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The effect of *Mycoplasma bovoculi* infection on bovine natural killer activity

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GENERAL INTRODUCTION

Infectious bovine keratoconjunctivitis (IBK), or pinkeye, is one of the most important ocular diseases of cattle, resulting in significant yearly economic losses in the cattle industry. Several factors which predispose cattle to *Moraxella bovis* - induced pinkeye have been identified. In each case, some damage to the protective conjunctiva occurs before the weakly invasive *Moraxella bovis* is able to initiate colonization of the corneal tissues. One such factor is an initial infection with *Mycoplasma bovoculi* (28, 81). A possible method of decreasing pinkeye infections in cattle herds, therefore, might be to control or eliminate the *M. bovoculi* infection before subsequent infection with *M. bovis* occurs. If the host's immune system could be stimulated to perform more effectively against this mycoplasma, resulting in a decreased period of colonization with reduced tissue damage, the opportunities for disease caused by *Moraxella bovis* infections would be greatly decreased.

The period of time needed for this primary mycoplasma infection to cause an effect on the pathogenicity of *M. bovis* is short. It has been observed that infection of cattle with *M. bovoculi* five to seven days prior to exposure with *M. bovis* resulted in increased incidence of disease in cattle (28, 80). It is well known that the adaptive branch of the immune system requires as much as ten to twenty days to mount a protective response upon primary exposure to an antigen. The secondary response develops much more rapidly, but the animal may have only a few days in which to combat the infection and prevent conjunctival damage due to mycoplasma colonization. Because the innate immune response does not have this lag period, stimulation of this branch of the immune system might prove to be more effective in the control of IBK. One type of native effector cell which has generated much

interest in recent years is the natural killer (NK) cell (34). There is growing evidence that these cells respond to some types of bacterial infections (29, 30, 48) as well as to the viral infections and host tumor cells against which they were originally characterized (79, 86, 92). One such infection is a respiratory mycoplasma infection in mice, which has been shown to augment NK activity (49, 50, 51). While most NK research has been conducted with mouse or human NK cells, NK activity has been demonstrated in cattle against established tumor cell lines, namely K562 and YAC-1, and virus-infected cell cultures (4, 10, 14, 67, 68).

The effect of ocular infections on the host immune system has not been as well investigated as those at other mucosal sites. No work has been published on the bovine NK response to ocular mycoplasma infections, and it is not known if a conjunctival infection will induce a response from this cell population. In addition, although the YAC-1 tumor cell line has been utilized effectively by other researchers, initial attempts to duplicate results which indicated the presence of a bovine NK cell population in this lab have been unsuccessful.

The main objectives of this research project were to 1) develop a working cytotoxicity assay for use in cattle against an established tumor cell line recognized as an NK target; 2) determine the effect of in vivo ocular mycoplasma infections on NK activity, both systemically and, if possible, in the conjunctiva, and 3) determine if NK cells are present in the conjunctiva of control and infected calves.

Explanation of Thesis Format

This thesis has been written following the alternate thesis format. A general review of the literature is followed by a paper being submitted for publication to Comparative Immunology, Microbiology, and Infectious Diseases.

This candidate was the principal author of the paper. A summary of the entire thesis, and one appendix are also included. The references cited in the General Introduction, Literature Review, and General Summary follow the General Summary.

LITERATURE REVIEW

Natural Killer Cells

Types of cytolytic effector cells

Exactly what are natural killer cells? They have classically been defined as a heterogeneous population of morphologically large granular lymphocytes (LGL) which exhibit spontaneous cytolytic activity in a non-MHC restricted manner against a variety of target cell types (34, 76, 99). These targets have included host tumor cells, defined tumor cell lines, virus-infected cells, and more recently, bacteria-infected cells. However, this definition has proved to be too vague in many instances, resulting in frequent use of the term "natural killer-like" cells or activity. To complicate matters further, recent work by several researchers has indicated a population of cells which seem to be T lymphocytes, yet exhibit non-MHC restricted cytotoxicity against recognized targets (37, 52, 71). As increasingly detailed studies have been conducted to evaluate surface antigens, target recognition structures, response to cytokines, and mode of action, there is a clear need for a more specific definition.

It has been posited that three types of cytolytic cells actually exist, contradicting the classical definition which included the $CD8^+$ cytotoxic T lymphocyte, and the natural killer cell. This reclassification includes specific definitions of these two types along with a provision for a subpopulation of non-MHC restricted cytotoxic T lymphocytes (56).

The classic cytotoxic T lymphocyte is characterized primarily by the fact that it has an antigen-specific function developed only after exposure to the antigen, and

that target recognition is possible only in the context of its own MHC Class I surface antigen. Mature T lymphocytes express the CD3/Ti surface complex, through which target recognition occurs. Transcription of Ti genes must occur, and so mRNA must also be present. Mature T cytotoxic cells also express several other surface antigens that identify this group of effector cells. These include T1, T3, T8, T11, and T12. Antibodies against specific surface antigens common to T lymphocytes can block their cytolytic activity. These include antibodies which recognize the CD3 (T3) molecule, the pan-T CD2 (T11) molecule, and the CD8 (T8) structure found on T cells with cytolytic capabilities (56, 76).

The second type of T cell which exhibits cytolytic activity has frequently been labelled a T cell with NK-like activity (37, 52, 71). These cells were identified in studies which made use of NK-like cloned cell lines in the early 1980's, and more recently by researchers investigating $CD8^+$T cell responses to extracellular *Toxoplasma* parasites (43). These cells also express the full complement of mature T lymphocyte surface antigens including the CD3/Ti complex. Anti-CD3 antibody inhibits their activity as with other cytotoxic T cells, yet they have been shown not to be restricted by MHC compatibility (37, 43, 52, 75). Further evidence for this group of effector cells comes from the observation that some T helper clones maintained in vitro spontaneously develop the ability to effect non-MHC restricted cytotoxicity against NK-sensitive targets (71).

The definition of the natural killer cell also includes the trait of not being restricted by MHC compatibility for the purpose of target recognition. These cells are capable of exhibiting cytolytic activity without prior sensitization to the antigens recognized as targets (34). As these are not T lymphocytes, they do not express the CD3/Ti receptor complex, and do not produce Ti mRNA (75). They do,

however, express several surface antigens which are present in high concentrations on these cells. These include NKH1, which has been shown to identify virtually all active NK cells and is also referred to as Leu 7 or CD56 (36,53); and CD16, which is a receptor for the constant fragment of IgG (FcRγ) (52). A more detailed listing of other surface markers will be discussed under the section entitled "Surface Antigens". The target recognition structure for these cells is as yet unidentified, but antibodies against CD3 have no effect on the cytolytic activity of these cells, unlike both types of T lymphocytes. Anti-CD2 antibodies have also been shown to have no effect on the activity of these cells (56). While all cells with NK activity are characterized morphologically as large granular lymphocytes with an abundant cytoplasm containing azurophilic granules, not every LGL has NK activity. Therefore, it seems clear that attempting to define these cells on the basis of activity or morphology alone is insufficient. Within this new classification, natural killer cells still remain a varied and heterogeneous population of cells.

General characteristics

Now that a basic definition of the NK population of cells has been provided, this can be expanded upon with reviews of more detailed specifics of such features as NK surface antigens, functions in vivo, mechanisms of cytotoxicity, regulation of activity, and the NK role in anti-bacterial defense of the host.

As stated previously, NK cells are morphologically large granular lymphocytes that possess the ability to lyse tumor cells without prior sensitization. In addition, they have no memory capacity, since they are a part of the native immune system in the host (76, 99). They are not phagocytes, and do not adhere to plastic; two traits which are used to distinguish them from macrophages (99).

Electron microscopy has been used to gain a better idea of the exact characteristics of the organelles within these cells. NK cells have an eccentric nucleus, with a low nuclear to cytoplasmic ratio (23, 99). The golgi, mitochondria, centrioles, and granules are clustered (12, 35). Rough endoplasmic reticulum is sparse, but the presence of many cytoplasmic vesicles is common (35). The cytoplasm of these cells is frequently seen flowing outwards, much like a pseudopod, and the electron-dense granules can be seen congregating in these areas (23, 35).

The granule type and number varies among the different host species examined. By electron microscopy, they have been identified as .05-.10μm in diameter. There are usually between 2 and 8 granules on average per cell, but within every NK cell line examined, there are always a few cells which contained no visible granules (12). Under high magnifications, either a parallel tubular array of granule content, or hexagonal crystalline structures may be observed (12, 32).

Natural killer activity has been identified in every vertebrate species examined so far. These include humans and mice, by far the most thoroughly studied species, and also hamsters, rats, chickens, guinea pigs, miniature swine, and cattle (11, 15, 30, 51, 99). This broad range would seem to indicate that this population of effector cells is one that has been well-conserved as a basic, first-line defense in vetebrates. There are, however, many differences in the effector cells among these species. One such difference is the age at which NK activity begins to be demonstrated. In mice, rats, and chickens, NK activity is much lower at birth than in adult animals; while in humans there is no such detectable change in levels of activity (99).

Natural killer cells have a unique distribution in the host, far different from that of T or B lymphocytes, or monocytes. The areas most heavily populated with NK cells are the peripheral blood and spleen, and this pattern holds true for both mice and humans. The lymph nodes, peritoneum, bone marrow, and lungs are some of the other sites in which NK cells are routinely found, but in much lower concentrations than those seen in the spleen or peripheral blood. The liver, intestinal epithelium, and lamina propria are sites which may contain NK cells, but if present, the numbers are very low, typically being identified as small numbers of isolated cells. The thymus, however, is a site which has consistently been found to be negative for any indication of NK cell presence (3, 74, 99). In those non-lymphoid organs which do show small numbers of cells positively stained for NK surface markers, there seem to be typical locations in which they reside. For example, positive cells have been found along capillaries and in the, skin, uterus, prostate, vagina, mammary glands, and basal and subepithelial layers of the esophagus (74).

Many of these sites have been examined quantitatively to determine the percentages of NK cells present. As many as 10-20% of the peripheral blood mononuclear cells in humans are NK cells (12, 42). In a bronchial alveolar lavage, where only 5-10% of the total cells obtained are lymphocytes, as much as 40% of these can be NK cells, although this varies with the individual being tested (3). Cells stained as NK positive were found to make up 5% of the cells within the bronchus-associated lymphoid tissue, and 6% of the periarteriolar lymphatic sheath region of the spleen, but only less than 1% of the germinal centers of the spleen and lymph node. The amount of NK activity in these various locations has been found to correlate closely with the percentage of LGL present. The peripheral

blood shows greater activity levels than the spleen, which is correspondingly greater than the lymph nodes, which have more activity than the thymus (74).

Not much is known about the traffic patterns of NK cells in vivo. It has been determined, however, that they are not a part of the recirculating lymphocyte population because no NK cells have yet been isolated from the thoracic duct which drains the lymph vessels into the circulating bloodstream. There is also some evidence that NK cells do not appear to require specialized epithelium to emigrate from the vasculature. Rather, they are selectively attracted by stimuli, much like a macrophage. It has been shown in vitro that NK cells have a positive chemotactic response to interleukin-2, interferon β, and C5a (3).

Surface antigens

This topic, maybe more than any other involving NK cells, is still very confusing and unclear. This is largely due to the fact that there is no universally accepted method for the designation of NK surface antigens. Currently there are several in use, and these include the CD, Leu, HNK, and OK designations, among others which are used more infrequently. It is difficult to try to interpret which are actually representing the same surface structure from one system to the next. By far the most commonly used, however, are the CD and Leu systems.

So is there a pan-NK marker? One that is specific for NK cells only and recognizes the entire population of these cells? Unfortunately, the answer to this question is unclear. Several researchers claim that Leu 11 is one such marker, that it recognizes all LGL, and therefore all NK cells (42, 54, 82). There is some evidence which indicates that the Leu 11^- population of cells contains no NK activity, so by default, then, all cells with NK activity are Leu 11^+ (54). CD16 is

another name for this structure. Supporters of this marker claim that not only all NK activity, but all antibody-dependent-cell-mediated cytotoxicity associated with this cell population lies in cells positive for expression of this marker (52). Both the size and function of this structure have identified it as the receptor for the constant fragment of IgG on these cells (52).

