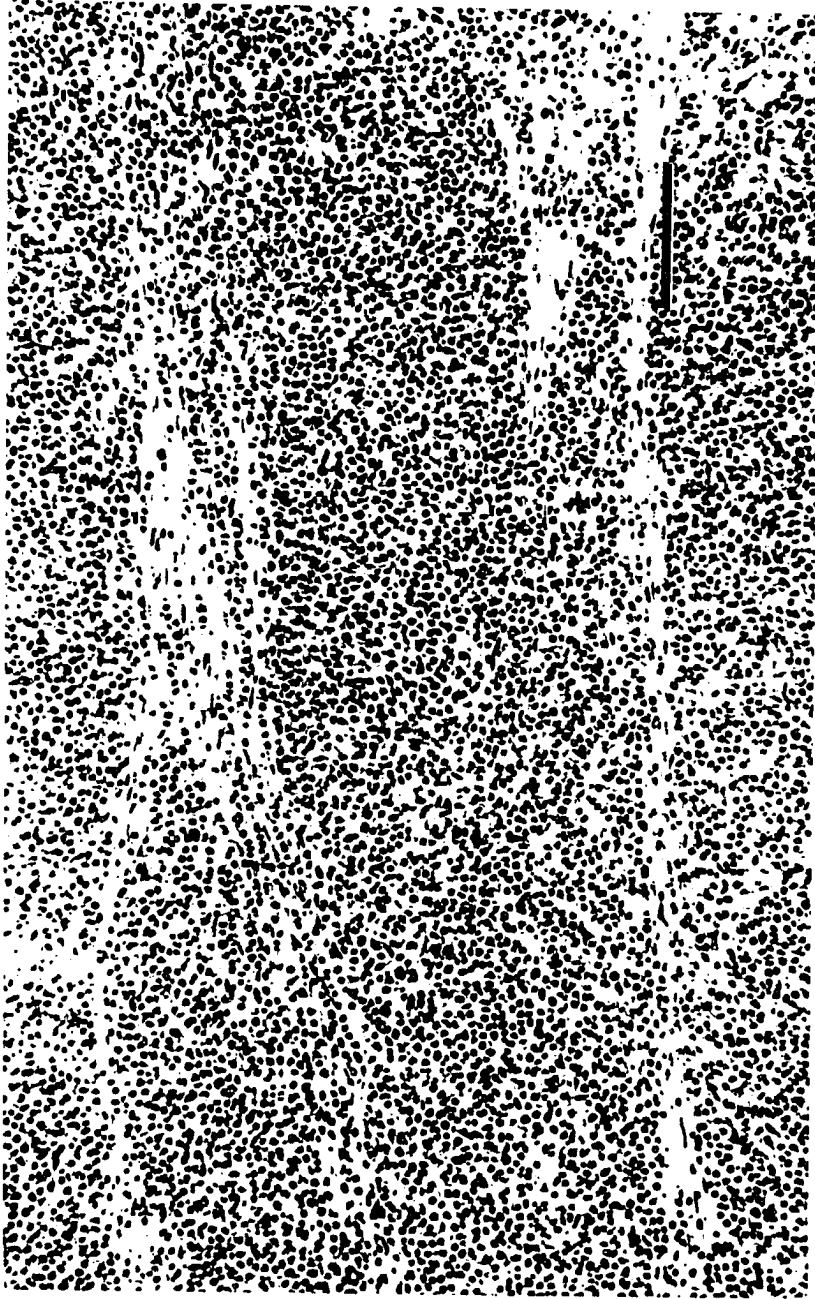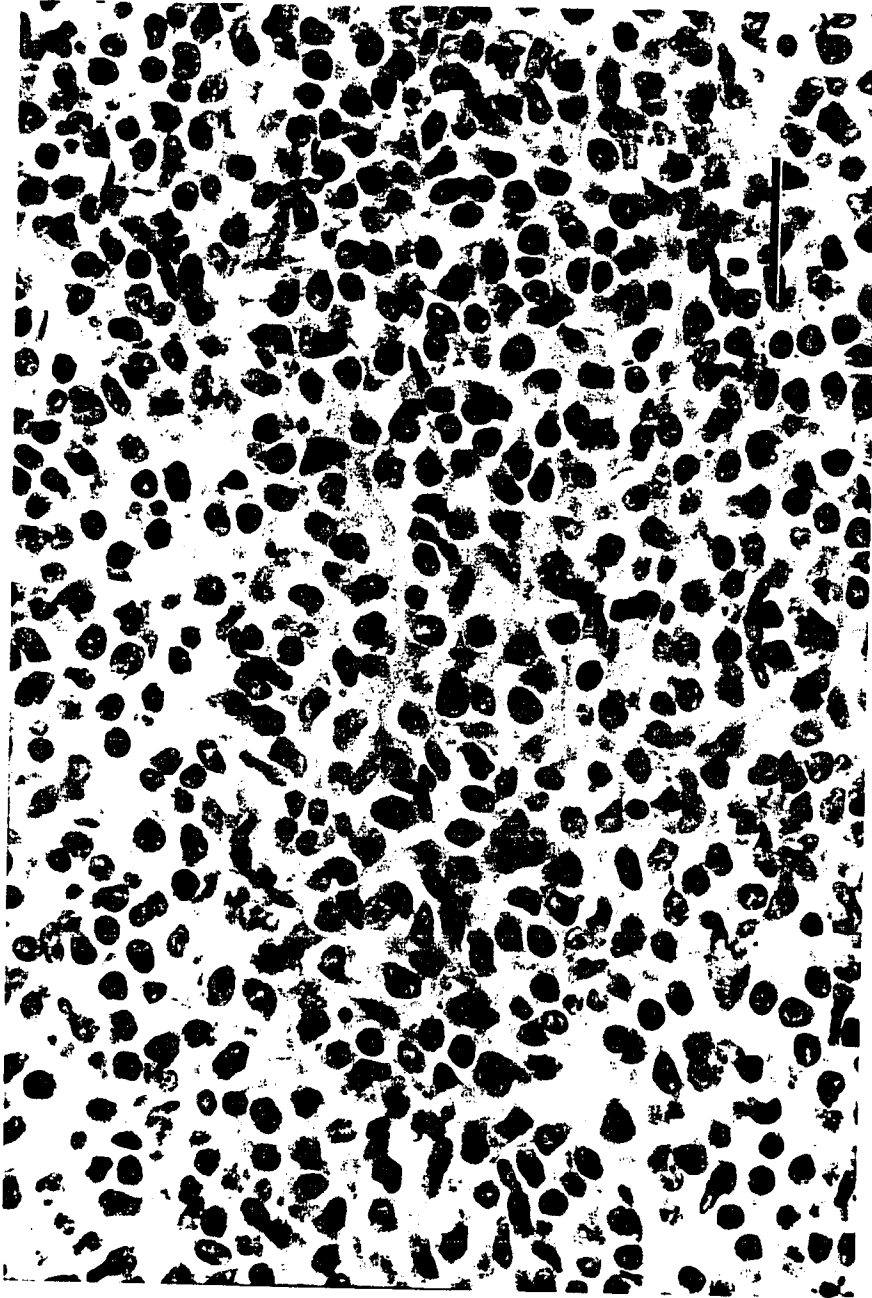
Figure 2.   Thoracic lymph node. Large lymphocytes with indistinct
                cytoplasmic borders in a diffuse pattern effacing normal
                lymph node architecture. Cellular characteristics are similar
                to those in the thymic neoplasm. Harris' hematoxylin and
                eosin stain. Bar = 60 μm.

lymphosarcoma associated with BLV infection in adult cattle, adult cutaneous lymphosarcoma, calfhood multicentric and calfhood thymic lymphosarcoma. This case of lymphosarcoma does not fit well into any of these classic syndromes. The calf was older than generally seen in the calfhood multicentric form, and liver, spleen, and bone marrow were not involved. Unlike the usual thymic form, numerous lymph nodes posterior to the diaphragm were also infiltrated. The case also does not fit the recently described familial form of thymic lymphosarcoma, in either the pattern of tumors or in the cell type involved.[5] This case, therefore, raises the possibility that BIV infection may, like HIV-1 and FIV infection, predispose animals to the development of atypical lymphosarcomas.

The mechanisms responsible for the development of lymphomas in lentivirus infected animals and humans are unclear. A recent report raises the possibility that HIV may directly induce oncogene expression in a subset of lymphomas [9]; whether this mechanism occurs in BIV or other lentiviruses is currently unknown. Immunosuppression and the failure of immune surveillance are also believed to contribute to the induction of tumors by lentiviruses.[1,4] Chronic immune stimulation may also predispose to the development of mutations in immune cells.[1,4] In this animal, immunosuppression does not appear to have been a major cause of tumor initiation, although it may have developed later as a consequence of the tumors. Lymphopenia and reduction of all lymphocyte subsets were seen at day 190; however, there was no evidence for immunosuppression before the development of tumors. A slight decrease in $\gamma\delta$ T cells and B cells was seen on day 112, prior to the onset of clinical signs; the significance of this is unknown. Immune function parameters measured included neutrophil function, monocyte function, and lymphocyte blastogenesis. All neutrophil and monocyte function tests in this calf (including tests of phagocytosis, migration, and cytotoxicity) were normal both before and after the onset of clinical signs. Lymphocyte blastogenesis was, however, increased in response to phytohemagglutinin (PHA), Pokeweed mitogen (PWM) and/or

concanavalin A (ConA) on all dates examined. There was no evidence of opportunistic infections.

In this calf, therefore, chronic immune stimulation may be a more likely contributor to the development of lymphosarcoma. Follicular hyperplasia seen in early stages of BIV infection in this and other cases [2,11], as well as enhanced lymphocyte blastogenesis, lend support to this hypothesis. In an interesting parallel, Callanan et. al. [1] have reported the development of a multicentric B cell lymphosarcoma in a young cat experimentally infected with FIV. The tumor in this cat was presumed to arise as the result of B cell activation during FIV infection. Similarly, lymphosarcoma in the calf in this report could potentially have originated from a T cell infected or activated by BIV. The target cell(s) for BIV are currently unknown, although virus can be recovered from unfractionated mononuclear cells and from isolated monocytes. It is intriguing that, in this calf, tumor cells were immature T cells, with a $CD2^+$ $CD4^-$ $CD8^-$ phenotype.

