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DETECTION OF CANINE CYTOKINES USING MESSAGE AMPLIFICATION PHENOTYPING

by

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ABSTRACT

CANINE CYTOKINE MAPPING USING
HUMAN AND MURINE PRIMERS

by

Dana Renee Atkinson

Message amplification phenotyping (MAPPing) involves Polymerase Chain Reaction (PCR) amplification of complimentary Deoxyribonucleic Acid (cDNA) reversely transcribed from Ribonucleic Acid (RNA) using primers specific for discrete cellular products. Murine positive control cDNA was purchased; human and canine cDNA were prepared from non-stimulated and phytohemagglutinin stimulated lymphocytes. The cDNA was amplified with primer sets, based on human and murine sequence data, for B-actin, Cluster Designation 4 (CD4), Cluster Designation 8 (CD8), Interleukin-2 (IL-2), gamma Interferon (gIFN), Tumor Necrosis Factor Alpha and Beta (TNF-A, TNF-B). Results represent optimal conditions for MAPPing canines. The fidelity of amplified products was confirmed by sequencing and comparing canine sequences to published sequences. Canine and human CD4 are 75% homologous; canine and human IL-2 are 81% homologous; canine and human gIFN are 75% homologous; canine and murine TNF-B are 92% homologous. Results indicate that canine messages can be MAPPed using human and/or murine primer sets.

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INTRODUCTION

The primary goal of this project was to develop the MAPPing technique for canine cytokines and surface markers using human and/or murine primers. For this project, RNA was isolated from T lymphocytes and amplified using primer sets for the cytokines IL-2, gIFN, TNF-A and TNF-B as well as the surface markers CD4 and CD8. B-actin was used as a positive control. A secondary goal of this project was to obtain canine sequence information because other than gIFN there is currently no information available (1).

In 1989, Brenner, et. al., incorporated the PCR into a technique called MAPPing (2). MAPPing is a process which involves isolation of messenger RNA (mRNA) followed by reverse transcription of the mRNA to produce cDNA and subsequent amplification of the cDNA by the PCR (2). This procedure can be used on cells of any type and can determine the presence of any mRNA by using specific primers in the PCR (2).

The PCR is a 3 step process which uses a primer set specific for Deoxyribonucleic Acid (DNA) regions flanking a DNA sequence of interest and results in amplification of the DNA sequence. The DNA has a helical structure consisting of two complimentary strands, the sense and the anti-sense.

One of the primers is complementary to the flanking region at one end of the sequence on the sense strand while the other primer is complementary to the flanking region at the other end of the sequence on the anti-sense strand (3). The reaction mixture consists of template DNA, both primers, nucleotides, buffer, water and Thermus aquaticus (Taq) DNA Polymerase. The first step of PCR involves denaturing the double stranded DNA at 95°C for one minute. The second step involves annealing the primers at 50-60°C for usually one minute. The third step involves extending the primers by Taq DNA Polymerase addition of nucleotides at 72°C for usually one minute. The Taq DNA Polymerase adds nucleotides to the primer using the DNA strands as templates and because primers are annealed to each strand, a copy of the double stranded DNA is produced. The PCR is usually repeated for 30-40 cycles. Because the primers flank the sequence of interest, the process ultimately leads to exponential amplification of the DNA sequence of interest (3).

There is evidence that the PCR primers may not need to be species specific, especially when dealing with highly conserved gene sequences (4). PCR primers for amplification of mitochondrial DNA have been designed that amplified DNA from over 100 species of animals, even though there are large differences among the mitochondrial DNA of animal species (4). This suggests that PCR primers for genes may amplify cross species. The primer annealing temperature greatly affects the efficiency of the PCR and the optimal