Another marker which is expressed on the majority of NK cells is the HNK-1or Leu 7 antigen, also referred to as CD56 (40, 42, 52, 53, 75). This can be used to identify NK cells, but it is also expressed on the myelin-associated group of myelin sheaths, on some T_H cells, and on myeloid leukemia cells (53). An interesting aside is the fact that antibody to this antigen is thought to be one autoantibody produced in Multiple Sclerosis. In patients with MS, NK activity is very low, perhaps indicating that the individual's NK cells are being destroyed (36). One problem with the use of this as a pan-NK marker is that it does not appear to be on all NK cells, and that anti-Leu 7 plus complement only results in a 5% decrease in observed NK activity (42, 60, 82). There are researchers who refute this, and state that it is found on all active NK cells, and that over 95% of observed NK activity is found within this population of cells (36).

Asialo GM1 is yet another surface structure on some NK cell populations. First identified on murine NK cells, this is a glycosphingolipid which is expressed in high quantities on these cells (43). Antibody against this structure eliminates NK activity in mice. GM1 is found as an element in the brain, and it was discovered by Kasai et al. that rabbits immunized with this substance preferentially produce antibody to Asialo GM1. Anti-Asialo GM1 does react with a small percentage of peripheral T lymphocytes, and so can not be used to give a completely pure sample of NK cells (43).

It may prove easier to identify these cells by markers which they do not express. It is generally agreed upon that NK cells do not express the CD3, CD4, or CD8 antigens common to mature T lymphocytes (75). They also never express the Leu -10, -12, or -14 antigens found on mature B lymphocytes (55). Much confusion has arisen in the past by the presence of apparent NK cells which showed expression of the CD3 T cell receptor complex, as was previously mentioned. By following the current theory of T cells which can exhibit non-MHC restricted killing, these cells would not be labelled as NK cells.

Within cells that express these surface markers, there exist many subpopulations defined by the presence or absence of certain markers. For example, within the population of Leu7$^+$ cells, there are two distinct groups, one of which is NK active, the other of which is not. The latter have been identified as expressing Leu 1, Leu 2, and Leu 4 (54). Leu 1 is common to all T and B lymphocytes, Leu 2 is also known as CD8, which is expressed on the suppressor-cytotoxic group of T cells, and Leu 4 is synonymous with the CD3 receptor complex on T cells (48). The remaining cells which tested originally negative for the expression of Leu 7 can also demonstrate NK activity. In fact, these negative cells may make up as much as 10-40% of the total NK activity seen in an individual (54).

Another example of subpopulations of effector cells was defined by Warren and Skipsey, who determined that there existed differing amounts of expression of the CD16 antigen, and that these levels corresponded to observed activity and response to interleukin-2 (IL-2), a known regulator of NK cells. Cells which expressed large amounts of CD16 were identified by them to be resting NK cells, which had at one point been previously activated. Cells with no CD16 expression, but that were positive for other NK markers, such as CD56, were identified as a

highly activated population of cells, and responded well to IL-2 stimulation. Cells that had an intermediate expression of CD16 were also resting, but these were found to be unable to respond to IL-2 in vitro without a second stimulatory signal from an activated T lymphocyte (98). It does seem rather unlikely that NK cells should require stimulatory signals from activated T cells, and there has been no supporting data published to confirm this theory. The work done on varying levels of CD16 expression does seem to be quite sound, and agrees with the type of antigen expression seen in other cell populations.

At this point, the best way to definitively define NK cells would be those cells which expressed both Leu 11 and Leu 7 jointly on their surface. These double positive cells encompass all NK active cells, and those cells which are negative for both markers have been shown to have no NK activity. There are, of course, subsets of all four of the possible combinations of these two markers, and not all four can be identified in every individual tested (54).

To complicate matters, a group of normal- sized lymphocytes have been identified which are $CD3^-$ $CD16^+$ $CD56^+$, contain no cytoplasmic granules, and yet express comparable NK activity against recognized targets to LGL (40). Although their surface marker expression and cytolytic activity seem to place them in the population of NK cells, their morphology is not in agreement with any current definition of NK cells. Inverardi and Bach have suggested that because they do express known NK surface markers and demonstrate NK activity, they should be classified as small, agranular lymphocytes. They have suggested that these cells may be actually be precursors of the circulating LGL (40).

The designation system for surface antigens in mice is, by contrast, much less confusing. The positively expressed markers common to the majority of murine NK

cells are Ly 5, NK 1, NK 2, and Asialo GM1. The markers found on subsets of NK cells are FcR, and Thy 1, the T cell marker. Lyt 1 and Lyt 2 are not expressed on murine NK cells (73).

To summarize and hopefully help clarify the matter of which markers are found on NK cells, and which are not, most common antigens can be divided into three groups. The first comprise those found on most NK cells: Leu 11 or CD16, Leu 7 also referred to as NKH-1 or CD56, and the complement receptors CR1and CR3 (54, 55, 75, 82). The second are those which are found on subpopulations of NK cells:

Asialo GM1, Leu 2, Leu 5 (the erythrocyte rosetting antigen), and Leu 8, which is also found on granulocytes and monocytes (42, 43, 54, 55). The third group consists of those markers not found on NK cells: Leu1, Leu 3 or CD3, and Leu 4 (54, 73, 75, 98).

Functions in vivo

The classical explanation of the role of NK cells in vivo was one of simply a cytotoxic effector cell, active against tumor cells and virus-infected cells. Much research in the field has opened new roles for these cells in vivo. While cytotoxic activity against tumors and virus-infected cells receive new interest, activity against bacteria is also being investigated. There is also mounting evidence for their role as a mediator of the immune response, and a possible role in protection or regulation of uterine activity during pregnancy.

The role of NK cells in viral infections has been well documented. Viral infections, probably through induced host production of interferon (INF), activate the NK population in vivo. NK cells here are a host defense mechanism by which

virus-infected cells are destroyed by the host. Thus further replication of the virus, and spread to adjacent uninfected host cells is prevented. It is known that infected cells release interferon, which not only helps make adjacent host cells resistant to virus, but also activates the NK cells used by the body to eliminate these virus-infected cells (76). This establishes a coordinated regulation of the immune response (76).

In vivo work with Influenza-infected mice has clearly indicated that while not totally eliminating these infections, NK cells can serve to limit infection until the host's adaptive immune responses become activated. If anti-Asialo GMI, an antibody which abrogates NK activity, is administered before exposure of mice to the virus, much greater mortality is noted. It is believed that both the local NK population of the lung and systemic NK cells work to control this infection (92).

Work in humans with a killed trivalent influenza vaccine has shown that NK cells do not need viral glycoprotein expression on host cell surfaces to respond with increased activity. In one trial, five of five individuals showed at least a 20% increase in NK activity after exposure to the killed virus vaccine, and this activity remained elevated for over 30 days (86). These results contradict other work which has typically shown a short-lived peak of activity occurring several days after exposure to a stimulus. It was concluded by these researchers that NK cells may serve to limit viral infections by acting as an early defense mechanism.

Most recent work involving NK cells and tumors has centered around the use of adoptive immunotherapy. This is a technique in which host anti-tumor cells are removed from the body, stimulated in vitro, and returned to the individual to combat metastatic tumors. Part of the functional definition of NK cells has long been their spontaneous activity against tumor cells (34). Dr. Steven Rosenberg has done

most of the work which has defined the use of adoptive immunotherapy (22,79). One of his more impressive indications for future possibilities of cancer control involved the use of lymphokine-activated-killer (LAK) cells. Large granular lymphocytes were removed from peripheral blood and treated with Interleukin-2, a potent stimulator of NK cells. In 11 of 25 patients treated, there was a decrease in tumor volume of greater than 50% (79).

Other researchers have also been involved in cancer research utilizing NK cells. There is some indication that local pulmonary NK populations may be important in controlling malignancies in the lung. Research utilizing IL-2 activated LAK cells in vivo compared the cytolytic activity of pleural and splenic NK populations in mice. It was found that either systemic or intrapleural administration of IL-2 augmented the activity of pleural NK cells against tumor cell targets in vitro (27). Work by Yokoyama et al. indicated that NK populations in vivo are important in the regulation and control of metastasis of intra-ocular tumors. It was found that elimination of NK activity with anti-Asialo GM1 greatly increased metastasis of these tumors, while augmentation of their activity with IFN decreased observed metastasis (101).

The possibility that NK cells may be able to directly recognize and kill bacteria suggests important new roles for these cells in vivo. To date, it has been shown that both Gram $^{+}$ and Gram $^{-}$ organisms can activate NK cells. By figuring in a correction factor to account for bacterial multiplication during in vitro assays, Garcia-Penarrubia et al. found there was a net reduction in bacterial numbers, indicating direct killing of bacteria had occured. *Staphylococcus epidermidis*, *Salmonella typhi* and *Escherichia coli* were shown to be NK targets. The cells responsible for this activity were found to be Leu 11^{+}. Leu 11^{-} cells showed no

anti-bacterial activity. Enrichment of effector populations for Leu 11^+ cells increased the levels of activity seen. Visualization of the anti-bacterial process through TEM showed cellular processes contacting *E. coli* after only five minutes of incubation. Bacterial ghosts were noted after 90 minutes (29, 30).

Incubation of peripheral LGL with glutaraldehyde fixed *Salmonella* resulted in augmented NK cytolysis similar to that seen with interleukin-2, and caused a slight proliferation of effector cells as well. It was found that these LGL were positive for the expression of NKH-1. Within this group of cells, there were both $CD3^+$ and $CD3^-$ cells. All cells activated by bacteria were contained in the $CD3^-$ subpopulation (94).

Direct activity against *Cryptococcus neoformans* was shown to inhibit net growth of this organism in vitro. Beige mice, which have defective neutrophil, macrophage and NK function, had more *C. neoformans* in their lungs by day 3 post infection than normal mice. Adoptive transfer of NK cells caused an immediate decrease in the numbers of fungi (85).

Research by Lai et al. has indicated that pulmonary mycoplasma infections caused by *M. pulmonis* in mice are controlled by NK cells. In vitro cytotoxicity assays have demonstrated elevated levels of activity in response to these infections. The role of NK cells in the host immune response was determined by elimination of host NK activity with anti-Asialo GM1, which resulted in increased severity of the mycoplasma infection. Treatment of mice with inducers of NK activation accordingly resulted in decreased length and severity of infection (49, 50, 51).

In addition, it has been demonstrated that NK cells can also mediate the indirect killing of bacteria. One way this occurs, in the case of intracellular pathogens such

as *Shigella*, is the preferential recognition and killing of infected cells. It is thought that NK cells can recognize the altered cell membrane of these cells, resulting in their lysis. This would then expose the bacteria to the immune system, allowing complement, antibody, or phagocytic cells to participate in bacterial elimination. Therefore, NK cells in this case may serve to aid other immune mechanisms rather than to directly kill the organisms. The effector cells responsible for this lysis were found to be Leu 11^+ (48)

Another route by which NK cells can mediate indirect killing is through the production of cytokines which then activate other immune cells. As a general rule, contact with tumors, virus, mycoplasmas, or bacteria can induce the production of IFN in leukocytes (99). It has been shown that NK cells can produce all three types of IFN, depending on the stimuli. Virus and mycoplasma-free tumor cells induce them to produce IFNα. Staphylococcal enterotoxin A, and some mitogens such as PHA, induce the production of IFNβ, while virus or IL-2 can induce production of IFN γ (99).

Shigella flexneri and *Salmonella typhimurium* also induce NK cells to produce IFNγ. This immune interferon can then act on other cells to increase the bactericidal activities of host phagocytic cells. At the same time, IFNα also makes epithelial cells more resistant to invasion by *Salmonella* or *Shigella* (47). As with viral infections, the produced IFN also sets up an autocrine feedback system which then further increases NK activity (99).

In studies with *Listeria monocytogenes*, it was shown that early IFNα levels peaked at 24 hours post infection, long before T cells would have time to respond. T cells are usually thought to be the main producers of IFN in the body.