The atypical nature of this lymphosarcoma, the absence of BLV, and the presence of replicating BIV, suggest that BIV infection may have predisposed the calf to the development of lymphosarcoma. Case studies or experiments on larger numbers of animals will be necessary to prove or disprove a causal relationship; however, this case does raise the possibility that some cases of atypical bovine lymphosarcoma may be associated with BIV infection.

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GENERAL CONCLUSIONS

From the results presented in this dissertation, it appears that BIV virus does affect macrophage function. Treatment of normal monocytes with supernatants from BIV-infected cells *in vitro* was shown to affect monocyte phagocytosis of opsonized *Staphylococcus aureus*, random and chemotactic migration, and ADCC function (1). The increases in phagocytosis, random migration, and chemotaxis appear to be due to the BIV Gag protein (2). The molecule(s) responsible for the suppression of ADCC activity are not known, but do not appear to be Gag products (2).

The effects of Gag on monocyte function are unexpected, but not unprecedented. The HIV p24 (capsid) protein was recently shown to affect macrophage responses to interferon (3), as well as the induction of lymphocyte proliferative responses to antigens (4,5). The effect of HIV p24 was traced to a conserved 7 amino acid peptide RGSDIAG (5). A homologous peptide, KTDEIIG, is present in a similar position (amino acids 239-246) in the p26 protein of BIV-R29 (6). These two peptides are 71% homologous, when conservative amino acid substitutions are included in the analysis: there are 2 identical amino acids and 3 conservative amino acid substitutions. In addition, a proline is present immediately 5' to both peptides. The effects of the BIV peptide have not yet been tested on immune function, and other epitopes of the BIV Gag proteins may be responsible for the effects of BIV on chemotaxis and phagocytosis. Nevertheless, it is intriguing that this particular amino acid sequence has been conserved between the Gag proteins of BIV and HIV, particularly as the overall amino acid homology between these proteins is only 48-55%, when conservative amino acid substitutions are included (6).

Immunomodulatory effects have not been reported for the Gag proteins of lentiviruses other than HIV or BIV, and most of the research on HIV p24 effects is relatively recent. It is not surprising that the effects of these proteins on immune function have not been studied. The Gag (core) proteins are located beneath the

envelope of mature retroviral virions; due to this structural position, they appear to be unlikely candidates for immunomodulatory agents. The Gag proteins are, however, also one of the major proteins produced in infected cells (7,8), and circulating Gag antigens may be found in the blood in some lentivirus infections (9,10). As BIV and HIV Gag proteins (3-5) both affect monocyte function, it is quite likely that Gag proteins from lentiviruses other than HIV and BIV can also affect immune functions.

The Gag proteins of BIV and HIV share a common structural role and might, therefore, be expected have similar effects on the immune system. This does not, however, appear to be the case for either monocyte phagocytosis or chemotaxis. Direct *in vitro* treatment of macrophages with HIV virus suppresses phagocytosis and chemotaxis (11-14); these effects appear to be mainly due to envelope and transmembrane protein interactions (11,12,14,15) and not to gp24 (12,15). In contrast, direct treatment with BIV virus increased phagocytosis, random migration, and chemotaxis in our studies, and these effects appeared to be mediated by the BIV Gag protein(s) (1,2). Although both HIV (16,17) and BIV appear to suppress ADCC function, the mechanism for this effect has not been determined for either virus. Data is not available on the effects of other lentiviruses on macrophage function *in vitro*; however, the early *in vivo* effect of FIV appears to be an enhancement of antimicrobial function (18). In this respect, FIV appears to mimic the effects of BIV seen *in vitro*, rather than HIV.

BIV and HIV viruses also appear to have very different effects on monocyte function *in vivo*. HIV infection suppresses monocyte chemotaxis and phagocytosis *in vivo* (14,15,16,19-22); however, the only consistent effect of BIV infection in experimentally infected animals was a tendency (P = 0.06) for the suppression of phagocytosis during the first 4 to 8 months PI (1). During the last 2 years monocyte function in these animals was studied, monocyte function appeared to be entirely normal (1). One other study was published, during the course of this project, describing monocyte function in BIV infected animals (23). In this study, three

sequentially infected animals were examined for monocyte function defects early in infection and monocyte chemiluminescence, chemotaxis, and phagocytosis of latex particles were found to be suppressed. Our studies in infected animals are consistent with the report of decreased monocyte phagocytosis, but do not confirm that chemotaxis is suppressed (1).

Finally, the functional changes seen in monocytes treated with BIV *in vitro* were not seen in monocytes from the infected animals. In fact, BIV enhanced monocyte phagocytosis and migration *in vitro*, but tended to suppress monocyte phagocytosis (but not affect chemotaxis, ADCC or random migration) for a few months *in vivo* (1). The difference between these *in vivo* and *in vitro* results may be a result of differences in the amount of Gag that monocytes are exposed to *in vivo* and *in vitro*. Production of the R29 virus *in vivo* is probably fairly low, as a low percentage of leukocytes appear to be infected in the blood (24). Circulating virus proteins in the blood, including Gag, would be expected to be correspondingly low. Furthermore, infection *in vivo* may also result in alterations in cytokine production, or other effects which overwhelm any effect of low levels of Gag. Slight increases in phagocytosis and migration induced by low levels of Gag could easily be masked by such effects. The early effects of BIV infection seen by Onuma et al (23) may also have been due to the induction of cytokines such as interferon. Currently, it is not known whether the effect of BIV on ADCC function *in vitro* is due to induction of cytokines, to other cellular molecules such as prostaglandin E_2, or to a virus protein other than Gag.

One final consideration is whether BIV (or Gag) production in the tissues is at sufficiently high levels to affect tissue macrophage function. Production of the related (25) HIV virus does appear to be upregulated in the tissues, as compared to the blood (26,27). The corresponding information is not available for BIV infection; however, there is evidence for the production of BIV Gag in lymph nodes (28). It is, therefore, possible that macrophage function is altered in the tissues, without effects on blood monocytes. It is not known whether alterations in tissue macrophage

function, particularly local suppression of ADCC, had any part to play in the unusual lymphosarcoma seen in one of the experimentally infected calves in this project. In this calf, blood monocyte function did not appear to be abnormal (29); however, tissue macrophages may have been affected without such alterations being reflected in blood monocyte function. The persistent monocytosis seen in this calf is occasionally seen with tumors (30); yet it is tempting to speculate that it may have been more than a nonspecific response to inflammation, in this animal. The finding that BIV may affect monocyte functions also raises the possibility that BIV might also affect the function of other immune cells such as natural killer cells and Tc cells, which appear to have greater importance in cancer immunosurveillance. Further research is necessary to determine whether BIV Gag affects leukocytes other than monocytes.

Additional research is also necessary to determine whether the newer isolates of BIV (31) affect macrophage function identically to the R29 virus. These viruses could not be examined in the current study, as they are not easily grown in cell culture. However, recombinant proteins based on their Gag sequences might be constructed and examined for effects on macrophage function. At present, it is not known which portion of the BIV Gag proteins affect macrophage function (although, from the effects of the recombinant Gag, the active factor appears to reside either in, or close to, the p26 capsid protein). If this information becomes available, the newer lentiviruses could also be examined for sequence similarities or differences in this/ these areas of the genome.

At the time this project was begun, BIV had been proposed as a model for AIDS. During the course of this study, it was found that neither the *in vivo* nor the *in vitro* effects of HIV and BIV in macrophages appear to be the same. Furthermore, published studies of the effect of BIV on other leukocytes have demonstrated only subtle alterations in cell function (32,33). The only direct parallels to HIV infection which have been found are follicular hyperplasia in lymph nodes (24,34), and production of virus proteins within lymph nodes (28). Whether

BIV (like HIV) causes clinical immunodeficiency, however, remains to be determined. Studies have not reported clinical immunodeficiency in animals experimentally infected with several different isolates of BIV (1,23,24,31-35) This does not, however, prove that clinical immunodeficiency could not be seen under other conditions, such as concurrent disease or physiologic stress, which might activate BIV virus. Most experimental studies on BIV infection have examined short-term infections of several weeks or months; our study on the long-term effects of BIV (of which these experiments were a part) appears to be unique. The effects of BIV infection on the induction of lymphosarcoma also remain to be explored. Finally, the value of research on BIV does not rest on solely on the discovery of similarities to HIV. The discovery that Gag affects macrophage function in both BIV and HIV (3-5) demonstrates how intriguing parallels may be found between lentiviruses that, in other respects, appear to be dissimilar.

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ACKNOWLEDGEMENTS

me so much more. You may no longer be able to do the things you used to, or even understand much of what I say - but I'm still glad you're my dad. This one's for you.

APPENDIX A: MACROPHAGE FUNCTION ASSAYS

Monocyte phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) assays were developed as modifications of previously published assays of neutrophil function (1). Assays were optimized by varying the reagent concentrations, changing incubation conditions and increasing incubation times. The blind well chamber chemotaxis assay is a modification of procedures used for human and mouse macrophages (2,3); the primary alterations in this assay were changes in incubation times and the use of different chemoattractants, as bovine leukocytes do not appear to have receptors for the bacterial fMLP (N-formyl-methionyl-leucyl-phenylalanine) peptide. This appendix describes the monocyte assays in greater detail than is presented in the materials and methods sections of the research papers. Additionally, the results of monocyte function assays in BIV-infected animal #808 are presented.