annealing temperature can be predicted by the length of the primer and the guanine and cytosine content. Optimal annealing temperatures for each of the primer sets used in this project for the specific species are 61^{o}C; however, decreasing the annealing temperature for any primer set allows for amplification of sequences to which the primers do not match perfectly. This usually results in undesirable non-specific PCR products; however, in this project it made amplification of the canine sequences possible using human and murine primers. The Taq "polymerase requires absolute matching of the primer to the template only in the last few bases of the 3' end of the" primers (4). There is also evidence for species cross reactivity of nucleic acids as seen by isolation of murine nucleic acids using human probes (5,6,7,8,9). In addition, there is evidence for species cross reactivity of cytokines as seen by cell responses to the cytokines (7,12,13). For example, "No species specificity has been observed for the TNF activities isolated from such sources as rabbits, mice, rats, and humans" (7). "This activity was discovered originally in the sera of mice and rabbits injected first with Mycobacterium bovis strain bacillus Calmette-Guerin (BCG) or other immunostimulatory agents, and subsequently with endotoxin. Serum from such animals causes haemorrhagic necrosis and in some cases complete regression of certain transplanted tumours in mice" (13). Another example of species cross reactivity of cytokines was noted when human

recombinant (Hr) interleukin-2-I^{125} was evaluated for its effects on equine, caprine, ovine, canine and feline peripheral blood lymphocytes; "HrIL-2-I^{125} induced lymphocyte proliferation in all animals tested" (12). gIFN, however, is known to exhibit species specificity (6).

PCR has many advantages over protein assays, such as immunoassays and bioassays, and other RNA assays, including northern or dot blots and in situ hybridization, which have been used in the past to detect cytokine gene products (14). Immunoassays like radioimmunoassays (RIA's), immunoradiometric assays (IRMA's) and enzyme linked immunosorbent assays (ELISA's) are quick and easy to perform because they may be used to detect cytokines in biological fluids (14,15). They are sensitive and molecule specific because the antibody used for binding is specific for the cytokine to be detected. The specificity allows these assays to be quantitative because the amount of antibody bound can be related to the amount of cytokine present (14,15). The quantities detected by these assays are related to the immunoreactivities of the particular cytokines; however, these quantities may be quite different than those related to the bioreactivities of the cytokines. For example, the presence of soluble receptors to which the cytokines can bind may decrease the immunoreactive quantities enough that they may be detected as a low quantity or not detected at all; however, the bioreactivity quantities may remain unchanged. Also, the presence binding

proteins which can inhibit cytokines can decrease the bioreactive quantities of the cytokines enough that they may actually have little or no effect on the immune system; however, the immunoreactive quantities may or may not be changed (14,15). The difference between the immunoreactive and bioreactive quantities is particularly a problem because cytokines are active at such low concentrations, 10^{-10}-10^{-15}. These assays are not good for the identification of cell types producing cytokines not only because biological fluids are often used but also because of the difficulty in differentiating between secretory and target cells. Membrane bound forms of cytokines may be present on secretory cells while cytokines bound to their specific receptors will be present of target cells (14,15). Bioassays are more widely used than immunoassays because they are based on bioreactive levels of the intact proteins. They are, however, not molecule specific. This poses a problem particularly for quantitation because cytokines are not produced in isolation (14,16). Bioassays also cannot detect cell surface cytokines because cell culture supernatants are assayed for bioreactivity (14,16). Bioassays differ for each cytokine, therefore it can be difficult to maintain assays for a great variety of cytokines and to perform them all at once (14,16).

The more widely used RNA assays do not have the disadvantage of depending on the immunoreactive or bioreactive levels of proteins for detection while at the

same time they are molecule specific because probes used are specific for nucleic acid sequences. The RNA assays do detect the expression of cytokine genes; however, it should be remembered that this does not ensure translation of biologically active proteins, it only gives a good indication of what proteins cells are attempting to produce. For the blotting techniques, such as Northern blotting and dot blotting, quantitation of mRNA is also possible by the extent of probe binding (14,17). Northern blotting has an added advantage over dot blotting in that length of mRNA can also be verified (14,17). The greatest disadvantage of the blotting techniques is the requirement for large amounts of mRNA, up to 15 ug's, to assay for the great variety of cytokines. Because of this, large numbers of cells are also needed and this limits the sample type on which the blotting techniques can be used (14,17). In situ hybridization can detect gene expression in single cells, allowing the technique to be used on a wider range of cell samples, and it can define cell types producing cytokines; however, great disadvantages of this technique are that the samples need to be fixed on a microscope slide and that the sensitivity has not been determined (14,17).