Interestingly, in this study antibody to IFN given just before or after infection had the effect of greatly increasing the severity of the disease. The mechanism for this is unclear, but probably involves the effect of IFN on host epithelial and immune cells (100).

Aside from IFN, NK cells produce other cytokines in lesser amounts. The most important of these is Tumor Necrosis Factor (TNF). Tumor Necrosis Factor production has been induced in LGL after exposure to *Candida albicans*. The cell phenotype responsible here was $CD2^+$ $CD11^+$ $CD16^+$ NKH-1^+ Leu 7^-, typical of a NK subset. In addition, TNF levels showed a peak at 24 hours post infection, very much like NK-produced IFN levels seen by other researchers (19). TNF is usually produced in vivo by macrophages or monocytes. It has a huge array of functions, only a few of which are the hemorrhagic necrosis of tumors, increase in phagocytosis by neutrophils, induction of the respiratory burst in neutrophils, activation of T cells (although not as strong as with IL-1), and increase of anti-parasitic capabilities of eosinophils (57).

Other regulatory roles have recently been suggested for NK cells. One is that of an antigen presenting cell for some types of antigen. This type of activity could stimulate T helper cells and thus T cytotoxic cells, demonstrating a cooperative effort with the adaptive immune system. The other role is a suppressive one for the soluble antigen-induced T cell production of IL-2 (95).

The possible involvement of NK cells during pregnancy is a fairly new idea. The identification of granulated metrial gland cells in the uterus of pregnant mice, which resemble NK cells, has led to formation of this theory. It was found that LGL with granules containing perforin and serine esterases, usually only found in active killer cells, and positive for Asialo GMI and Thy 1, were present at the implantation

site in the uterus. Their function at this time is unclear, but it is believed that they may kill aberrant placental cells, protect the fetus from viral infections, or regulate immune functions through cytokine production (69, 70).

It is clear that more work needs to be done in the area of NK functions in vivo. The inter-relationships between these cells and other host immune cells in particular needs further investigation. NK cells are being found to have a much greater role in host defense than was previously believed.

Mechanism of cytotoxicity

This is another area of NK function which has been the subject of intense research. The exact mechanism by which NK cells recognize and lyse their targets can be broken down into a few basic steps, generally agreed upon by most of the scientific community. Briefly, the first step which occurs is NK cell recognition of and binding to a potential target through a highly specific mechanism. This leads to the activation of the NK cell. Activation is an intracellular process, involving the rearrangement of several cytoplasmic organelles with a general redirection towards the site of attachment. The release of cytoplasmic granules follows, and their exocytosed contents bind to the target cell membrane. There they cause lysis of the cell, most probably through the formation of transmembrane channels which cause the loss of cell volume control in the target (34). Although it is generally agreed upon that perforin molecules are released from the NK cell resulting in lysis of targets, some researchers have indicated that a soluble lytic factor or even Tumor Necrosis Factor α may also play a role (8, 60).

The occurence of initial NK cell binding alone does not ensure that lysis of the recognized cell will follow. It has been observed that NK active cells frequently bind to fibroblasts without causing subsequent lysis. What then are the necessary steps that occur between the moment of contact, and activation of the effector to initiate killing? It has been observed that the binding of the two cells is dependent upon magnesium, and can be blocked by both proteolytic enzymes and cycloheximide (46, 99). This would seem to indicate that the binding process requires the de novo synthesis of some protein molecule by the NK cell to complete binding. Through experimental usage of cytochalasin B, it was discovered that binding also requires complete function of the cytoskeleton (99).

The composition of the target structure is a topic of debate. Some research has indicated it may be a glycoprotein, largely due to data involving YAC-1 cell line variants. YAC-1 cells are usually NK targets, but these variant cells have an increased amount of sialic acid on their surfaces, and it was discovered that they did not support the binding of NK cells (99). Other research has indicated that possibly the transferrin receptor is a target structure. It has been documented that NK-sensitive targets are often rapidly proliferating cell types, and these cells are known to have increased surface expression of transferrin receptor (46). This does seem to be rather circumstantial evidence, however. Target receptor expression may be influenced by a genetic factor. Work by other researchers has shown that fusion of NK-sensitive YAC-1 cells with resistant cells results in progeny which are also resistant to NK lysis (46). Another hypothesis is that the viral glycoproteins expressed on the surface of infected host cells may serve as target recognition structures for NK cells (46).

Once binding does occur, the membranes of the two cells come into close contact with each other, forming zones of apposition, or pockets, where they meet . The pockets formed are on average 20nm in length, and 0.5-1μm in width. It has been suggested that cytoplasmic granules are exocytosed into these pockets (12).

After binding, if the bound cell is recognized as a target, activation of the NK cell follows. The cytoplasmic organelles, including the golgi, centrioles, and granules, respond immediately by lining up at the point of contact, while at the same time, the nucleus is re-oriented away from this location (12). Several chemical reactions also occur at this time. Membrane fluidity is increased by the reactions of methyltransferases, and inhibitors of this reaction have been shown to inhibit lysis. The binding of calcium to calmodulin is also required for activation and subsequent lysis of targets; during activation, the intracellular calcium levels increase dramatically (42).

The result of NK activation is the exocytosis of cytoplasmic granules into the pockets formed between the contacting membranes of the two cells. Just what exactly are the contents of these granules which mediate target cell lysis? The main constituent is perforin, a 70,000 MW protein (31, 89, 90). Perforin has been shown to have both sequence and functional homology with C9 of the complement cascade (31, 90). Perforin has been identified within these granules in a monomer form, but activity is known to occur through a polymeric form of the molecule. It has been determined that one NK cell contains enough perforin to mediate the lysis of one target cell (72). The other main ingredients of the granules are two serine esterases with tryptic serine esterase and carboxypeptidase activities. These molecules are found within the granules as macromolecules complexed to a proteoglycan (42, 60).

After exocytosis of granules occurs, lysis of the target cell follows if conditions are conducive. Released perforin monomers bind to the host membrane through the binding of phosphorylcholine molecules (76). Several regulatory mechanisms control this process in vivo. An internal calcium flux across the effector cell membrane is required for lysis, as perforin monomers can only polymerize in the presence of increased levels of calcium (90). Once polymerized, the size and structure of the pores produced by the transmembrane insertion of the active perforin molecule are very similar to those seen with the membrane attack complex of the complement cascade. As observed by EM, they are approximately 15-20nm in diameter (72). It should be noted that the NK cell does not need to contact the target at the time of lysis. It is able to initiate this process, and leave to attack another cell. Interferon has been shown to increase the rate of NK recycling, as this process is called (46).

The formation of these channels causes a loss in the cell volume control of the target, and lysis is the end result. Cells regulate their volume control through the selective permeability of their cell membrane, and the action of Na^+K^+ ATPase pumps. These pumps regulate the ion concentrations within the cell, establishing huge concentration gradients against the exterior. If these ion pumps fail, ion gradients are destroyed and irreversible damage to the cell can occur. K^+ rushes out of the cell, Na^+ enters the cell, and an osmotic water gain within the cell results. Levels of ATP drop, stimulating glycolysis. After a period of time, both glycogen stores and the internal pH of the cell drop. This is followed by intracellular release of lysosomal contents. At the same time, water infusion causes dilation of the endoplasmic reticulum, the ribosomes detach, and protein synthesis stops. Defects in the cell membrane may be due to the loss of phospholipids caused by the

activation of calcium dependent endogenous phopholipases, and to the decreased reacylation and de novo synthesis of these molecules (18). Lysis of the target cell results.

To date, only one transmembrane signalling protein, NKR-P1, has been identified as playing a role in the activation process. A 60 kD disulfide-linked dimer has been found in high concentrations on the surface of NK cells. It has a high degree of homology with lectin related proteins with receptor characteristics. It is believed to trigger the activation and lytic processes of NK cells when it is cross-linked (32).

This is the basic mechanism of NK-mediated target lysis as outlined by Ortaldo and Herberman (34), and it has been the unchallenged standard for many years. However, several alternate mechanisms have recently been suggested. One of these has been put forth by Bonavida (8), and centers around the release of a soluble cytotoxic factor from NK cells (NKCF). This molecule has been identified as being of a lower molecular weight than perforin. It is thought to allow NK-mediated lysis of target cells without direct cell to cell contact, as a possible alternate mechanism for lysis. A major problem with this idea is that there have been no identified regulatory mechanisms by which NK activation and granule exocytosis are controlled, that do not require cell contact for initiation of the process. However, work by Garcia-Penarrubia et al. utilizing Boyden's chambers, which physically separated bacteria from NK cells, has indicated that contact with the target cell may not be necessary for activation of effector cells to occur. These researchers hypothesize that a soluble factor released from bacteria stimulated NK cells. They also provided evidence for the release of a cytotoxic factor from the NK cells which passed through the .45μm filter used in the chamber (29). As the perforin-

containing granules of NK cells measure only .10μm, this still does not provide strong evidence for the presence of Bonavida's soluble NKCF. A second mechanism is that of the NK role in antibody-dependent-cell-mediated cytotoxicity (ADCC). NK cells express a receptor for the constant fragment of IgG (76). This is thought to enable their binding to targets via receptor binding of attached antibodies (56). This would also provide NK cells with an alternate mechanism for lysis, and has more documented support than the Bonavida mechanism. Functioning as mediators of ADCC would allow NK cells to work cooperatively with the adaptive immune branch to continue their role in the control of infections.

One intriguing question is why are NK cells not lysed by their own cytolytic molecules? Many theories have been suggested , but none has seemed to achieve widespread acceptance. It is known that the serine esterase molecules contained in the cytoplasmic granules are complexed to a proteoglycan. This molecule is a negatively charged Chondroitin Sulfate A, and at the low pH found within the granules, it binds the serine esterase molecules. This serves to immobilize the granule contents while in the cytoplasm of the NK cell (42, 76). Another idea focuses around the homology of perforin to C9. Homologous Restriction Factor and C8 Binding Protein are already found on the surfaces of host cells to protect them from lysis induced by activation of the complement cascade. It is thought that the great degree of homology seen between C9 and perforin allows these two structures to also act in the protection of host cells from the activity of perforin (89).

Probably the most intruiging theory is that revolving around the hexagonal lattices observed in the interior of NK granules. It has been observed that perforin monomers within granules are bound to lipids which are arranged in these lattice

structures. Upon activation, these lattices have been observed to change into bilayer membrane vesicles by EM. These vesicles are thought to then transport the perforin monomers out of the NK cell into the space created between the effector and the target. At this point, the monomers are released from the vesicles and insert into the target membrane (12). This may explain why perforin monomers do not polymerize inside the cell, but what prevents released monomers from inserting into the NK membrane? Several studies have found that killer cell lines, both NK and cytotoxic T lymphocyte, were resistant to lysis by the addition of perforin to the culture media as compared to T helper and tumor cell lines, which were lysed. It has also been noticed that killer cells which are actively producing perforin are even more resistant to lysis than killer cells which are in a resting state (72, 89). This would seem to indicate that some aspect of their membrane structure prevents the insertion of perforin monomers, or their polymerization once inserted.

Regulation of activity

NK cell activity is regulated by a complex interaction of cytokines and other host cells. Although much of this is not yet clearly understood, it is another subject of intense investigation. If stimulatory effects could be defined and applied successfully in vivo, the response of these cells to targets might be greatly increased. More efficient lysis and regulation of other host cells could reduce infections even further, providing an even better initial defense mechanism for the host.

The best known regulator of NK activity is interleukin-2 (IL-2). This cytokine is generally produced by primed T helper cells which have received the double stimuli of antigen presented in the context of Class II MHC, and IL-1 produced by

activated macrophages. It should be pointed out here that many cells can function as antigen presentors, and that virtually every nucleated cell is able to produce Il-1. Therefore, stimulation of T helper cells can occur rapidly in virtually every part of the host. Once activated, T helpers begin to secrete IL-2, a 15.5 kD peptide which has unique biological activities (76).