Isolation of Bovine Monocytes

Reagents:

1) 75 cm^2 tissue culture flasks (Corning Co., Corning, NY)

2) Siliconized glass centrifuge tubes, 25 ml and 50 ml

3) Erythrocyte lysing solution (1)

4) Restoring solution (1)

5) Hanks balanced salt solution (Gibco Laboratories, Chagrin Falls, OH)

6) RPMI 1640 (Gibco Laboratories, Chagrin Falls, OH) with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (37°C)

7) RPMI 1640, plain (37°C)

8) Warm (37°C) phosphate buffered saline, pH 7.2 (PBS)

9) Medium M199 (Gibco Laboratories, Chagrin Falls, OH) with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin

Procedure:

1) Collect 225 ml whole blood into acid citrate dextrose anticoagulant (1) and centrifuge at 1500 x g for 30 minutes. Remove 12 ml buffy coat (approximately 1-5 x 10^8 mononuclear cells, with some neutrophil and erythrocyte contamination) into a 50 ml siliconized glass centrifuge tube.

2) Lyse the erythrocytes by adding 24 ml of cold (4°C) lysing solution for 1 minute, then add 12 ml restoring solution. Centrifuge cold (4°C) for 15 minutes at 100 g, and discard the supernatant. Some cell pellets will still contain erythrocytes, but the majority of the cells should be leukocytes.

3) Resuspend the cell pellets and wash the cells once at 100 x g for 15 minutes in Hanks balanced salt solution. Discard the supernatant.

4) Add 25 to 30 ml of RPMI 1640 with 10% fetal calf serum (FCS) and antibiotics to each tissue culture flask. Incubate the flasks in a 37°C, 5% CO_2 humidified incubator for 15 to 30 minutes (this step can be done while cells are being centrifuged), then add the cell pellet from one animal to each flask.

5) Incubate the flasks in a 37°C, 5% CO_2 humidified incubator for 1.5 hours.

6) Wash the adherent cells 5 times (with a 12 ml pipette) with warm (37°C) RPMI medium (plain), then once with warm PBS. Before the first wash, vigorously shake the flasks (side to side) to resuspend loosely adherent cells. Remove this medium, then pipette approximately 12 ml fresh RPMI medium onto the cells, shake the flask lightly, and discard the medium. Repeat 4 more times. The medium from the last wash should be clear.

7) Add 12 ml warm (37°C) PBS to each flask and incubate the flasks in a 37°C, 5% CO_2 humidified incubator for 45 minutes. Every 10-15 minutes, gently tap the sides of the flasks to dislodge the cells.

8) At the end of the 45 minute incubation, collect the PBS, with the cells which have detached, in a 25 ml siliconized glass centrifuge tube, and centrifuge for 15 minutes at 100 g. Discard the supernatant, and resuspend the cells in the small amount of PBS which remains in each tube (approximately 100 - 500 μl) by

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vortexing gently.

9) Remove a small sample of the cell pellet to count the cells (10 μl cell sample in 70 μl PBS works well). Resuspend the cells in M199 with FCS and antibiotics at the desired concentration (usually 2- 5 x 10⁶ cells/ml).

Phagocytosis Assay - Ingestion of *Staphylococcus aureus*

Principle:

This assay measures the specific ingestion of heat-killed, radioactively labelled *Staphylococcus aureus* which has been opsonized with anti-*S. aureus* antibody. After ingestion of bacteria by monocytes, extracellular *S. aureus* are lysed with lysostaphin, and the radioactivity associated with internalized bacteria is measured.

Reagents:

1) ^{125}I- iododeoxyuridine labeled *Staphylococcus aureus* (1)

2) Bovine anti- *Staphylococcus aureus* serum (1) diluted 1:10 in Earles balanced salt solution (Gibco Laboratories, Chagrin Falls, OH)

3) Medium M199 (Gibco Laboratories, Chagrin Falls, OH) with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin

4) Cold (4°C) phosphate buffered saline (PBS)

5) Lysostaphin (Sigma Chemical Co, St. Louis, MO); 1 unit/ml in PBS

6) 12 x 75 mm polystyrene tubes (Becton-Dickinson Labware, Lincoln Park, NJ)

Procedure:

Preparation of samples

Place in duplicate 12 x 75 mm plastic tubes: 5.0 - 10.0 x 10⁵ monocytes in 350 μl medium M199 with 10% fetal calf serum (FCS) and 100 U/ml penicillin, and 100 μg/ml streptomycin (bacteria:monocyte ratio of approximately 50:1 to 150:1, depending on the cell concentration chosen). Add monocytes to sterile sample tubes

at least 1 hour prior to assay, to allow monocytes to adhere to tubes.

Preparation of standard and background tubes (2 each):

Add 350 μl M199 with 10% FCS and 1% penicillin/ streptomycin per tube.

Assay

1) At the start of the assay, add to all tubes:

25μl of ^{125}I- iododeoxyuridine labeled *S. aureus*

50 μl of a 1:10 dilution of bovine anti-*S. aureus* serum

2) Incubate tubes for 6 hours in a 37°C 5% CO_2 humidified incubator. This assay can also be done overnight (12-16 hours), using 50 μl of *S. aureus* per tube.

3) Vortex briefly to resuspend unphagocytosed *S. aureus*.

4) Add 0.5 ml of phosphate buffered saline (PBS) containing 1 unit/ml of lysostaphin to sample and background but not standard tubes. Incubate for 30 minutes in a 37°C shaking water bath, to lyse unphagocytosed *S. aureus*.

5) Add 2.0 ml cold PBS to sample and background tubes to stop the lysostaphin reaction.

6) Wash cells by centrifugation at 1250 x g at 4°C for 10 minutes.

7) Remove supernatant by aspiration, and discard into radioactive waste.

8) Wash cell pellets one additional time by cold centrifugation with 2.0 ml cold PBS.

9) Remove supernatant by aspiration, and discard into radioactive waste without disturbing the cell pellet.

10) Determine the remaining radioactivity for standard, background, and sample tubes in a gamma counter set for iodine. Results = average of 2 replicates. Percent ingestion = ((cpm in sample tube) - (cpm in background tube)) ÷ ((cpm in standard tube) - (cpm in background tube)) x 100.

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

Principle:

Antibody-dependent cell-mediated cytotoxicity is measured as the percent specific lysis of ^{51}Cr-labelled chicken erythrocytes (cRBC) coated with a subagglutinating dose of bovine anti-cRBC antibodies. Lysis of cRBC releases ^{51}Cr into the medium; cell supernatants are collected and the amount of released ^{51}Cr is determined.

Reagents:

1) Sterile 96-well round bottom microtiter plate - at least 300 μl capacity per well (Becton-Dickinson Labware, Lincoln Park, NJ)

2) Chicken erythrocytes (cRBC) standardized to 12.5 x 10^6 cells/ml in phosphate buffered saline. 0.5-1 ml of blood is collected with a heparinized syringe from a healthy chicken and stored in an equal volume of Alsievers solution at 4°C for up to 2 weeks. This cRBC stock is diluted to 12.5 x 10^6 cells/ml in PBS. Diluted cRBC may be stored up to 2 weeks at 4°C, or until spontaneous lysis of erythrocytes is observed.

3) Bovine anti-cRBC serum diluted in M199. Preparation of bovine anti-cRBC serum has been previously described (1). The dilution of each lot of anti-cRBC serum must be standardized for the ADCC assay; with the current lot, a dilution of 1:10 is optimal for monocytes and macrophages.

4) 0.1 % Triton X in distilled H$_2$O

5) Medium M199 (Gibco Laboratories, Chagrin Falls, OH) with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin

Procedure:

Radioactive labelling of cRBC

Add 1 ml of diluted cRBC to a 15 ml conical centrifuge tube, with 12-14 ml PBS. One ml of cRBC will prepare one 96-well plate. Centrifuge for 10 minutes at

300 g, discard the supernatant, and resuspend the cRBC pellet in 0.3-0.5 ml M199 with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Add 50 μCi of sodium chromate (Amersham, Arlington Heights, IL) and incubate overnight in a 37°C, 5% CO_2 humidified incubator. The amount of sodium chromate may be increased up to 200 μCi with shorter incubation times. (Shorter incubation times will result in higher backgrounds for the assay.)

Wash labelled cRBC 2 times with 12-14 ml PBS, by centrifugation for 10 minutes at 300 g. Discard the supernatant into the radioactive waste. Resuspend labelled cRBC in 10 ml M199 with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Assay

1) Sample wells: Dilute monocytes at 3.5-5.0 x 10^6 cells/ml in medium M199 with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Aliquot monocytes to duplicate wells of a sterile 96 well plate at 100 μl per well and allow the cells to adhere (30 minutes to overnight).

2) Add (per duplicate or triplicate wells):

    a) for standards: 150μl 0.1 % Triton X in distilled water

    b) for background wells: 50 μl anti-cRBC antibody and 100 μl M199 with fetal calf serum and antibiotics.

    d) for sample wells: 50 μl anti-cRBC antibody to the 100 μl of monocytes.

3) Add to all wells: 100 μl ^{51}Cr labelled cRBC

4) Incubate for 6 hours in a 37° C 5% CO_2 humidified incubator, with no lid on the plate.

5) Harvest supernatant with a filter harvesting system (Skatron Inc, Sterling, VA) or centrifuge at 300 x g for 10 minutes, then remove 150 μl of the supernatant and transfer to tubes.

6) Count supernatant in a gamma counter set for chromium 51. Results = average of 2 replicates. ADCC = [((cpm in sample wells) - (cpm background)) $\div$ ((cpm in standard wells) - (cpm background))] x 100.

Random and Chemotactic Migration

Principle:

In the chemotaxis assay, monocytes migrate from the top chamber of a blind well chamber through a filter toward a chemoattractant placed in the bottom well of the chamber. In the random migration assay, medium is placed in the bottom of the blind well chamber, and the spontaneous migration of monocytes is measured. With a polycarbonate filter and relatively short incubation times, migrated monocytes can be found on the lower surface of the filter. The number of monocytes which have migrated is measured by staining the filter and counting monocyte numbers under a microscope.

Reagents:

1) 48-well microchemotaxis chamber (Neuroprobe, Rockville, MD)

2) 5.0 μ pore size polycarbonate filters for chamber (Nucleopore, Pleasanton, CA)

3) Medium M199 (Gibco Laboratories, Chagrin Falls, OH) with 10% fetal calf
      serum, 100 U/ml penicillin, and 100 μg/ml streptomycin

4) Chemoattractant:

      Chemotaxis: 10% zymosan activated serum (1) in medium M199 with 10%
           FCS and antibiotics.