The most notable advantage of the PCR over these assays is its sensitivity. Because of the exponential amplification, low copy number mRNA transcripts, such as cytokines, can be detected (18,19). Little RNA is needed for synthesis of cDNA used in the PCR, therefore a large

variety of cytokines may screened for at one time. Because of this, few cells are also needed, allowing the technique to be used on cells that cannot be obtained in large enough quantity for other assays (18,20). The technique is very specific because the primer sequences used in the PCR are based on the DNA sequence data. The technique has the unique advantage of storing cDNA and eliminating RNA degradation by contaminating RNases. Because the technique can be completed in two days, it provides for rapid detection of a great variety of cytokines (18). Until recently, the major disadvantage of this technique was the inability to quantitate RNA; however, use of an internal standard in the PCR now allows for quantitation (20). One remaining disadvantage is possible contamination of the PCR reaction, leading to false positives (18). With careful handling of the cDNA, however, this can be avoided.

Investigators have used this technique to detect cytokine mRNA's from T lymphocytes, B lymphocytes, and macrophages stimulated <u>in vitro</u> as well as for the detection of mutations and gene inheritance (20,21,22,23). In addition, some have begun to use this technique in the study of biological specimens including synovial fluid from rheumatoid arthritis patients and lesion biopsies from leprosy patients (20,24,25,26). Also, some investigators have identified subpopulations of cells in a mixed population and have defined cytokine profiles for these subpopulations (26,27). The technique has not only been

used to detect cytokine messages from T lymphocytes but there is a good correlation between message levels detected and proteins produced. (14,28).

T lymphocytes are very important immunoregulatory cells because of their role in both the humoral and the cellular immune pathways of the acquired immune system (29,30). The immune pathways become activated when a "foreign" or "non-self" substance termed antigen (Ag) gain access into a host by defeating the physical barriers and overcoming the non-specific defenses of the innate immune system (29,30). In order for T lymphocytes to recognize an Ag, the Ag must be digested into fragments (29,30). The processed Ag, in association with Major Histocompatibility (MHC) molecules, can be recognized by the T cell receptor (TCR; 29,30). The TCR's of helper T lymphocytes recognize Ag in association with MHC class II molecules with the aid of CD4 surface markers (29,30). MHC class II molecules are found on cells such as macrophages and monocytes, B lymphocytes and T lymphocytes (29,30). Macrophages work as Ag presenting cells (APC's); they digest Ag into fragments which are transported to the cell's surface and become associated with MHC class II molecules for recognition by the TCR's of helper T lymphocytes (29,30). When the TCR's of resting helper T lymphocytes recognize processed Ag on the surface of the macrophage or APC, the helper T lymphocytes become activated (29,30). Activated helper T lymphocytes can then interact with B lymphocytes to initiate the humoral immune

pathway and antibody (Ab) production (29,30). Activated helper T lymphocytes can also interact with other helper T lymphocytes and even cytotoxic T lymphocytes to initiate the cellular immune pathway and target cell lysis; fully differentiated cytotoxic T lymphocytes, however, can cause target cell lysis directly without helper T lymphocytes (29,30). The TCR's of cytotoxic T lymphocytes recognize Ag in association with MHC class I molecules with the aid of CD8 surface markers (29,30). MHC class I molecules are found on all nucleated cells (29,30). When a cell becomes infected with a virus, viral antigenic fragments become associated with MHC class I molecules and are transported to the cell's surface for recognition by the TCR's cytotoxic T lymphocytes (29,30). When the TCR's of cytotoxic T lymphocytes recognize processed Ag on a cell's surface, the cytotoxic T lymphocytes target the cell for lysis (29,30). Cytotoxic T lymphocytes destroy tumor cells and grafted cells in the same manner (29,30).