It is well documented that IL-2 activates NK cells (7, 9, 33, 97). Much of the increase in activity is a result of these LGL differentiating into "lymphokine activated killer" (LAK) cells. LAK cells are able to kill many targets which were previously resistant to NK lysis, or were unrecognizable as targets. They also exhibit greater levels of activity, and it is this fact of which advantage is taken in adoptive immunotherapy for cancer patients (79). Mycobacteria-infected monocytes are targets of endogenous NK activity. The levels of cytotoxicity are generally low, however. After exposure of LGL to IL-2, a great increase in the level of cytolysis exhibited against these infected cells are developed by activated effector cells (7).

The progenitors of LAK cells have been shown to be LGL with a typical NK surface antigen expression ($CD8^-$ Asialo $GM1^+$) and the capability to effect YAC-1 cell lysis. Upon exposure to IL-2, these LAK cells become able to adhere to plastic, unlike normal NK cells, and undergo an increased proliferation. This may result in up to a 30-100x increase in cell numbers within 4 days in culture (5). Giemsa staining of these cells still showed a typical LGL morphology, although cells were slightly larger overall than originally (97). The proliferation seen is a result of NK cells undergoing blastogenesis as a response to IL-2 exposure (5).

The effects of IL-2 are short-lived. LAK cells cultured in vitro did not show continued proliferation when adoptively transferred to irradiated mice unless additional IL-2 was added. To determine this, dividing cells in vivo were labelled

with ^{125}I-labelled iododeoxyuridine, which incorporates selectively into the DNA of dividing cells (22).

It has always been believed that the entire population, or at least a large majority of NK cells responded to IL-2. New evidence has revealed that this is not universally true. The IL-2 receptor is comprised of 2 chains, one of 75KD, and the other of 55KD. The former is thought to possess the signal transduction functions, and has a large intracytoplasmic domain. It is capable of intermediate affinity binding of IL-2. The smaller chain facilitates IL-2 binding, creating a high affinity receptor when combined with the first. Only NK cells with a high density of NKH-1 expression consitutively express both chains. They respond to IL-2 exposure with great increases in proliferation. Other NK cells express only the 75KD protein, and have a much smaller proliferative response (9). It is not known if increases in activity correspond to those noted for proliferation.

Another well known regulator of NK activity is interferon. Many of its effects on NK responses have been covered elsewhere in this thesis, but further specifics will be summarized here. Interferon is a 15-45KD glycoprotein. It was initially identified as being produced by virus-infected cells, giving resistance in a non-specific manner to nearby uninfected host cells. There are three types of interferon:

- α: produced by leukocytes stimulated with either virus or synthetic mitogens.
- β: produced by non-leukocytic cells, such as fibroblasts or epithelial cells, in response to virus or synthetic mitogens.
- γ: produced by T cells after secondary stimulation or lectin stimulation.

Interferon has many functions in vivo, and these range from inhibition of tumor growth, to alteration of cell surfaces (76, 99). It can also have suppressive effects

on some immune responses. For example, IFN has been shown to reverse the stimulatory effects of IL-2 on cytotoxic T cells mediating delayed type hypersensitivity. Interferon prevention of increased capillary permeability is suspected in this instance (65). Of specific interest here are its abilities to activate NK cells, induce NK blastogenesis, and, paradoxically, to protect targets from NK-mediated lysis (99).

Natural killer cells respond to IFN exposure by increasing the expression of surface FcR (CD16), lysing previously resistant targets, and by decreasing their sensitivity to inhibition of activity by anti-Asialo GM1 (99). Many viral infections which are known to induce host IFN production have also been shown to result in increased NK activity. This is probably due to the direct effects of IFN.on NK cells.

All three types of IFN can stimulate NK cells, and this activation has been shown to be dose-dependent. Activation can occur within one hour, or may be even faster if large amounts of IFN are used. This elevated NK activity remains for only about two days unless re-exposure to IFN occurs (99). Work by Djeu and Herberman has indicated that in mice with low endogenous levels of NK activity, exposure to stimulatory agents such as Poly I:C or IFN resulted in immediate increases in activity. This augmented activity peaked after about 3 days, and returned to normal baseline levels by about 7-10 days after exposure. Anti-IFN abrogates this observed increase in activity (20).

Interferon is also believed to play a role in the differentiation and maturation of NK cells. Progenitor NK cells when exposed to IFN, differentiate into endogenous NK cells with low IFN receptor expression. Further IFN exposure results in increased receptor expression, activation, and ultimately blastogenesis and proliferation (99).

Natural killer cells are also able to produce IFN themselves. Depending on the source of stimuli, all three types of IFN can be produced by these cells. Lectins, virus, mycoplasmas and tumor cells are all able to induce IFNα secretion by NK cells (99). Exposure of NK cells to *Shigella*-infected cells also results in the production of IFN (47). The combination of *L. monocytogenes* and TNF exposure has also been shown to induce IFN production by NK cells (100).

Interferon does not always stimulate NK cells, however. If concentrations of IFN become too high, NK activity becomes suppressed. This is an internal down-regulating mechanism which helps to control NK activity. The suppression of NK activity by interferon also occurs indirectly through prostaglandins. Macrophages stimulated by IFN produce prostaglandins, and these substances increase levels of cAMP in cells. This serves to decrease NK activity, which requires high ratios of cGMP to cAMP internally (99).

In addition, as previously mentioned, IFN can also protect targets from NK cells by making them resistant to lysis. For example, NK sensitive monocytes, when treated with IFNα for 2 hours, became resistant to lysis by NK cells for up to 3 days (6). This resistance mechanism, although undefined, does seem to occur post binding of NK cells to the target. In this study recognition and binding were observed to occur normally (23).

Other less-known regulators of NK cells are macrophages, T cells, and manganese chloride (13, 63, 91). Macrophages cultured for 5-7 days in vitro have been shown to cause increased NK activity. Longer periods in culture caused depression of NK activity upon exposure. It has also been noted that the ratio of macrophages to NK cells is important, as low ratios favored stimulation, while high ratios favored suppression of NK activity (13). T cells which are IL-2

activated have been shown to decrease the augmented activity of LAK cells. This response is thought to be partially accountable for the limited success of adoptive immunotherapy treatment (63). Manganese chloride, in the presence of macrophages, was shown to increase NK activity in vitro without causing proliferation of these cells. It was concluded that IFN had a role in the increased cytolysis observed, as it was identified in the culture medium of treated cells, and addition of anti-IFN eliminated this response (41). Manganese chloride is required in trace amounts for many biological functions, and its role as an immunomodulator is currently being investigated (74).

So, while the two classical regulators of NK activity, IL-2 and IFN are probably the most common in vivo, many other regulatory mechanisms are being discovered. It is not unexpected that cells which express so many functions in vivo should be regulated by several independent pathways.

Evidence for bovine NK activity

NK responses in cattle, and domestic animals in general, have not received as much attention as those in mice or humans. Little work is available which conclusively describes NK activity in cattle. Campos and Rossi outlined bovine natural cytotoxic activity in the early 1980's (10, 11). Bielefeldt-Ohmann, Davis and Babiuk investigated bovine natural cytotoxicity against both tumor cell targets, and BHV-1 infected bovine fibroblasts (4). Cook and Splitter have conducted more recent work showing bovine NK cytotoxicity directed against several different virus infected cell lines (17). Much of the problem of investigating bovine NK responses lies in the unavailability of monoclonal antibodies to bovine NK surface antigens.

Without definition by both functional and phenotypic methods, effector cells can not accurately be labelled as NK cells. Although these reports do not contain information

on surface antigen expression of effector cells, it is obvious that the cells in question are bovine NK cells. Isolation of peripheral blood large granular lymphocytes on density-specific gradients, as utilized by these researchers, is the most widely used method of enriching for NK activity. In other species studied, the purity of LGL in these preparations reaches over 98% as tested by surface marker analysis (63).

Work with isolated effector cells has included lymphokine activation and subsequent noted augmentation of activity. Targets used included the standard NK targets K562 and YAC-1 cells, and a third human tumor cell line HSB-2. It was found that effector cells stimulated in vitro with IL-2 were able to directly lyse K562 and YAC-1 cells. However, the addition of specific antibody was required for the lysis of HSB-2 cells. Assays involving bovine embryonic kidney cultures with the infectious bovine rhinotracheitis (IBR) virus also showed direct lytic activity by the LAK cells generated in vitro. Peaks of activity were observed that followed the typical NK response of being short-lived and early. Maximal lytic levels were demonstrated by day 3 after initial stimulation, with activity decreasing to normal levels by day 7 post infection (10). Other work has investigated the ability of purified effector cells to lyse a variety of virus-infected bovine cells (11). Only those bovine cell cultures infected with parainfluenza virus 3 were recognized as targets by the effector cells. Cultures infected with IBR virus or non-cytopathic bovine diarrhea virus were not lysed by freshly isolated bovine effector cells (9). These results reinforce the expected trend of LAK cells demonstrating greater

activity against a wider variety of targets as compared to unstimulated killer cells, and corresponded closely with those predicted for any defined NK cell population.

Work by Splitter et al. has shown that BHV-1 glycoproteins are recognized by these effector cells as targets and induce cytolytic activity against infected cells. This was done without the use of whole virus, by the utilization of expression vectors for individual viral glycoproteins. It clearly demonstrated that isolated effector cells from cattle responded in an antigen-specific manner to identified targets, as the cells not containing viral proteins were not recognized as targets (68).

In a study investigating natural cytotoxic activity in cattle against YAC-1 cells, all ages of cattle were found to respond equally. The youngest animal was one month of age and the oldest used was three years of age. These researchers found that while lytic acitvity occurred within three hours during the in vitro cytotoxicity assay, increasing incubation times to eighteen hours increased the specific lysis seen as much as five fold. BHV-1 infected bovine fibroblasts were also recognized as targets by effector cells. However, it was concluded that the natural cytotoxic cells present were of a monocyte lineage, due to the fact that they were plastic adherant, and their activity was diminished when treated with complement plus anti-monocyte monoclonal antibodies (4). The observed adherance to plastic in their effector cell population suggests that they were not utilizing a population enriched for NK cells.

These researchers have constructed the necessary basis for continued research into and characterization of bovine NK cells and their roles in host protection during infection.

Mycoplasmas and Their Infections

General characteristics

Mycoplasmas are the smallest prokaryotic organisms capable of self-replication. They belong to the order Mycoplasmatales, family Mycoplasmataceae, class Mollicutes. Mollicutes are defined by their characteristic lack of a cell wall. Many are commensal or opportunistic inhabitants of mucus membranes, with few being true pathogens.

Mycoplasmas are only 200-300nm in size, their cell membrane measures 8-10nm in thickness. Because they do not have a rigid cell wall, their shape is pleomorphic. Their circular ds DNA is the smallest genome of any self-replicating prokaryote, at only 5 x 10^5KDa (15, 41).

Mycoplasmas have limited biosynthetic abilities, and thus strict nutritional requirements (15). Most require cholesterol, which is needed for membrane synthesis. They lack the enzymatic pathways used to produce de novo purines or pyrimidines, so these must be provided as well . Most culture media require the addition of yeast extract, serum, and nucleic acids to support the growth of these organisms (50).

The metabolism of mycoplasmas can either be fermentative, deriving ATP from sugars by glycolysis, or non-fermentative. This utilizes the arginine dihydrolase pathway for the production of ATP.

Mycoplasmas establish chronic infections at mucosal sites, and this suggests that some mechanism is present by which they evade host immune responses (16). Several pathogenic factors for these bacteria have been identified. It has been observed that killed *M. fermentans* is toxic if injected into a host, inducing

symptoms similar to those seen with endotoxemia. Mycoplasmas have the ability to adhere to and colonize cell surfaces, a characteristic necessary for the establishment of a close relationship with the host cell from which they obtain their metabolites. Alteration of host antigens may occur; combined with the similarity of mycoplasma and host antigens , this may help to initiate an autoimmune response in the host. In addition, tissue damage has been shown to occur at attachment sites due to the accumulation of metabolites such as hydrogen peroxide and ammonia. Depletion of host arginine and nucleic acids also occurs, due to parasitic utilization by the mycoplasma (50).