      Random migration: Medium M199 with 10% FCS and antibiotics.

5) Diff Quik stain (American Scientific Products, Columbia, MD)

Procedure: (run wells in duplicate)

1) Add 26 μl chemoattractant or medium, per well, to bottom chamber wells. The chemoattractant or medium should be vortexed briefly, prior to use, to prevent the later formation of air bubbles within the chamber.

2) Insert a filter, and assemble the blind well chamber.

3) Incubate blind well chamber in a 37° C 5% CO_2 humidified incubator for 30 minutes to equilibrate the solutions, with a slide over the chamber to prevent

evaporation.

4) Add macrophages : 25-50 μl per chamber of a 2.0 to 5.0 x 10^6 macrophage/ml in M199 with 10% FCS and antibiotics. Do not use antimycotics in the migration medium, as they inhibit migration.

5) Incubate for 1.5 hours in a 37° C 5% CO_2 humidified incubator.

6) Remove the filter, and scrape cells off the top (unmigrated) edge of the filter, using the edge of a glass slide or other cell-scraping apparatus. Stain the filter with a modified hematoxylin-eosin stain (Diff Quik) stain.

7) Examine the filter with the oil immersion objective of a light microscope - determine the number of cells per 2-10 high power fields (hpf) per sample. Two high power fields can be counted if cells are evenly distributed across the filter; more fields may need to be counted if the cell distribution is uneven or cell numbers are low (less than 15-20 cells/ high power field). Migration = cells/hpf (average of 2 replicates).

Monocyte Function Assays
Animal #808

Animal #808 is a Holstein steer infected with 1.3 x 10^4 syncytium forming units of monocyte-derived BIV virus (R29 strain) from a calf with lymphosarcoma (4,5). BIV virus has been consistently isolated from the monocytes of animal #808 for the first 2.5 years post-inoculation (PI). Monocyte function assays were repeated every 2-4 weeks PI, starting at 5 weeks PI and continuing for the next 6 months. The function of monocytes from animal #808 was compared to three retrovirus-negative, age matched Holstein calves. As seen in table 1, there was no consistent significant (P < 0.05) difference between functional assays in animal #808 and the three control animals. (The occasional significant (P < 0.05) differences from the controls are not unexpected, when a large number of statistical comparisons have been made.)

It is interesting, however, to note that monocytes from calf #808 had

Table 1: Monocyte function in calf #808, compared to three age, sex and breed matched controls.

Weeks PI	*Staphylococcus aureus* Ingestion Controls (Mean ± SD)	808	ADCC Controls (Mean ± SD)	808	Random Migration Controls (Mean ± SD)	808	Chemotaxis Controls (Mean ± SD)	808
5	12 ± 9	25	26 ± 18	33	NA	NA	NA	NA
13	16 ± 6	21	52 ± 7	18*	8 ± 6	5	38 ± 18	26*
14	28 ± 7	31	NA	NA	17 ± 12	23	35 ± 18	56
18	22 ± 3	25	19[a]	11	7[a]	5	64[a]	40
19	NA	NA	NA	NA	14 ± 6	8	67 ± 16	51
21	28 ± 2	27	NA	NA	17 ± 14	18	46 ± 28	67
27	18 ± 12	22	37[a]	35	7[a]	11	39[a]	52
27	17 ± 1	20*	12 ± 3	5*	NA	NA	NA	NA
31	32 ± 2	33	10 ± 6	4	15 ± 7	3	96 ± 51	70
33	NA	NA	16 ± 10	11	32 ± 2	10*	84 ± 12	37*
35	37 ± 4	32	NA	NA	15 ± 9	19	65 ± 2	72*

Values marked with an asterisk (*) are at least 2 standard deviations different from the mean for the control animals, on that date. For controls, n = 3, except data marked with [a], where values were only available from 2 animals, and no standard deviation can be computed. Abbreviations: SD = standard deviation. NA = data not available.

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consistently lower ADCC function (on 6 of 7 dates), and higher *Staphylococcus aureus* ingestion (on 7 of 9 dates) than the mean for the 3 control animals, even though this was not statistically significant. These observations are consistent with the effect of BIV on normal macrophage function *in vitro* (4,6); however, it is also possible that calf #808 had a natural tendency to have higher than average phagocytic function and lower than average ADCC function, unrelated to his BIV infection status. No patterns were visible for random and chemotactic migration of monocytes from calf #808.

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APPENDIX B: RESPONSES OF CULTURED BOVINE MONOCYTES TO BOVINE ALPHA AND GAMMA INTERFERON

This is the text and graphs from a poster presented at the 70th Conference of Research Workers in Animal Disease (1990).

Abstract

Bovine blood monocytes were isolated by adherence to plasma-coated tissue culture flasks and cultured for 1 to 5 days to determine whether time in culture affects the responsiveness of monocytes to interferons. On days 1, 2 and 4 of culture, aliquots of monocytes were removed, treated for 15 hours with recombinant bovine alpha or gamma interferon, and the following function assays performed: random migration, chemotaxis, antibody-dependent cell cytotoxicity (ADCC) and antibody-independent cell cytotoxicity (AICC). Chicken erythrocytes were used as target cells in cytotoxicity assays. Some but not all monocyte functions changed with time in culture. Both random and chemotactic migration decreased by the fifth day in culture. Variable responses to interferon treatment were seen, depending on the particular assay performed. Most animals consistently responded to both alpha and gamma interferon with decreased random and chemotactic migration on all 3 days; however, not all animals had increased ADCC activity in response to interferon. Some monocytes which were initially unresponsive to interferon in ADCC assays became responsive with increased time in culture. AICC was only occasionally seen in a few individuals and did not change with interferon treatment. According to these results, bovine monocyte responses to interferon may vary depending on the functional assay performed and may change with time in culture.

Introduction

Macrophage functions are modulated both by cytokines, particularly the interferons, and by the degree of macrophage maturity. Increased cytotoxicity,

increased expression of Fc receptors, and decreased cell migration are among the functions associated with interferon activation (1). The degree of monocyte maturity may influence the response to interferon. Although some of the responses of human and mouse interferons during aging in culture have been studied (2,3), few studies have been carried out on other species. We have, therefore, examined the responses of bovine monocytes to bovine recombinant alpha and gamma interferon during aging in culture.

Materials and Methods

Monocyte isolation and culture

Bovine mononuclear cell fractions were isolated from the buffy coat of 12 healthy adult Holstein steers and the red cells lysed by flash lysis using distilled water. Tissue culture flasks were prepared by coating each flask with autologous plasma for 30 minutes, then adding culture medium consisting of RPMI 1640 (Gibco Laboratories, Chagrin Falls, OH) supplemented 10% fetal calf serum, and antibiotics (50 U/ml penicillin, 0.05 mg/ml streptomycin) to each flask; the final concentration of autologous plasma was 20%. The mononuclear cells from each animal were divided into 3 tissue culture flasks and incubated for 1 to 2 hours at 39°C in a 5% CO_2 humidified incubator. After incubation, the adherent cell populations were washed three times with warm RPMI 1640 containing 10% FCS and antibiotics.

On days 1, 2 and 4, the monocytes from one flask were detached for each animal (4). The culture medium was removed, and the adherent cells washed once with warm phosphate buffered saline (PBS). The medium was replaced with fresh warm PBS and the cells incubated for 30 minutes at 39°C, with occasional brief agitation. The detached cells were washed once at 120 x g for 15 minutes, and the cell numbers adjusted to 5.0 x 10^6 cells/ml in medium M199 (Gibco Laboratories, Chagrin Falls, OH) supplemented with 10% FCS and antibiotics. The monocytes or monocyte-derived macrophages were aliquoted into individual wells (ADCC) or

siliconized glass tubes (migration) and treated overnight (12-15 hours) with various concentrations of recombinant bovine Interferon alpha (lot 13/1035/1, gift of Ciba-Geigy, Basel, Switzerland) or interferon gamma (lot AE62, gift of Ciba-Geigy, Basel, Switzerland) or medium alone. Functional assays were performed on days 2, 3 and 5.

Migration

Random and chemotactic migration were performed in a 48-well blind well chamber (Neuroprobe, Rockville, MD) using 5.0 μm pore size polycarbonate filters (Nucleopore, Pleasanton, CA). Medium M199 or 10% zymosan activated serum were used as chemoattractants for random and chemotactic migration, respectively. All migration assays were performed in duplicate, using 2.5 x 10^5 monocytes or macrophages per well. Samples were incubated for 2 hours at 39°C in a 5% CO_2 humidified incubator. After 2 hours, the filters were stained with a modified hematoxylin-eosin stain (Diff Quik, American Scientific Products, Columbia, MD) and cells that had migrated to the bottom of the filter were counted in 10 high power fields. Results are expressed as the average number of cells per high power field for 2 replicates per animal.

Cytotoxicity Assays

Antibody dependent cell-mediated cytotoxicity (ADCC) and antibody-independent cell cytotoxicity (AICC) were performed in 96 well microtiter plates (Becton-Dickinson Labware, Lincoln Park, NJ): 2.5 x 10^5 monocytes or macrophages at a macrophage to target cell ratio of 20:1 were added to triplicate wells with or without anti-cRBC antibody. ^{51}Cr labelled chicken erythrocytes (cRBC) were used as target cells (5). Standard wells containing cRBC lysed with 0.1% Triton X in distilled water, and background wells containing cRBC with or without antibody and no added monocytes, were run on each plate. The plates were centrifuged at 400 x *g* for 5 minutes, then incubated at 39°C in a 5% CO_2 humidified incubator for 6

hours. The supernatant was harvested and the percent of radioactivity released from the labelled cRBC determined. Percent ADCC or AICC is defined as ((radioactivity in sample wells - radioactivity in background wells) ÷ (radioactivity in standard wells - radioactivity in background wells) x 100).