T lymphocytes originate in the bone marrow and move to the thymus where they mature after which they move to the lymph nodes (29,30). The initial presentation of a specific Ag to T lymphocytes and T lymphocyte activation occurs in the lymph nodes; however, T lymphocytes also comprise 60-80% of the peripheral blood lymphocytes (29,30). T lymphocytes in the peripheral blood can therefore recognize the Ag due to a previous exposure and become activated again (29,30). Upon activation, T lymphocytes produce proteins termed

lymphokines, which are part of the broader group cytokines (29,30).

Normal immune responses and the immune cells involved are coordinated in part by these soluble protein mediators termed cytokines. Cytokines are not constitutively produced but are produced by various cells only after activation (14). Cytokines are induced by the presence of antigen and interaction of the antigen with specific receptors on the cells like the interaction between Ag on the APC's surface and the TCR. Cytokines, however, are not functionally antigen specific (29).

The Ag induced activation of lymphocytes which gives rise to cytokines *in vivo* can be achieved *in vitro* by culturing lymphocytes with specific Ag or with non-specific mitogens which bind to cell surface carbohydrate residues (30). Mitogen induced activation does not require recognition of the mitogen by the TCR in association with MHC molecules. Mitogen activation does not require previous exposure although it is believed to mimic lymphocyte activation by specific Ag *in vivo* (29,30). Mitogen induced activation *in vitro* has been shown to result in the production of cytokines (30).

Because of their role in the normal immune response many investigators desire to detect cytokines in order to dissect the immune response in certain abnormal and diseased states. Detection is difficult, however, because cytokines are translated from low copy number mRNA transcripts (30). T

lymphocyte cytokines are even more difficult to detect than cytokines from other cells because they are transiently produced in a lesser quantity and because a great variety may be produced at one time; however, the MAPPing technique has been successful at detecting human T lymphocyte cytokines (14,20). The primary goal of this project was to develop the MAPPing technique to detect the canine T lymphocyte cytokines IL-2, gIFN, TNF-A and TNF-B as well as the canine T lymphocyte surface markers CD4 and CD8.

Interleukin-2

IL-2, also called T cell growth factor, is produced by Th cells in response to stimulation by either Ag or lectin and peak expression is seen 6 hours after induction (5,20,31). The major effects of IL-2 are promotion of T and B cell division as well as activation of monocytes and lymphokine activated killer cells (LAK's; 29). Human IL-2 mRNA has been isolated from Concanavalin A (ConA) stimulated cells of a human leukemic T-cell line Jurkat-111 and the cDNA has been cloned, expressed and sequenced. The nucleic acid sequence of human IL-2 cDNA is 812 base pairs (bp) and the deduced mature protein is 133 amino acids (31). Murine IL-2 mRNA has been isolated from Phytohemagglutinin (PHA) stimulated cells of a mouse lymphoma cell line LBRM-33 using a human IL-2 cDNA probe. The cDNA has been cloned, expressed and sequenced. The nucleic acid sequence of murine cDNA IL-2 is 940 bp and the deduced mature protein is

149 amino acids (5). Human and murine IL-2 are 76% homologous at the nucleic acid level while being 63% homologous at the protein level (5). The 25 bp human IL-2 sense primer anneals beginning at base 48 and the 25 bp human IL-2 anti-sense primer anneals ending at base 505; the resulting PCR amplified human IL-2 is 458 base pairs (31). The 23 bp murine IL-2 sense primer anneals beginning at base 48 and the 25 bp murine IL-2 anti-sense primer anneals ending at base 549; the resulting PCR amplified murine IL-2 is 502 bp (5).