Interaction with host immune system

The interaction of mycoplasmas with the immune system is complex. Some host cells and responses are suppressed by these organisms while others are stimulated. Much work has been done in this area to define specific host responses to these organisms.

Mycoplasma arthritidis secretes a soluble factor, MAM, which is mitogenic for T cells and induces IFNα production by them (15). MAM has been shown to act on T and B cells as a type of superantigen. This means it does not require processing and presentation by accessory cells to stimulate these lymphocytes. MAM is able to directly bind to the variable β chain of the T cell receptor, and cause expansion of all cells with this Vβ receptor chain, regardless of the specificity of the α chain of the receptor complex. MAM binds to B cell receptors in much the same way, and induces polyclonal activation in these cells (15, 26).

Mycoplasma interactions with host complement have been found to rely on the virulence of the species involved. In an examination of mycoplasma sensitivity to

complement-mediated lysis, only 1 of 15 species examined were sensitive. This was an avirulent laboratory culture strain. This observed resistance of virulent strains was thought to be due to failure of the mycoplasma to activate the complement cascade (38).

A common feature of chronic mycoplasma infections is an accumulation of neutrophils and macrophages at the site. These cells are present, but their phagocytic abilities are limited, and the disease persists. There are many conflicting reports on whether opsonizing antibodies are required for phagocytosis of these bacteria. It has been shown that unactivated alveolar macrophages and lacteal neutrophils can not kill *M.dispar* or *M.bovis* without the help of specific antibody (39).

Another characteristic of mycoplasmas is their ability to colonize and proliferate on the surfaces of macrophages. By an unknown mechanism, they are able to suppress phagocytosis until an opsonizing antibody is added. They are also able to suppress the phagocytosis of other bacteria, such as *E. coli*, by mycoplasma-infected macrophages (16).

Not all mycoplasmas have suppressive actions on host functions. For example, *M. capricolum* and *M. gallisepticum* membranes induce blastogenesis in murine B cells, and activate bone marrow macrophages to secrete TNFα (88). Mycoplasmas have also been shown to induce Class II MHC antigen expression on host cells (93). This may help lead to the development of autoimmune disorders. Increased Class II antigen expression is seen in most autoimmune diseases. When mycoplasma colonization eventually stimulates macrophage activity, some destruction of host tissue occurs at infection sites. Abnormally high levels of Class II antigen present combine with host antigen expression on macrophage surfaces,

and in this manner macrophages may end up presenting self antigens to T cells which then become activated.

As reviewed earlier, mycoplasmas have been shown to also stimulate NK activity (49, 50, 51, 58). Mycoplasma infection induces IL-2, IFNα, and IFNβ production in the host, which then augment NK activity. It has been demonstrated in mice that infection with *M. pulmonis* augments NK cells, and these cells then respond with increased activity in vitro against YAC-1 tumor cells. Lai et al. have concluded that this increase is largely due to IFN production by other cells stimulated by the mycoplasma infection (49, 50, 51). In this case, it is not thought that the mycoplasma itself is directly stimulating to the NK cells of the mice. This increased IFN level also stimulates macrophages, which in their activated state have been shown to kill mycoplasmas without the help of specific antibody (50).

Mycoplasma-infected cells are also more sensitive to lysis by NK cells. It has been shown that while lysing mycoplasma infected cells, NK cells secrete TNF. This TNF may help other host immune cells lyse those cells infected with mycoplasmas (58). Other evidence has suggested that NK cells are only able to lyse those cultures in vitro which are already contaminated with mycoplasma (16). Given the scope of cell types susceptible to NK-mediated lysis, this hardly seems accurate.

The mycoplasma of specific interest in this work was *Mycoplasma bovoculi* . This mycoplasma colonizes the conjunctival membranes of the eyes of cattle, initiating a close attachment to the conjunctival epithelium (83). *M. bovoculi* has been shown to cause a mild conjunctivitis in cattle (81). In one study of clinically normal cattle, *M. bovoculi* was isolated from 45% of tested animals. This illustrates

its widespread presence in herds (2). *M. bovoculi* is a predisposing agent for *Moraxella bovis*-induced infectious bovine keratoconjunctivitis (IBK) increasing the length of colonization of this organism on the ocular surface (28, 80). However, Kelly et al. found that only six of thirty-one calves with IBK demonstrated the presence of *M. bovoculi* in their eyes (45). This is not surprising, as many other predisposing factors for this disease exist.

Infectious bovine keratoconjunctivitis is a common disease of cattle (96). This contagious disease is seen clinically as an inflammation of the eyelids, conjunctiva, and cornea of cattle. Early symptoms include excessive tearing, and sensitivity to light. Eventually, opaque ulcerations may form on the cornea which may enlarge and ulcerate if not treated. Blindness may result, but even if it does not, the pain associated with infection leads to lethargy and loss of appetite. This results in either reduced weight gain, or loss of milk production, causing significant economic problems in the industry (96).

The immunoregulatory effects of *M. bovoculi* have not been well investigated, probably because it is not generally thought to be as pathogenic as other mycoplasmas. Consequently, many responses of host immune cells to this organism have not been investigated completely Its role as a stimulator of NK responses, as seen with *M. pulmonis* in mice, is also unknown, and remains to be determined.

PART I. MYCOPLASMA-AUGMENTED BOVINE NATURAL KILLER ACTIVITY

Mycoplasma-augmented bovine natural killer activity

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ABSTRACT

The effect of conjunctival *Mycoplasma bovoculi* infection on local and systemic natural killer activity was studied in cattle. A standard in vitro assay against the mouse tumor cell line, YAC-1, was used to measure increases in effector cell activity. Local responses in the eye were investigated by examination of stained exfoliative conjunctival cells and tissue biopsies. An increase in systemic cytolytic activity was seen in infected calves, and maximal values were observed on the second day post infection (DPI). An elevation in the number of large granular lymphocytes was noted in the conjunctival samples, and maximal numbers were again observed at two days post infection. It is proposed that natural killer cell activity may play a role in the initial host responses of cattle to *M. bovoculi* infection.

Key words: natural killer, mycoplasma, cattle, ocular, mucosal.

INTRODUCTION

Mycoplasmas are microorganisms characterized by the lack of a cell wall. They require close association with host cells to supply needed metabolites for survival, and cause chronic infections at mucosal sites. Mycoplasmas have both suppressive and stimulatory effects on many host immune responses (2, 5). *M. bovoculi* is a causative agent of chronic conjunctivitis in cattle (19), and has been identified as a predisposing agent for infectious bovine keratoconjunctivitis, or pinkeye (7, 18).

It is well documented that natural killer activity is one of the immune responses stimulated by mycoplasma infections in mice (12, 13, 14, 15). Natural killer (NK) cells are morphologically large granular lymphocytes (LGL) with the ability to spontaneously lyse target cells without prior sensitization, and they are not restricted by MHC-compatibility requirements (9, 21). Recent work has indicated that many bacterial infections augment NK activity, and that subsequent secretion of cytokines from these activated effector cells may increase the anti-bacterial capabilities of other host cells (4,10, 21). In addition, there is also evidence that NK cells may have a more direct role in these infections through the actual lysis of extracellular bacteria or infected cells (8, 11).

Although NK activity has been observed in cattle (1,18), it has not been as thoroughly investigated as in mice or humans. Because expression of known surface antigens has not yet been identified in cattle, the effector cells responsible for this activity can not truly be referred to as NK cells.

In the present study, we investigated the effect of *M. bovoculi* infections on peripheral blood NK activity in cattle. Because little is known about the presence of cells with NK activity in the eye, changes in large granular lymphocyte numbers as a response to the infection were also noted.

MATERIALS AND METHODS

Calves

Six Hereford calves, aged 3-5 months, were used for the cytotoxicity assays and conjunctival scraping samples. Prior to use in this study, all calves were shown to be negative for the presence of *M. bovoculi* in both eyes as determined by culture of eye swabs and IFA on conjunctival exfoliative samples. Calves were randomly placed in control or experimental groups. Three Hereford calves were used to obtain conjunctival biopsy samples at approximately 20 day intervals to allow sufficient time for healing between samplings. All calves were kept in individual isolation rooms.

Inoculation and Confirmation of Infection

Clone-purified *Mycoplasma bovoculi* strain FS8-7 at passage 9 in modified Friis broth without thallium acetate, was used to inoculate experimental animals. The suspension containing 3×10^8 color changing units (CCU) was slowly instilled into each conjunctival sac to allow adherance of the mycoplasmas to the conjunctiva. Control animals received the sterile medium only. Conjunctival swabs from calves were cultured as dilutions in modified Friis broth to determine mycoplasma colonization levels of control and infected animals.

Effector Cell Isolation for in vitro Assays

This procedure was a modification of the method of Campos and Rossi (1).

Peripheral blood, collected by venipuncture, was treated with acid citrate. This was then diluted 1: 3.5 with Phosphate Buffered Saline (pH 7.3), layered over Histopaque (specific gravity 1.083), and centrifuged at 400g for 45 minutes. The interface was harvested and washed 3 times in Hanks BSS ($Ca^{++}$and Mg^{++} -free). The concentration was adjusted to 2.5 x 10^6 cells/ml and incubated overnight at 37°C and 7% CO_2 in modified Medium 199 (Medium 199 with 15% Fetal Bovine Serum, 50 μM β-ME, and 50 U/ml each of penicillin, kanamycin, and streptomycin). Non-adherent cells were retained, washed once in modified Medium 199, and their viability determined by Trypan Blue exclusion.

Target Cells

The mouse lymphoma cell line YAC-1 was maintained in RPMI 1640 supplemented by 10% FBS and 50 U/ml each of penicillin, kanamycin, and streptomycin at 37°C and 7% CO_2. Twenty-four hours prior to labelling, cultures were split and fresh medium added to ensure log phase growth. Target cells were labelled with radioactive Sodium Chromate (^{51}Cr) by exposure of 1.5 x 10^6 cells to 100 μCi ^{51}Cr overnight at 37°C in 3 ml of supplemented RPMI 1640 on a rocker platform. The cells were then washed 3X and finally resuspended in modified Medium199.

Cytotoxicity Assay

Assays were carried out using effector cells from the overnight incubation at Effector:Target ratios of 80:1 and 40:1. A 100 μl volume of effector cells was incubated with 100 μl of labelled targets at 37°C for 4 hours in a 96 well flat bottom plate. Each combination of effector and target cells was run in triplicate. Maximum

release of ^{51}Cr was determined by addition of 100µl 2% NP40 to wells containing only target cells, spontaneous release of ^{51}Cr was determined by addition of 100µl of modified Medium199 to additional wells containing only targets. Supernatant was harvested using a supernatant collection system (Skatron Instruments, Sterling, VA), and samples were counted in a gamma counter. The specific amount of target lysis in experimental wells was determined as :

(experimental cpm - spontaneous cpm / total cpm) x 100.

Throughout the experiments, spontaneous release of ^{51} Cr was less than 12% of the total cpm.

Conjunctival Exfoliative Samples

Samples were collected with sterile steel spatulas by gently scraping the conjunctiva, and then spread onto glass slides. Slides were fixed and stained by the Diff-Quick procedure (Am. Scientific Products, McGaw Park, Il.). Evaluations were standardized to 80 epithelial cells counted per calf at each sampling date. The numbers of LGL, lymphocytes, and neutrophils seen in these fields were also counted. Slides were counted by a third party in random order to maintain impartiality of the examiner. Results are expressed as the number of LGL per 80 epithelial cells.

Tissue Samples

A second group of calves which were not being sampled for conjunctival scrapings were used to obtain biopsies at 20 day intervals before and during ocular infection with *M. bovoculi*. To obtain samples, calves were anesthetized and

a small area of conjunctiva removed with the use of a sterile forceps and scalpel. Tissue samples were fixed in 10% buffered formalin for 30 minutes and stored in 70% ethanol until processing. Paraffin-embedded sections were cut and stained with hematoxylin and eosin.