Statistical Analysis

Each animal served as its own control; interferon treated monocytes and macrophages were compared to control cells using an analysis of variance blocked by animal. All samples were included in the analysis for significance, independent of whether the animals were classified as responders or non-responders to interferon.

Results

Monocyte purity and viability

Monocyte populations were at least 84% pure (average purity 93% ± 4%) by nonspecific esterase staining (6) and eosin-hematoxylin staining; the contaminating cells were mainly eosinophils and small lymphocytes. Viability by trypan blue staining was 93% ± 3%.

ADCC

In the ADCC assay, overall responsiveness to the interferons appeared to increase with time in culture (Figure 1); however, individual cattle were highly variable in their responsiveness to alpha or gamma interferon (figure 2). We have defined responders as cattle whose ADCC values increased to greater than 125% of the control value with interferon treatment. Responses up to 376% of control values were seen. Three animals remained responders on all 3 days examined and 3 animals remained non-responders. In the remaining 6 animals, a trend was seen for increasing numbers of responders to interferon γ and decreasing numbers of responders to interferon α, with longer time in culture. Additional experiments with larger numbers of animals would be necessary to confirm these observations.
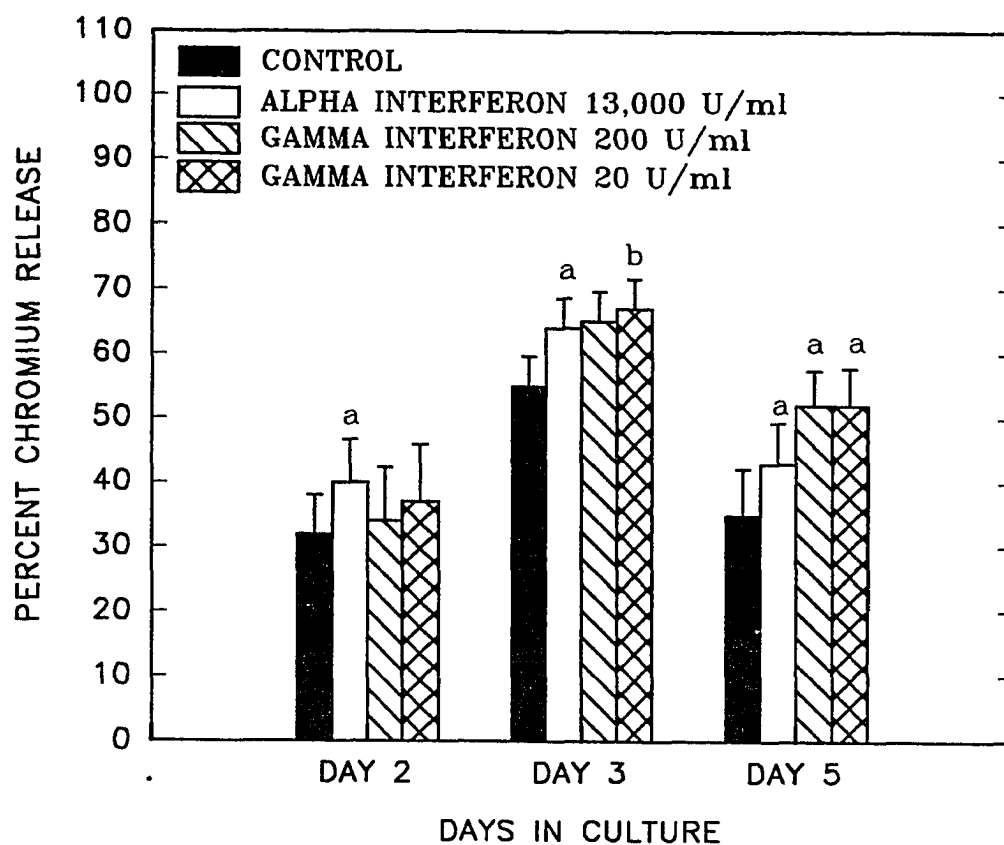
Figure 1: Means and standard errors for ADCC of control and interferon treated monocytes on days 2, 3 and 5 of culture (n = 12). a: value is significantly different from control at P < 0.05. b: value is different from control at P < 0.10.

Alpha Interferon

Number of responders

Day 2: 6/12
Day 3: 3/12
Day 5: 4/12

Gamma Interferon 20 U/ml

Number of responders

Day 2: 4/11
Day 3: 6/12
Day 5: 6/12

Gamma Interferon 200 U/ml

Number of responders

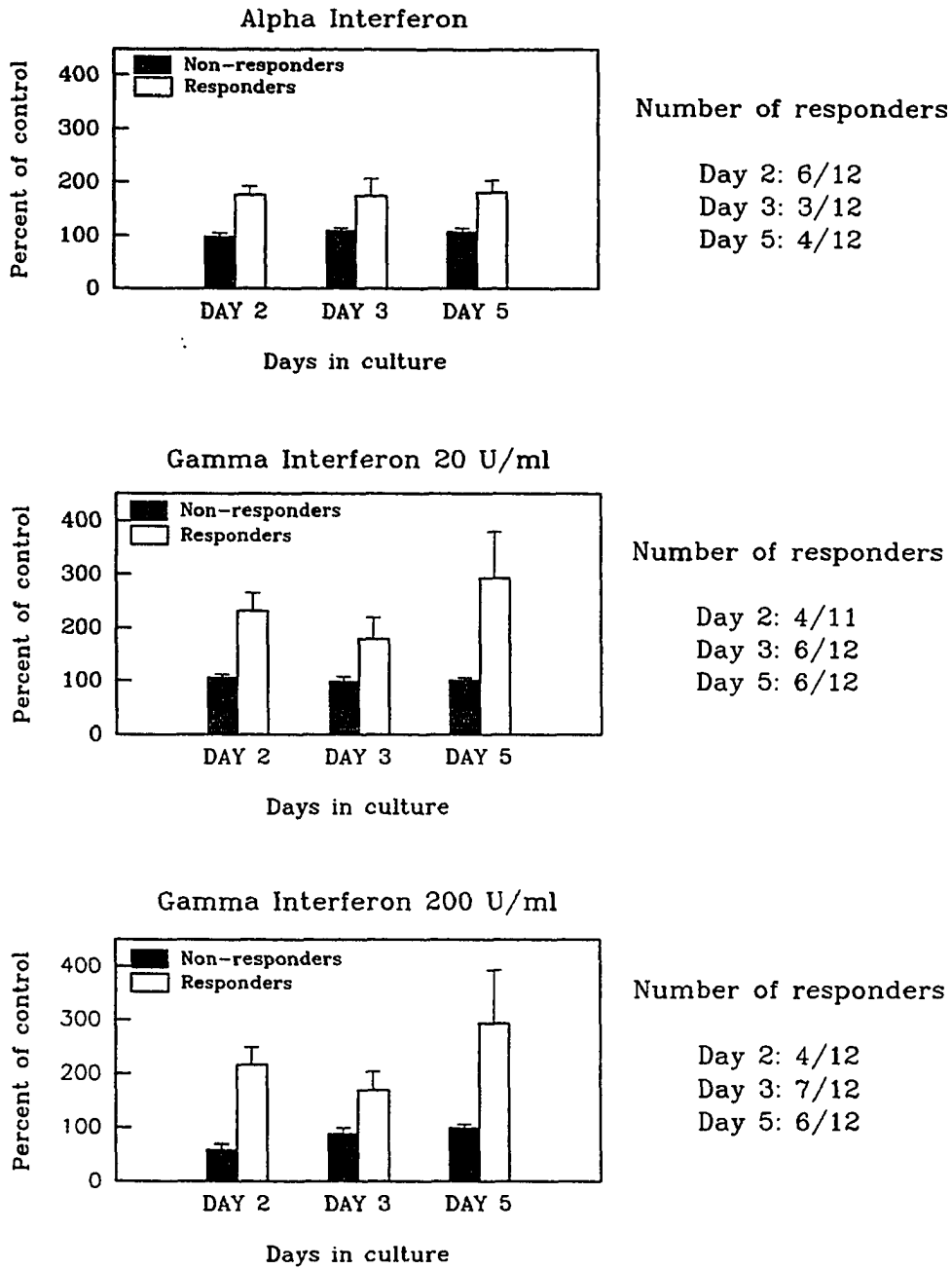Day 2: 4/12
Day 3: 7/12
Day 5: 6/12

Figure 2: Means and standard errors for ADCC function, with animals divided into groups of responders and non-responders to alpha or gamma interferon. Responder = ADCC (treated monocytes) ÷ ADCC (control) > 125%.

AICC

AICC by bovine monocytes against cRBC has not been consistently seen in our laboratory in this or previous experiments. The AICC levels in this experiment were generally very low: most animals had values of less than 3%, although a few animals had control values of up to 25%. AICC values were not influenced by interferon treatment (figure 3).

Migration

All samples that had mononuclear phagocyte migration of at least 2 cells per high power field consistently responded to alpha and gamma interferon with decreased random and chemotactic migration (figures 4 and 5). This response was most pronounced with 200 U/ml of interferon γ, but decreased levels of migration inhibition were seen with 20 U/ml interferon γ or 13,000 U/ml interferon α, Variability in migration was high, both between animals and on different days; however, in general, migration began to decrease on day 3 and reached low levels by day 5. For this reason, samples assayed on days 3 and 5 were less likely to reach statistical significance. By day 3, two thirds of the animals had low levels of control migration that could not be influenced by interferon, although the cells that did migrate were inhibited by interferon.