Gamma Interferon

gIFN is called "immune" or type II interferon to distinguish it from type I interferons, alpha and beta interferon. gIFN is also produced by Th cells in response to stimulation by either Ag or mitogen and peak levels appear at 6 hours after induction as well (20,32). gIFN production depends upon IL-1 produced by macrophages and is regulated by IL-2 (32). gIFN is a good immunoregulator of lymphocytes and monocytes and its major effects include induction of class I MHC molecules and class II MHC molecules and enhancement of B lymphocyte maturation (29). Human gIFN mRNA has been isolated from Staphylococcal enterotoxin A (SEA) stimulated human splenocytes. The cDNA has been cloned, expressed and sequenced. The nucleic acid sequence of human gIFN cDNA is 1170 bp and the deduced mature protein is 146 amino acids (32). Murine gIFN has

been isolated from a recombinant murine-lambda phage library using a human gIFN probe containing the coding region of human gIFN and the gene has been expressed. Murine gIFN RNA has been isolated from the expression vector and the cDNA has been sequenced. The nucleic acid sequence is 1192 bp and the deduced mature protein is 136 amino acids (6). Human and murine gIFN are 64% homologous at the nucleic acid level while being only 40% homologous at the protein level (6). The 29 bp human gIFN sense primer anneals beginning at base 92 and the 26 bp human gIFN anti-sense primer anneals ending at base 585; the resulting PCR amplified human gIFN is 494 bp (32). The 24 bp murine gIFN sense primer anneals beginning at base 71 and the 23 bp murine gIFN anti-sense primer anneals ending at base 530; the resulting PCR amplified murine gIFN is 460 bp (6).

Tumor Necrosis Factor-Alpha

TNF-A, or cachectin, is produced mainly from macrophages which distinguishes it from TNF-B. TNF-A can be produced in response to mitogen and peak levels of mRNA appear within 4 hours of induction (13). The major effects of TNF-A include activation of macrophages, granulocytes and tissue cells; increased leukocyte and endothelial cell adhesion; cachexia and induction of class II MHC molecules on somatic cells (29). Human TNF-A mRNA has been isolated from a 4B-phorbol 12B-myristate 13A-acetate (PMA) stimulated human promyelocytic leukemia cell line HL-60. The cDNA has been

cloned, expressed and sequenced. The nucleic acid sequence of human TNF-A cDNA is 1642 bp and the deduced mature protein is 157 amino acids (13). Murine TNF-A mRNA has been isolated from a PMA stimulated murine monocytic tumor cell line PU5-1.8 using a human TNF-A cDNA probe. The cDNA has been cloned, expressed and sequenced. The nucleic acid sequence is 1638 bp and the deduced mature protein is 156 amino acids (7). Human and murine TNF-A are 78% homologous at the nucleic acid level and 79% homologous at the protein level (7). The 24 bp human TNF-A sense primer anneals beginning at base 153 and the 26 bp human TNF-A anti-sense primer anneals ending at base 847; the resulting PCR amplified human TNF-A is 695 bp (13). The 24 bp murine TNF-A sense primer anneals beginning at base 144 and the 23 bp murine TNF-A anti-sense primer anneals ending at base 835; the resulting PCR amplified murine TNF-A is 692 bp (7).

Tumor Necrosis Factor-Beta

TNF-B, or lymphotoxin, is produced by T lymphocytes in response to mitogen stimulation 24-48 hours after induction (33). The major effects of TNF-B are the same as those of TNF-A; both are well known for tumor cell cytolysis and anti-proliferative effects on tumors (13,29). The human TNF-B gene has been isolated from a human genomic-lambda library using three synthetic probes homologous to the cDNA encoding for amino terminal residues 1-34, 35-84 and 85-155. The nucleic acid sequence is 3037 bp and the deduced mature

protein is 171 amino acids (33). The murine TNF-B gene has been isolated using a human TNF-B cDNA probe; the nucleic acid sequence is 3219 bp and the deduced mature protein is 169 amino acids (34). Human and murine TNF-B are 77% homologous at the nucleic acid level and 81% homologous at the protein level (8,33). The 24 bp human TNF-B sense primer anneals beginning at base 1276 and the 25 bp human TNF-B anti-sense primer anneals ending at base 2218; the resulting PCR amplified human TNF-B is 610 bp (33). The 21 bp murine TNF-B sense primer anneals beginning at base 1633 and the 24 bp murine TNF-B anti-sense primer anneals ending at base 2217; the resulting PCR amplified murine TNF-B for genomic DNA is 584 bp (8). PCR amplification of murine TNF-B cDNA results in a 278 bp product because of the excision from genomic DNA of 2 introns totaling 306 bp (8).