RESULTS

Colonization of Calf Conjunctiva with *M. bovoculi*

Calves were infected with *M. bovoculi* by instilling a suspension of approximately 3 x 10^8 organisms into each eye of the experimental animals. While the majority of this suspension did not remain on the eye surface, enough mycoplasma adhered to establish an infection. A mild conjunctivitis marked by a slight reddening of the eyes and increased tearing was noted in calves infected with *M. bovoculi*. The colonization curve (Figure 1) seen was typical of that expected for a chronic conjunctivitis (19). The number of mycoplasmas by 2 DPI had reached 10^4 CCU/ swab, and continued to increase for approximately 4 weeks, maintaining a level of 10^7CCU/ swab. The control calves receiving medium alone showed no signs of ocular irritation, and remained culture negative for mycoplasma.

Effect of *M. bovoculi* on systemic NK Activity

Although the local cell response in the eye was of particular interest, it was not feasible to sample effector cells from this site for use in the in vitro assay. Therefore, it was decided to examine whether a mucosal infection of the eye would stimulate circulating effector cells. A significant increase of NK activity was seen 2 DPI as shown in Table 1. This corresponds well with data from others which have shown an early, short-lived NK activity in response to bacterial infections, even

when bacterial presence continues (13,16). The baseline range of NK activity in uninfected calves was found to fluctuate routinely between 0 and 6% specific lysis.

Histological Examination of the Bovine Conjunctiva

To determine which locations of the conjunctiva might have significant accumulations of lymphocytes, tissue sections were sampled from a variety of conjunctival sites. Routine hemtoxylin and eosin staining of tissue sections allowed the visualization of lymphocytes in the conjunctiva, and this gave some indication of where LGL were likely to be found. Figures 2 and3 reflect the great variations seen in the conjunctiva from one area to another within the same uninfected, healthy calf. Large numbers of lymphocytes (Figure 3) were found in the area previously reported to contain CALT (6). The lymphoid tissues in these regions created folds in the conjunctiva; germinal centers were observed, and modified epithelium was noted over these sites. As these are likely areas in which antigen could be contained and presented, it would seem that these would also be good regions for NK cells to contact and be stimulated by antigen. The upper lateral surface of the eye is covered with a thin conjunctival membrane, and few lymphocytes were found in these tissue samples (Figure 2). This is the region from which conjunctival exfoliative samples were taken. No histological differences were noted between infected and control calves (Figure 5).

Detection of LGL in Conjunctival Scrapings

The *M. bovoculi* infection did induce a local lymphocyte response as seen by examination of stained conjunctival exfoliative cell samples. Figure 6 illustrates a

typical stained cytology sample obtained from an infected calf. A sharp increase in the numbers of LGL was detected by 2 DPI in infected calves. These numbers then decreased but remained slightly elevated over control animals throughout the course of the sampling (Figure 5). The initial peak response and subsequent decline correspond closely with results measuring systemic cytolytic activity of effector cells. Because the counts of control calves remained so consistently low, there was no indication that the sampling itself was a source of irritation in the eyes of the inoculated calves, and it was thus dismissed as the cause of the increased numbers of LGL noted.

A normal neutrophil response was also noted upon examination of the stained conjunctival exfoliative samples. Increased neutrophil numbers were observed in infected calves, and remained elevated above the numbers observed for control calves through 26 DPI (data not shown). This indicates the immune response observed did follow an expected pattern in the infected calves.

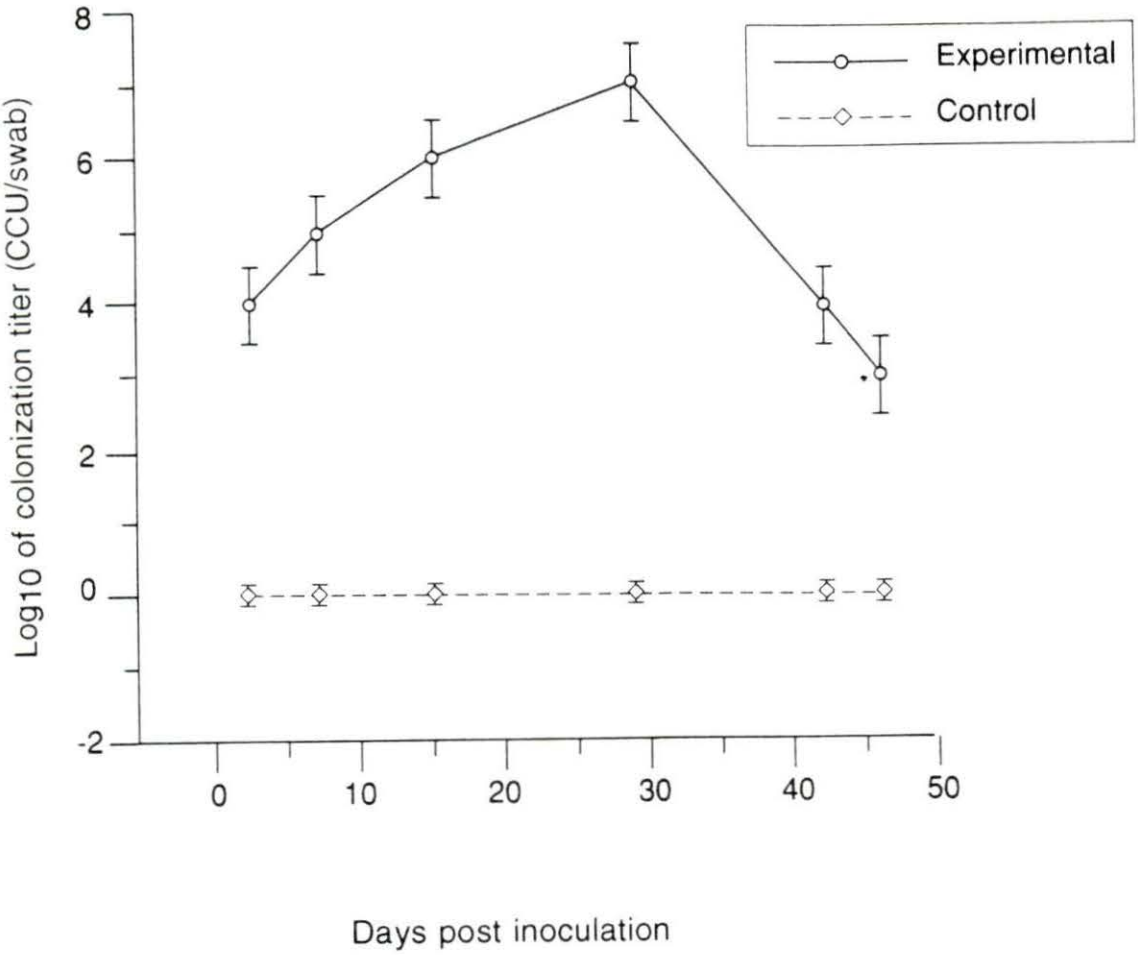


Figure 1. *M. bovoculi* colonization levels of calf conjunctiva as determined by culture of eye swabs (expressed as mean ± SEM; n= 3 calves). Experimental calves illustrated a typical colonization pattern, while control calves remained culture negative.

Table 1. Effect of *Mycoplasma bovoculi* on systemic natural killer activity

DPI	Infected calves	Control calves
	% specific lysis[a]	
0	2.95 ± 4.17	1.6 ±2.26
2	*10.37 ±3.19	4.97 ±0.25
7	0.50 ±0.71	2.65 ±0.64
9	3.61 ±3.72	5.50 ±5.50
14	1.28 ±1.48	3.17 ±2.49
30	3.93 ±3.28	2.77 ±0.72
40	0.60 ±1.04	0.10 ±0.16
42	1.31 ±1.07	0.17 ±0.15
44	0.33 ±0.30	1.84±2.16
46	7.33 ±4.72	2.77±2.40

[a]Results expressed are mean ± std deviation (n=3 calves). Effector: target ratio for given data was 80: 1.

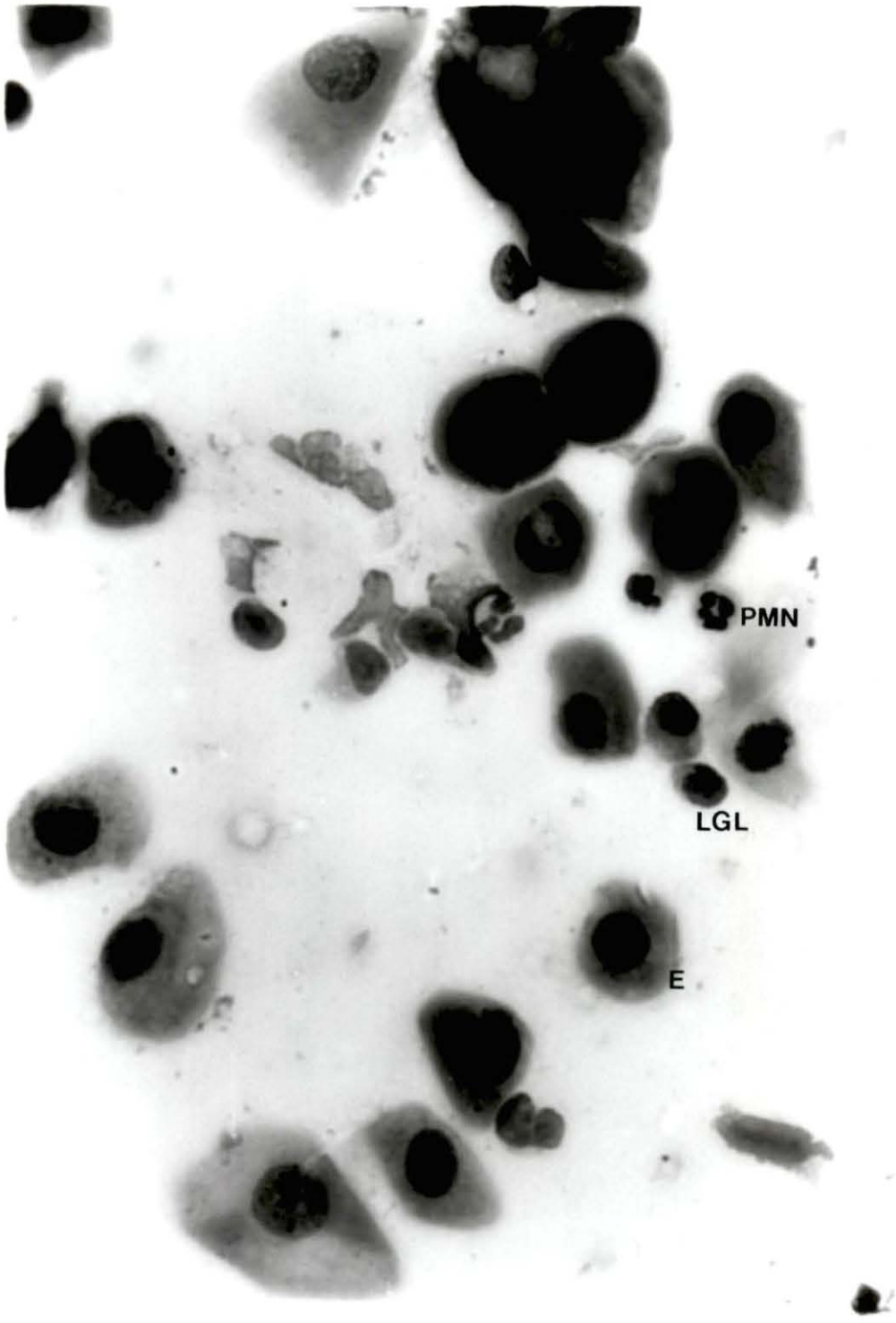
*significantly different from controls for the same DPI by the student's t test (P<.10).

Figure 2. Photomicrograph of a conjunctival section from a control calf. Conjunctival exfoliative samples were obtained from this region of the eye surface. Tissue is mainly connective tissue with few lymphocytes (LC), and a thin layer of epithelium.

Figure 3. Photomicrograph of a conjunctival section from a control calf. Region sampled is from the lower medial conjunctival sac and shows lymphoid follicules with germinal centers and modified epithelial mucosa (arrow).