Summary

We have found that the responsiveness of bovine mononuclear phagocytes to bovine alpha and gamma interferon is variable and dependent on the particular function tested, the individual animal, and the length of time the monocytes have been in culture. Recombinant bovine interferon treatment increased ADCC function in both monocytes and monocyte-derived macrophages; however, this response was not seen in all animals. Animals could be classified into two groups for ADCC function: responders and non-responders. Macrophages from some animals consistently remained responders or non-responders during the culture period;
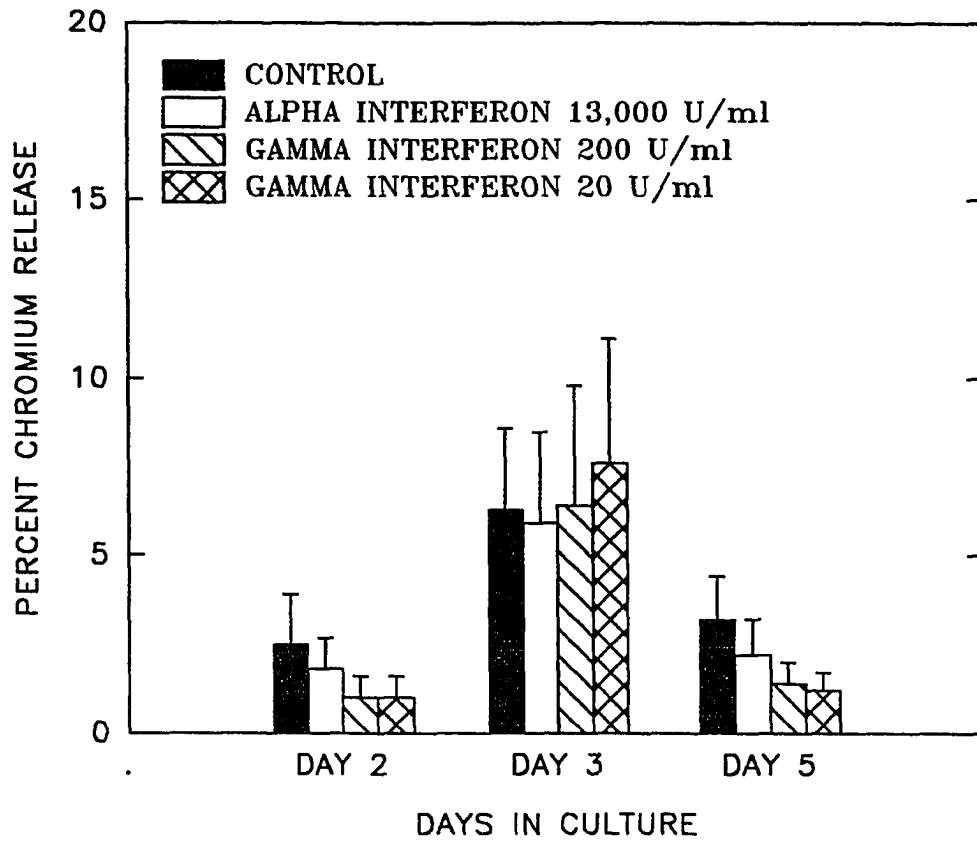
Figure 3: Means and standard errors for AICC of control and interferon treated monocytes on days 2, 3 and 5 of culture (n = 12). No values are significantly different from the control.

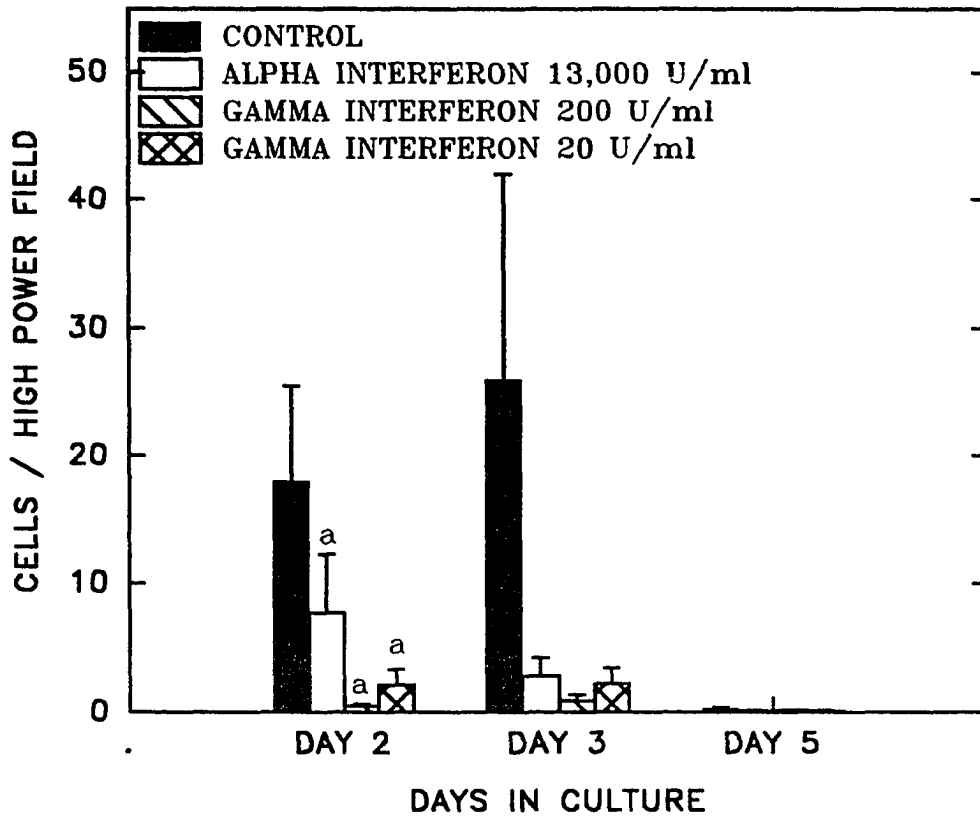Figure 4: Means and standard errors for random migration of control and interferon treated monocytes on days 2,3 and 5 of culture. Migration for each animal is expressed as the average number of cells per high power field in 2 replicates (n = 11 on day 2; n = 9 on day 3; n = 9 on day 5). a: value is significantly different from control at P < 0.05. b: value is different from control at P < 0.10.

Figure 5: Means and standard errors for chemotactic migration of control and interferon treated monocytes on days 2,3 and 5 of culture. Migration for each animal is expressed as the average number of cells per high power field in 2 replicates (n = 11 on day 2; n = 9 on day 3; n = 9 on day 5). a: value is significantly different from control at P < 0.05. b: value is different from control at P < 0.10.
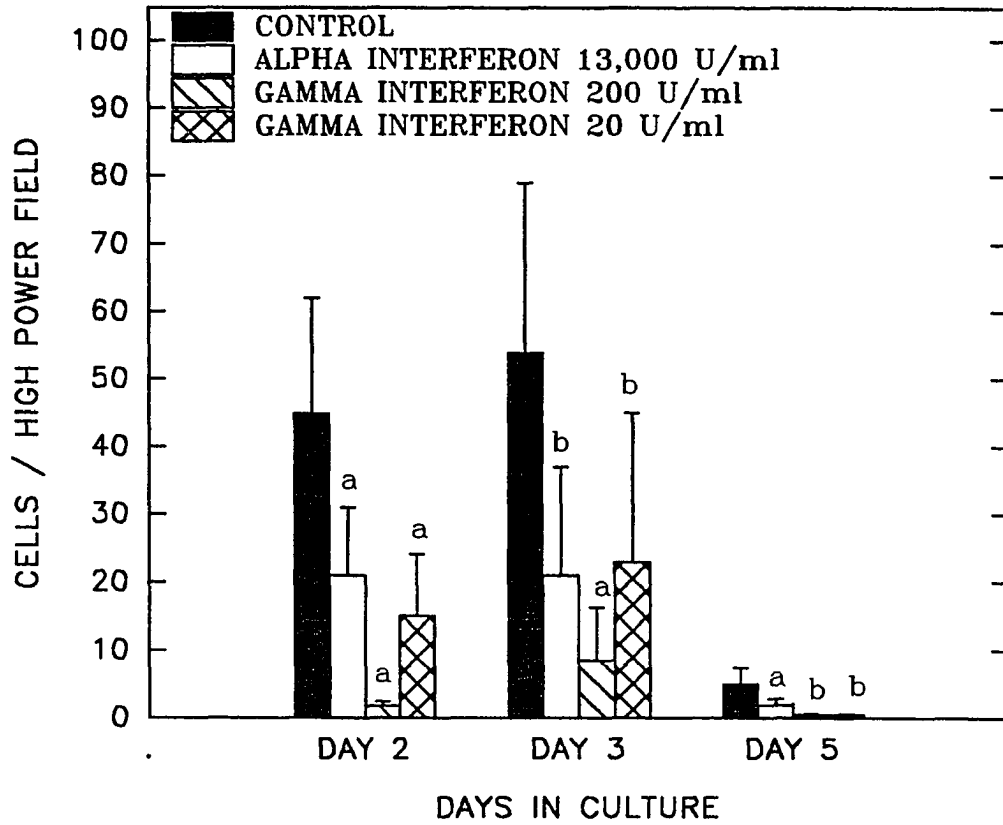
however, some cultured macrophages appeared to gain responsiveness to interferon gamma and lose responsiveness to interferon alpha with longer time in culture. Both 20 U/ml and 200 U/ml of recombinant bovine gamma interferon were equally effective in stimulating increased ADCC, independent of the length of time the monocytes had been cultured.

In contrast to the animal variability found in the ADCC assay, macrophage random and chemotactic migration was inhibited by both recombinant bovine alpha and gamma interferon in most animals. Migration inhibition was most effective with 200 U/ml of interferon γ, although a lesser degree of inhibition was seen with 20 U/ml interferon γ and 13,000 U/ ml interferon α. The response to interferons did not appear to change with time in culture, although the numbers of migrating cells tended to decrease after 3 days in culture and migration inhibition thereafter became difficult to measure.

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APPENDIX C: MACROPHAGE INFECTION WITH BIV *IN VITRO*

Infection of macrophages with cell-associated but not cell-free virus

BIV virus can infect a variety of primary bovine embryonic tissue explants, as well as canine thymocyte and embryonic rabbit epithelial cell lines (1). In these systems, cell-free virus is able to infect cells, although infection does appear to be more efficient with cell to cell transmission (1). Therefore, attempts were initially made to infect bovine monocytes and monocyte-derived macrophages with cell-free virus (multiplicity of infection approximately 10^{-4} to 10^{-5} syncytium forming units of BIV per monocyte). These experiments were unsuccessful. Macrophages from several different animals, infected after different periods of time in culture, did not become infected. Both unstimulated cultures and cultures stimulated with 5 μg/ml of lipopolysaccharide resisted infection. There were a few cultures from which low amounts of BIV was recovered after long periods (greater than 1 month); however, these macrophage cultures also showed an overgrowth of resembling fibroblasts, which cast doubt on which cells were harboring the virus.