Figure 4. Photomicrograph of a conjunctival section from an infected calf. Region sampled is from the lower medial conjunctival sac. The conjunctiva of infected calves did not show histological differences from comparable sections of control calves.

Figure 5. Photomicrograph of a stained conjunctival exfoliative sample, illustrating the morphology of a large granular lymphocyte (LGL), neutrophil (PMN), and epithelial cell (E).


PMN
LGL
E

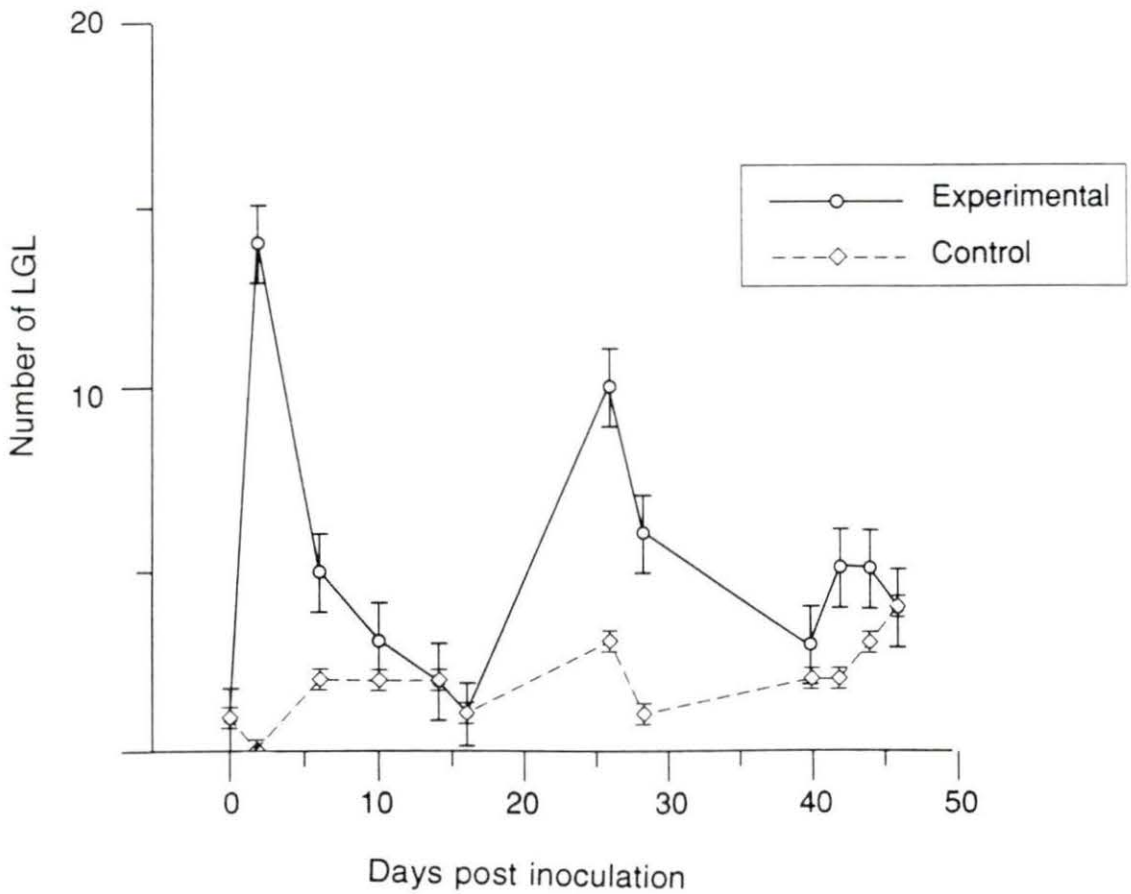


Figure 6. Numbers of large granular lymphocytes detected in conjunctival exfoliative samples from experimental and control calves. Each point is the mean number of LGL per 80 epithelial cells counted (mean ± SEM; n=3 calves). Experimental calves demonstrated an increase in the numbers of large granular lymphocytes in the conjunctiva, as compared to control calves.

DISCUSSION

Inoculation of the bovine conjunctiva with *M. bovoculi* causes a mild chronic conjunctivitis. This infection stimulates a population of host LGL which exhibit NK activity, and these cells respond with increased peripheral cytolytic activity and population increases within the conjunctiva.

There may be two reasons for the observed responses: 1) mycoplasma presence directly stimulates cells with NK activity or 2) mycoplasma indirectly cause stimulation of these cells through induced production of regulatory cytokines by other host cells. The first theory is not supported by our data, although several other bacteria have been shown to directly stimulate NK cells (7,10). It would be expected that if the mycoplasma themselves were directly stimulatory, then an increase in the number of mycoplasma should be followed by a corresponding increase in cytolytic activity of effector cells. This relationship was not observed. The cytolytic activity and increases in numbers of LGL observed had peaked and declined to normal levels while the numbers of mycoplasma were still relatively low (2 DPI titer of 10^4 CCU/swab). As the mycoplasma reached their highest level of colonization titer (10^7 CCU/swab) at 28 DPI , both the cytolytic activity and number of lymphocytes had returned to near normal levels. There was no clear correlation between the degree of mycoplasma colonization and the effector cell response. This suggests that the observed responses were induced by cytokine production from other host cells which were directly stimulated by mycoplasma presence. Other mycoplasma species are known to induce interferon (IFN), and IFN is a known regulator of NK cell activity (2,18). This suggests that IFN is a possible regulator of the observed reponses.

Interferon-induced NK activity may occur in as short a time as 1 hour in vitro, and is short-lived, as cells return to their normal activity levels within 18 hours unless reactivated. Interferon causes increased cytolytic activity, increased range of target recognition, proliferation, and even induced secretion of IFN in NK cells (18). It has been suggested that NK cell-produced IFN is important in the early host defense against bacterial infections, as elimination of either NK response or IFN with monoclonal antibodies results in increased severity of disease (4,11,12).

Further experimentation involving monitored IFN levels and NK activity as a result of *M. bovoculi* infection would be necessary to determine if this cytokine were responsible for the increased cytolytic activity and numbers of LGL observed.

The relatively low percent specific lysis observed in this assay as compared to other host effector- target systems may be explained in several ways (1,11). Human donors of NK cells have been shown to have great variation in normal endogenous levels of NK activity (8), and the animals selected for our trials may have had low levels of NK activity. Within the two groups of calves, variation of as much as 20% specific lysis for the same DPI was observed (data not shown). Other work with NK cytotoxic assays has shown a twofold increase in observed percent lysis with an increase in incubation time from four to eighteen hours (20). Alternately, there may be a species-specific response in recognition of different target cell lines. Comparisons using different cell lines, such as K562, and incubation times should be done to determine the optimal parameters for use in this assay.

There is little documentation which relates NK activity to ocular infections. Because NK cell presence has been demonstrated at other mucosal surfaces

(20,21), it was believed that they would also be found at the conjunctiva. Conjunctiva-associated lymphoid tissue (CALT) has been reported in other species at the medial region of the lower conjunctiva (6), so this was one area chosen for sampling. One problem with examination of tissue sections solely for active NK cells is the lack of established bovine monoclonal antibodies for use in immunohistochemistry. Attempts to label sections with an antibody against a mouse NK surface antigen were unsuccessful (data not shown).

A role for NK active effector cells in the response of cattle to *M. bovoculi* ocular infection was illustrated in this work. However, it must be noted that the increases in cytolytic activity and LGL numbers did not resolve this infection. One way to determine if the observed responses were protective would be to eliminate NK activity through the use of monoclonal antibodies to NK active cells, resulting in increased severity of disease and colonization titers. This is not possible at this time due to the lack of defined antibodies for this cell population in cattle. Another method would be to investigate the results obtained if known NK-inducers such as IFN were administered to cattle to further elevate NK activity. In this case, the length and severity of colonization should be decreased, as has been noted in mice (12).

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GENERAL SUMMARY

Three research goals were outlined in the Introduction section of this thesis. The first, to develop a working cytotoxicity assay for bovine NK active cells against an established cell line, was achieved. This was necessary in order to help define the population of effector cells obtained as being NK cells. The current definition has three criteria: morphology, function, and surface antigen expression. The visual morphology of the enriched cell population agreed with that of a LGL. The function was determined by the non-specific lysis of an established cell line known to be a target for NK cells. The third criterion could not be established due to the unavailability of monoclonal antibody (Mab) against specific surface antigens expressed in high density on the surface of bovine NK cells. Antigens unique to bovine NK cells have not yet been defined, and it was not feasible to run exhaustive comparisons with all known mouse or human Mabs to identify homologous markers. Identification of effector cells by surface antigen expression is necessary to ensure that $CD8^+$ cells are not present. Because T cells which lack MHC histocompatibility requirements have been identified, the possibility of lysis due to these cells should be eliminated.

The second goal was to determine the effect of an ocular *M. bovoculi* infection on bovine NK populations. The effect was observed as an increase in the measured cytolytic activity. This is one response displayed by NK cells activated by the presence of some external stimuli. This response is believed to be caused by cytokines. Cytokine production was very likely induced in the cattle by the presence of mycoplasma. The large number of mycoplasma inoculated into the

eye would clearly be sufficient to induce a host response. Mycoplasma-induced IFN production has been observed in other species, and this IFN could activate NK populations. Evidence of IFN production in the eye as a response to *M.bovoculi* inoculation should be documented. Characterization of a local NK response to the infection was not as clearly obtained. An attempt to specifically label LGL in the conjunctiva with antibody against the NK marker Asialo-GM1 failed (see Appendix A). Results were obtained which did show an increase in the number of LGL as a response to mycoplasma presence. This proliferation also agreed with results expected if IFN were the direct cause of effector cell activation.

Because immunohistochemical staining failed, it was not possible to achieve the third goal of determining the presence of NK cells in the eyes of calves. The only evidence obtained to support this theory is circumstantial. Hematoxylin and eosin staining of normal calf conjunctival sections revealed lymphoid regions in the eye, possibly similar to the CALT seen in turkeys. There is some evidence which indicates these lymphoid regions should contain NK cells. NK cells are a subpopulation of lymphocytes, but their traffic patterns in the host are not clear. Conjunctival scraping samples examined histologically did show low numbers of LGL in the eyes of normal calves, with increases seen in inoculated animals. Availability of needed antibody would resolve the question of NK presence in the eye, and specific antibody to markers which are expressed in high density upon NK activation would also demonstrate local responses to the mycoplasma.

Overall, this work provided good preliminary evidence for the presence of NK cells in the bovine eye, and the response of bovine effector cells with NK activity to ocular infection with *M. bovoculi* . Results obtained correlate well with published results of similar mycoplasma infections, and NK responses to bacterial stimuli.

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LAN.

APPENDIX A. IMMUNOHISTOCHEMICAL STAINING FOR NATURAL KILLER ANTIGENS IN BOVINE CONJUNCTIVA

This appendix summarizes an attempt to specifically stain bovine natural killer cells with the rabbit anti-Asialo GM1 polyclonal antibody by developing an IHC protocol for this tissue. NK cells are defined not only on the basis of their function, but also on the expression of recognised surface antigens unique to this population of effector cells. The use of monoclonal (Mab) or polyclonal antibody is required to identify these antigens. The most commonly used antibodies for human NK cells include: anti-CD16, anti-Leu 7, and anti-CD56. By far the most widely used for murine work is anti-Asialo GM1, which recognizes a glycosphingolipid on the surface of NK cells.

The two available Mabs for bovine work which could possibly have been of use here lacked the specificity needed to utilize them as a means of identifying NK cells. The mouse anti-bovine Null Mab (non-T, non-B) B7A1 (VMRD, Pullman, WA), was reportedly specific for non-T non-B lymphocytes. Further investigation with the producer of this Mab revealed that it had later been found to identify $CD8^+$ T cells. The other was an anti-bovine $\gamma\delta$ receptor for non-T non-B lymphocytes. Neither were specific for antigens expressed on active NK cells. Because many LGL fail to show NK activity, use of these antibodies would fail to accomplish desired results.

The procedure followed was an adaptation of the combined methods of Jane Fagerland (ISU Vet. Path.) and the provided instructions for use with the Vectastain ABC Kit (Vector Labs, Burlingame, CA) employed.