It was later observed that these fibroblast-like cells are not able to proliferate in a serum free macrophage medium (macrophage-SFM) (Gibco Laboratories, Chagrin Falls, OH). This observation allowed us to infect macrophages without the potential for overgrowth by the fibroblast-like cells. Furthermore, it was found that fetal bovine lung (FBL) cells did not survive in macrophage-SFM medium. Therefore, an experiment was designed to infect macrophages by short-term co-culture with BIV-infected FBL cells. Macrophages were established in culture in RPMI 1640 (Gibco Laboratories, Chagrin Falls, OH) with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. After 2-4 days, BIV-infected FBL cells were added to the culture. The cells were co-cultured for 1-2 days, then the medium was changed to macrophage-SFM. In some experiments, cultures were maintained for the entire period (from day 0) in macrophage-SFM. Control cultures, with normal macrophages and uninfected FBL cells, were established in parallel. FBL

cells disappeared by 11 to 20 days from the infected cultures, and more slowly from the uninfected cultures. Fibroblast-like cells were not seen.

Slow death of macrophages occurred in the infected cultures and very few cells (in either of 2 cultures from different animals) were left at the conclusion of one 3 week experiment. Some of these infected macrophages had been passaged onto uninfected macrophages from the same animal; these macrophage cultures were also very sparse. FBL cells had disappeared from these particular cultures by day 11 of culture; the remaining cells were determined by visual observation (on day 20) to be macrophages. Large quantities of virus were recovered from these few remaining macrophages by co-cultivation with FBL cells, in both cultures. FBL cells formed large numbers of BIV syncytia within 3 days of their addition to the infected macrophage cultures. Passage of the FBL cells was not necessary to recover the virus, indicating that a large quantity of virus was present.

Staining of infected macrophages and other cells with antibodies to BIV Gag proteins

Procedure

Cultures were established from a variety of BIV-infected and uninfected cells, in 6 well plates (Becton-Dickinson Labware, Lincoln Park, NJ). These cells included canine thymocytes infected with BIV, uninfected FBL cells, FBL cells infected with BIV, macrophages from experimentally infected and control animals, and macrophages infected *in vitro* with BIV by co-cultivation. Infected and uninfected cell cultures were fixed with either cold (4°C) methanol or cold 70% acetone/ 30% methanol fixative, and washed for 10 minutes in Hanks balanced salt solution (Gibco Laboratories, Chagrin Falls, OH) or phosphate buffered saline. Prior to staining, wells were blocked with normal goat serum (Pierce, Rockford, IL). Fixed cultures were incubated for 30 minutes at room temperature with rabbit anti-Gag or control serum (2) diluted in Hanks balanced salt solution. The anti-Gag antibodies detect BIV core (Gag) proteins. Bound primary antibodies were detected by staining

with avidin/biotin horseradish peroxidase kits which detect rabbit IgG (Pierce, Rockford, IL).

Results

Initial experiments demonstrated that rabbit anti-Gag antibodies, but not control antibodies, specifically stained BIV-infected canine thymocytes at dilutions of 1:200 or 1:1000. BIV-infected (but not uninfected) FBL cells could also be specifically stained with 1:200 rabbit anti-Gag serum. Macrophages infected *in vitro* by co-culture also appeared to be specifically stained by antibodies to the Gag proteins. In macrophages infected by co-culture, some individual cells stained darkly with anti-Gag but not control sera at dilutions of either 1:100 or 1:200. In some cases, the stain was dark enough to obscure cellular details.

Staining of macrophages from infected animals, however, yielded equivocal results. Individual macrophages in these cultures were not stained with either anti-Gag or control antibodies, at dilutions of 1:100 or 1:200. Macrophage syncytia stained faintly at 1:200 and more strongly at 1:100 with anti-Gag serum. The pattern of staining in macrophage cultures from BIV-infected animals #808 and #342 showed a relatively dark rim of stain around the periphery of the syncytia, a second area of dark staining in the center of macrophage syncytia (often surrounded by a halo of nuclei), and faint cytoplasmic staining. It is not clear, however, whether this staining was specific for BIV, as macrophage syncytia in uninfected cultures also stained, although more faintly, with anti-Gag antibodies. Light staining of syncytia was also seen with control antibodies. It appeared, therefore, that, at a dilution of 1:100, rabbit (particularly anti-Gag) antibodies were binding nonspecifically to a macrophage protein which was concentrated at the edge and perinuclear areas of syncytia. Some of this staining may have been specific for Gag, as the stain was darker in infected than control cells; however, as Gag proteins could not be detected in macrophages from infected animals at a dilution of 1:200, it was concluded that either Gag antigens were not present in these macrophages, or the

staining was not sensitive enough to detect them.

Summary

From these experiments, it appears that macrophages can be infected with BIV virus, if macrophages are co-cultivated with infected cells. Onuma et al (3) were also successful in infecting bovine macrophages by co-culture with irradiated BIV-infected bovine embryonic spleen cells. Macrophage infection with cell free virus appears to be difficult, at least with the R29 virus under the conditions used in these experiments. It is possible that different incubation conditions, an alternate virus source, or a higher multiplicity of infection may result in successful infection with cell free virus. It is not clear why macrophages appear to be more difficult to infect with cell free virus than are primary bovine cell lines (1). It is possible that these embryonic cell lines contain higher levels of the (unknown) BIV receptor than do macrophages and monocytes. Alternatively, the R29 virus may have adapted so well to *in vitro* cell culture that it has lost its receptor for macrophages. The newer isolates of BIV do not readily infect primary embryonic cell cultures (4); it appears, therefore, that the second hypothesis is more likely to be true. Finally, it is possible that the natural mode of macrophage infection with BIV is by cell to cell contact. The BIV-related (5) lentivirus HIV can spread very quickly by cell contact, without the production of mature virions (6). It would not be surprising if this proves to be the most effective mode for BIV transmission, as well.

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APPENDIX D: AN ANTIGEN CAPTURE ELISA FOR GAG p26 ANTIGEN

Background

Current methods for detecting BIV infected cells include direct staining for BIV Gag (core protein) antigens, detection of reverse transcriptase in cell supernatants, detection of viral DNA by PCR, *in situ* hybridization for mRNA, and virus isolation by co-cultivation with fetal bovine lung (FBL) cells. Of these procedures, the only assay which can easily be used on large numbers of samples is the reverse transcriptase assay; all of the other assays are limited in the number of samples which can be tested at one time. Reverse transcriptase activity has not, however, been detected in macrophage cultures from BIV infected animals, including those which contain virus by co-cultivation with FBL cells. Consequently, an antigen capture ELISA was developed to detect viral antigens in supernatants from BIV-infected macrophage cultures. The BIV p26 antigen was chosen as the target for detection, as the Gag proteins appear to be the major proteins produced in BIV-infected cell cultures (1), and the p26 protein also has less antigenic variability than the BIV envelope proteins (2). An additional consideration was the availability of both mouse monoclonal and rabbit polyclonal antibodies specific for the BIV Gag proteins.

Principle

BIV p26 antigen is captured by rabbit antiserum specific for the recombinant BIV protein Gag3. The bound antigen is detected with a mouse monoclonal IgG antibody against BIV p26, and an avidin-biotin/horseradish peroxidase detection system for mouse antibodies (Pierce, Rockford, IL).

Reagents

1) Immulon 4 ELISA plates

   (Dynatech Laboratories, Chantilly, VA)

2) *Carbonate binding Buffer - pH 9.5 - 9.7*

Add 0.159 g Na_2CO_3 and 0.293 gNaHCO$_3$ to 100 ml distilled H_2O. Adjust the pH, if necessary, with HCl or NaOH.

3) *Wash solution - Phosphate buffered saline* (pH 7)

Stock solution (10X):

monobasic $NaH_2PO_4.H_2O$	13.2 g
dibasic Na_2HPO_4	43.2 g
NaCl	340.0 g
H_2O	4 liters

Working solution (0.01 M PBS/ 0.05% Tween wash solution):

Dilute 400 ml 10X PBS stock solution with 3.6 liters distilled H_2O and add 2 ml Tween 20.

3) *Blocker*: 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in PBS with 0.05% Tween 20

4) *Antibodies*

Capture antibody: Polyclonal rabbit serum against the BIV Gag proteins was prepared by immunizing rabbits with a recombinant fusion protein (Gag3, provided by J. Isaacson). The Gag3 protein consists of the BIV p26 capsid protein, with small flanking regions of the nucleocapsid and matrix proteins, expressed as a trpE fusion protein (3). The optimum concentration in this ELISA for the serum collected 10/22/92 is 1:800 in carbonate buffer.

Detection antibody: The mouse monoclonal antibody against BIV p26 was prepared (4) and concentrated (5) as previously described. The optimal dilution for the 32X concentrate is 1:50 in Hanks balanced salt solution (Gibco Laboratories, Chagrin Falls, OH) with 1% fetal calf serum.

5) *Detection system*: Avidin-biotin horseradish peroxidase kit for mouse IgG (Pierce, Rockford, IL). The avidin-biotin system is available as a kit which has been optimized for the detection of mouse IgG. For this ELISA, a biotin-labelled rabbit anti-mouse IgG antibody (Pierce, Rockford, IL) (1:400 in Hanks balanced salt

solution) has been substituted for the horse anti-IgG provided in the kit, to reduce background staining.

6) *Substrate*: TMB-ELISA solution (Gibco Laboratories, Chagrin Falls, OH). TMB
= 3,3',5,5'- Tetramethylbenzidine dihydrochloride.