Tissue sections were obtained from sedated, anesthetized calves. Conjunctiva samples were fixed in 10% neutral buffered formalin for 30 minutes, and stored in 70% EtOH until processing. Slides were paraffin embedded and sectioned at a 5μm thickness. A diamond pen was used to encircle the fixed tissue sections. Slides were deparaffinized in the following sequence:

1) xylene 2 changes, 5 minutes each
2) absolute EtOH 2 changes, 3 minutes each
3) 95% EtOH 2 changes, 3 minutes each
4) 70% EtOH 1 change, 3 minutes and
5) distilled H_2O 5 minutes.

Next, slides were incubated for 30 minutes in 0.3% Methanol and washed in a buffer solution of 10mM Sodium phosophate at pH 7.5 and 0.9% saline solution (PBS) for 5 minutes. After this, slides were trypsinized in a 0.1% Trypsin solution, and washed in buffer again for 5 minutes. Normal blocking serum was then added to the slides to prohibit non-specific staining. This was carried out in a humid chamber, and allowed to incubate for 20 minutes. The excess was then shaken from slides, before returning them to another PBS wash. Primary antibody, anti - Asialo GM1, was added and allowed to incubate for 30 minutes in a humid chamber. A 10 minute buffer wash followed. After this, secondary biotinylated antibody, an anti-rabbit, was added to the slides and allowed to incubate for 30 minutes also. After another wash in PBS, the ABC reagents (Vectastain) were added to the slides. This solution contained an avidin-horseradish peroxidase conjugate which would complex to the biotin on the antibody, and the enzyme with which the chromogen substrate would react to create a color change. The chromogen used was diaminobenzidine (DAB), and a nickle solution was added to

increase the sensitivity of the reaction. A counterstain of Gill's hematoxylin was then used to provide the nuclear staining necessary for visualization of cells. Before mounting the slides with a coverslip, a dehydration process, the reverse of the deparaffinization outlined at the beginning, was completed.

Various primary antibody dilutions, from 1:2.5 to 1:500 were tried, as well as various times for trypsinization, 0-5 minutes, and lengths of incubation for the primary and secondary antibodies. Many initial attempts resulted in large amounts of non-specific staining by the DAB. High dilutions of anti-Asialo GM1 showed staining of the nuclei of epithelial cells, or very little color at all on the slides. No peripheral staining of the cytoplasm was observed. Control slides made of mouse spleen sections were also used, and these also failed to show areas of positive staining.

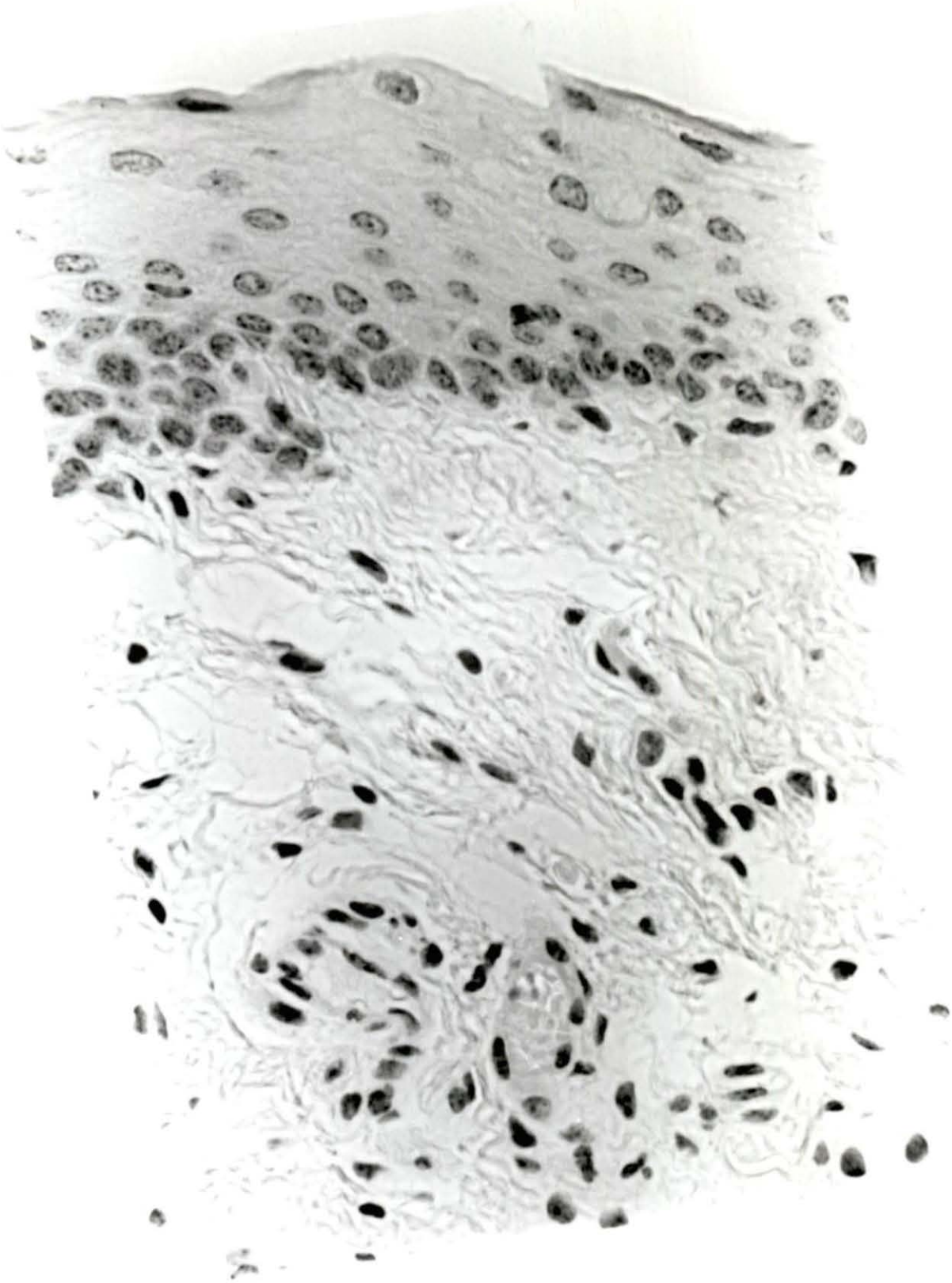
As a result of this, frozen sections were prepared in OCT blocks and cut on a cryostat. These also failed to show positive staining. Loss of tissue architecture was so great that clear viewing of stained secitons was difficult. However, even with a low dilution of primary antibody, 1:5, no positive staining was detected.

Results of the final method are shown in Figures 1, 2, and 3. These photos are indicative of the general results observed. All three sections are from the same calf. Results from the negative control procedure are shown in Figure 1. Use of the noraml blocking serum, primary antibody, secondary antibody, ABC reagents, and DAB resulted in abscence of non-specific staining. All observed stain is due to the hematoxylin counterstain. Figures 2 and 3 illustrate the non-specific DAB staining of the epithelial layer which resulted when the primary antibody was added. Instead of a clear ring of color around the periphery of individual cells, the entire

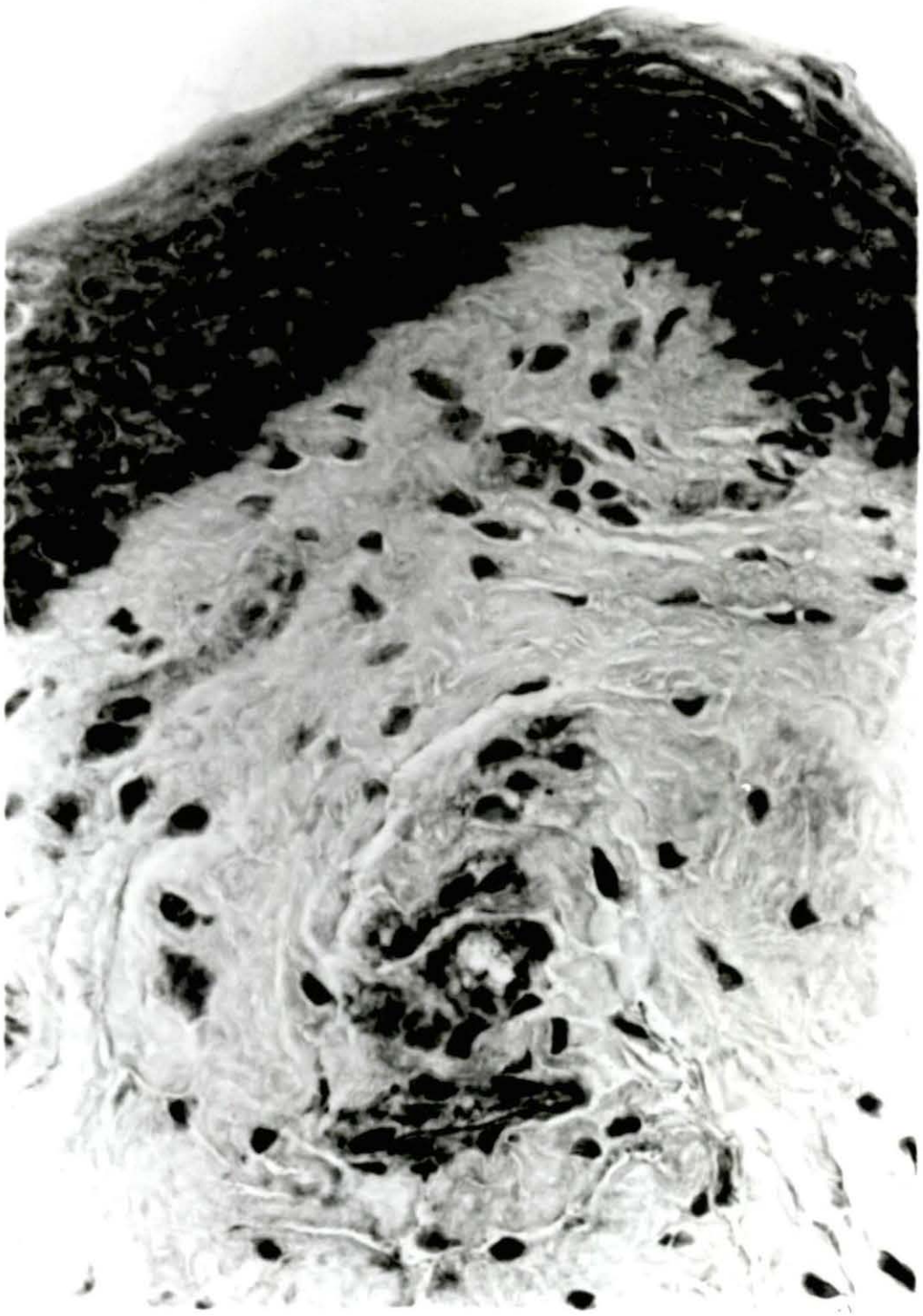
cytoplasm of many cells picked up the stain. In some areas of the conjunctiva, basal layers of the epithelium were darkly stained, while keratinized cells on the outer surface seemed to pick up less of the DAB. Other areas of the epithelium did not show this type of staining. Figure 3 contains a few cells with dark DAB staining around the periphery, but because the overall quality of the staining was poor, these could not definitively be called positive.

The control procedures indicate that the staining process developed was successful. A wide variety of conditions attempted failed to give clear positive NK staining. This may indicate that anti-Asialo GM1 is not the correct antibody for use in the staining of bovine conjunctival sections for NK cells.

A1. Photomicrograph of immunohistochemical stain of calf conjunctiva. Negative control- no primary antibody used. Counterstain with Gill's hematoxylin.



A2. Photomicrograph of immunohistochemical stain of calf conjunctiva. Primary antibody used was rabbit anti- Asialo GM1 at a 1:5 dilution. Epithelium exhibits severe non-specific staining.



A3. Photomicrograph of immunohistochemical stain of calf conjunctiva. Primary antibody used was a 1:5 dilution of anti-Asialo GM1. Questionable positive staining can be seen on several cells located in the submucosa (arrows).