7) *Stop solution for TMB:* 1 N H_2SO_4 in distilled water

8) *Test antigens for assay development*:

recombinant BIV Gag3 fusion protein (3), provided by J. Isaacson

recombinant trpE portion of the Gag3 fusion protein (control) (3)

supernatants from fetal bovine lung (FBL) cells infected with BIV

Procedure

Pre-treatment of cell supernatants

Cell supernatants do not show detectable p26 antigen unless they are treated with 2% Nonidet P40 (Sigma Chemical Co, St. Louis, MO) to lyse virions.

1. Prepare plates by binding capture antibody

Bind polyclonal anti-Gag antibody to ELISA plate(s) (100 μl per well; 1:800 in carbonate binding buffer) overnight at 4°C. Wash 5 times with PBS/Tween wash solution (250-300 μl/ well).

2. Block plates

Add blocker (150 μl 1% bovine serum albumin in PBS/Tween) to all wells. Incubate at 37°C for 45 minutes, then wash 5 times with PBS/Tween wash solution.

3. Bind samples

Add samples or standards (in duplicate) at 100 μl/ well, and incubate at 37°C for 45 minutes. Wash 5 times with PBS/Tween wash solution.

4. Detect bound antigen

Add 100 μl biotin-labelled rabbit anti-mouse IgG (diluted 1:400 in PBS or Hanks balanced salt solution) per well. Incubate at 37°C for 45 minutes. then wash 5 times with PBS/Tween wash solution. Add 50 μl avidin/biotin/horseradish peroxidase complex (see kit for instructions), and incubate at 37°C for 45 minutes. Wash 6 times with PBS/Tween wash solution.

5. Substrate

Add 100 μl TMB solution, and incubate for 25 minutes at room temperature. At the end of the incubation, add stop solution (1N H$_2$SO$_4$) at 25 μl per well.

6. Read plate: Read plates at 450 nm wavelength on an ELISA reader.

Parameters examined during assay development

Optimal antibody concentrations

Monoclonal anti-p26 antibodies were tested at dilutions of 1:10 to 1:320, and polyclonal anti-Gag rabbit antibodies at 1:200 to 1:12800. The optimal concentration was defined as the most dilute concentration of antibody which gave OD (optical density) readings equal to or higher than other antibody concentrations.

An irrelevant mouse monoclonal antibody (IgG specific for γδ T cells) was used to demonstrate that the anti-p26 mouse monoclonal was not binding non-specifically: OD values for the irrelevant monoclonal antibody were identical to wells with no antigen. Control rabbit antibodies (generated against the TrpE vector) did not bind BIV-infected FBL cell supernatants (OD values: 0.44, anti-Gag antibodies with BIV supernatants; 0.25, anti-trpE antibodies with BIV supernatants; 0.25-0.28, wells with control supernatants (no antigen) and either anti-trpE or anti-Gag antibodies).

Blockers

Several blocking solutions were tested, using recombinant Gag protein (Gag3) at 0.25 or 2.5 μg/ml as the antigen. The results from 2 dates, for 2.5 μg/ml recombinant Gag, are shown in table 1. Most blockers performed adequately; 1% bovine serum albumin (BSA) appeared to consistently have a slightly higher signal to background ratio than other blockers. Blotto blocker (2% powdered milk, 1% Triton X100, 50 mM Tris HCl (pH 8.5), 0.05% Tween 20, and 10 mM EDTA in PBS) was consistently poor. Gelatin gave erratic results, and was often a poor blocker.

Table 1: The effect of different blockers on detection of p26.

Experiment 1		Experiment 2	
Blocker	Signal/Background Ratio	Blocker	Signal/Background Ratio
Powdered milk		Bovine serum albumin (1%)	9.4
4%	5.0	Powdered milk (0.5%)	6.8 - 7.6
2%	5.4	Blotto	6.1
1%	5.0	Casein (1%)	8.5
0.5%	5.8	Superblock	8.2
Bovine serum albumin			
4%	5.2		
2%	5.2		
1%	6.1		
0.5%	5.7		
Blotto	4.5		
Gelatin (2%)	6.2		

Signal/ Background ratio = the OD for 2.5 μg/ml recombinant Gag3 protein ÷ the OD for wells with no antigen. All blockers except gelatin were diluted in phosphate buffered saline. Gelatin was diluted in carbonate buffer. Blotto = 2% powdered milk, 1% Triton X100, 50 mM Tris HCl (pH 8.5), 0.05% Tween 20, and 10 mM EDTA in PBS. Superblock is a proprietary solution from Pierce (Rockford, IL).

Incubation times

ELISAs with 60 minute incubations had slightly higher signal to background ratios than 30 minute incubations (table 2). An incubation period of 45 minutes (for all antigen-antibody and blocking steps) was chosen as a reasonable compromise between maximum detection of antigens and practical time limits.

Table 2: The effect of varying incubation time on detection of p26.

Incubation Time	Recombinant Gag3		BIV-infected FBL cell supernatants
	0.50 μg/ml	0.25 μg/ml	
30 minutes	9.3[a]	6.3	3.1
60 minutes	11.2	6.6	3.1

[a]Signal/background ratio (the OD reading for wells with antigen divided by the OD for wells with no antigen).

Detection methods : TMB, ABTS, and OPD substrates

Three different horseradish peroxidase ELISA substrates were tested: TMB (3,3',5,5'- Tetramethylbenzidine dihydrochloride), ABTS (2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulfonate)) and OPD (o-Phenylenediamine). Table 3 shows the results of an experiment comparing TMB and ABTS substrates. Although the signal/background ratios for TMB and ABTS are similar, the actual OD values for TMB were 5 times higher than for ABTS and it was, therefore, easier to detect positive samples with TMB. TMB was more sensitive than OPD; the signal to background ration for 2.5 μg/ml recombinant Gag protein was 1.5 times higher for TMB than for OPD.

Table 3: The signal/background ratios for ABTS and TMB ELISA substrates, for several antigen sources.

Substrate	Recombinant Gag3			BIV-infected FBL cell supernatants
	2.5 μg/ml	0.25 μg/ml	0.025 μg/ml	
ABTS	6.3[a]	2.5	1.1	1.1
TMB	6.9	2.6	1.1	1.1

Signal/ background ratio = the OD reading for wells with antigen divided by the OD for wells with no antigen.

Specificity for BIV p26 - cross-reactivity with other retroviruses

The reactivity of several other retroviruses was tested in the ELISA assay (Table 4). The viruses tested include equine infectious anemia (EIA), bovine leukemia virus (BLV), and bovine syncytial virus (BSV). The EIA virus tested was a stock solution of the MA-1 clone. The source for the BLV virus was culture supernatants of FLK-BLV persistently infected cells (provided by J.A. Miller). Both of these antigen solutions were cell supernatants, and contained virus concentrations much higher than are expected to be present in supernatants from BIV infected cells. Both viruses showed very slight reactivity in the p26 assay. The BSV virus antigen was a crude lysate of bovine embryonic spleen cells, used in the agar gel immunodiffusion (AGID) assay for BSV (provided by M.J. Van der Maaten); this particulate solution appeared to bind readily and non-specifically to surfaces.

Therefore, the reactivity of the BSV antigen in the p26 assay may have been due to non-specific binding.

Table 4: Detection of other retroviruses by the BIV p26 ELISA.

Antigen	OD	Antigen	OD
Recombinant BIV Gag3 0.25 μg/ml	2.1	Equine infectious anemia virus	0.16
None	0.11	Bovine leukemia virus*	0.21
		Bovine syncytial virus	
		1:4	0.20
		1:40	0.14
		1:400	0.13

*Bovine leukemia virus is a cell supernatant from FLK-BLV cell cultures; these cultures are co-infected with bovine leukemia virus and non-cytopathic bovine virus diarrhea virus. Equine infectious anemia virus is a stock solution of the MA-1 clone. BSV antigen is a lysate from BSV infected bovine embryonic spleen cells.

Results of p26 detection in macrophage supernatants

Although BIV p26 antigen could be detected in infected FBL cell supernatants, p26 was barely detectable or not detected in supernatants of macrophage cultures from infected animals (Table 5). Samples of macrophage supernatants examined on other dates were consistent with these results. No p26 antigen was detected in supernatants collected from macrophage cultures of 3 control, 4 BIV-infected, 5 BLV-infected, and 5 BIV/ BLV co-infected cattle. Macrophage supernatants from different treatment groups were not found to be significantly different from each other; furthermore, values were not significantly different from background (no antigen) levels. Some macrophage cultures were stimulated with LPS at 4 μg/ml; these supernatants also did not contain detectable p26.

Table 5: Detection of p26 in selected macrophage and FBL cell supernatants.

Sample	OD
Recombinant Gag3 - 0.25 μg/ml	2.05
No antigen	0.11
BIV-infected FBL cell supernatants	
Virus from animal #808	
11/25/92 culture	0.56
11/26/92 culture	0.50
Virus from animal #342	
11/25/92 culture	0.38
11/26/92 culture	0.45
Macrophage cultures from BIV infected animals	
Animal #356 (control)	0.11
Animal #808 (BIV infected)	0.15
Animal #341 (BIV infected)	0.12

Conclusions

Although the p26 capture ELISA does appear to detect recombinant BIV p26 antigens, its use for detecting p26 in cell supernatants appears to be limited. The ELISA could detect p26 in BIV-infected FBL cell culture supernatants; however, detection was marginal in some samples. Furthermore, this assay does not appear to be useful in the system for which it was developed. Although the assay was developed to detect p26 in infected macrophage cultures, levels of p26 appear to be very low in supernatants from these cells. Other evidence, including the inability to detect reverse transcriptase activity in macrophage cultures (data not shown), as well as the evidence that BIV is spread in bovine macrophage cultures mainly through cell contact (Appendix C), support the hypothesis that little virus (or

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viral protein) is released into macrophage supernatants. The sensitivity of this assay (detection limit approximately 0.025 μg/ml) could possibly be improved by purification of the antibodies used; however, this project was not pursued, due to the likelihood that p26 levels in macrophage supernatants would be difficult to detect, even with improved sensitivity of the assay.

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