Human TNF-A and human TNF-B are 59% homologous at the nucleic acid level and 39% homologous at the protein level (13,33) Murine TNF-A and murine TNF-B are 69% homologous at the nucleic acid level and 54% homologous at the protein level.

Cluster Designation 4

The CD4 molecule is expressed on helper T cells and mediates interaction of the TCR with MHC class II molecules on APC's cells. Human CD4 mRNA has been isolated from CD4 positive transformed fibroblasts and the cDNA has been expressed and sequenced (35). The nucleic acid sequence of

human CD4 cDNA is 1742 bp and the deduced mature protein is 435 amino acids (35). Murine L3T4 (CD4 in humans) cDNA has been isolated from a clonal T-cell library with a human CD4 cDNA probe (9). The cDNA sequence is 3119 bp and the deduced mature protein is 431 amino acids (9). Human and murine CD4 are 69% homologous at the nucleic acid level and 55% homologous at the protein level (9,35). The 23 bp human CD4 sense primer anneals beginning at base 1009 and the 27 bp human CD4 anti-sense primer anneals ending at base 1446; the resulting PCR amplified human CD4 is 437 bp (35). The 23 bp murine CD4 sense primer anneals beginning at base 171 and the murine CD4 anti-sense primer anneals ending at base 785; the resulting PCR amplified murine CD4 is 615 bp (9).

Cluster Designation 8

The CD8 molecule is expressed on cytotoxic T cells and mediates the interaction of the TCR with MHC class I molecules on target cells. Human CD8 has been isolated from a lambda gt 10 library from human Leu-2/T8 positive cells (36). The cDNA sequence is 1975 bp and the deduced mature protein is 214 amino acids (36). Murine lyt-3 (CD8 in humans) cDNA has been isolated from a lambda gt 10 cDNA library from murine cytotoxic T cells with a rat CD8 probe (10). The cDNA sequence is 1144 bp and the deduced mature protein is 192 amino acids (10,12). Human and murine CD8 are 62% homologous at the nucleic acid level and 45% homologous at the protein level (10,36). The 22 bp human

CD8 sense primer anneals beginning at base 93 and the 30 bp human CD8 anti-sense anneals ending at base 546; the resulting PCR amplified human CD8 is 454 bp (36). The 22 bp murine CD8 sense primer anneals beginning at base 39 and the 22 bp murine CD8 anti-sense anneals ending at base 551; the resulting PCR amplified murine CD8 is 513 bp (10).

Beta-Actin

B-actin is one of the two non-muscle actins; it is a cytoplasmic "house keeping" gene involved in a great variety of cell functions (37). Because B-actin is actively being produced in living cells, it serves as a good positive control. Human B-actin has been isolated from a human genomic library with human probes for the coding region and the 3' untranslated region (37). The DNA sequence is 2550 bp and the mature deduced protein is 374 amino acids (37,38). Murine B-actin cDNA has been isolated from a mouse lymphocyte cDNA library using a probe for murine adult skeletal muscle alpha actin (39). Human and murine B-actin are 90% homologous at the nucleic acid level and 99% homologous at the protein level (37,38,39,40). The 21 bp human B-actin sense primer anneals beginning at base 1 and the 30 bp human B-actin anti-sense primer anneals ending at base 1908; the resulting PCR amplified human B-actin for genomic DNA is 1908 bp (37). PCR amplification of human B-actin cDNA is 1126 because of the excision of 3 introns totaling 782 bp. The 21 bp murine B-actin sense primer

anneals beginning at base 25 and the 24 bp murine B-actin anti-sense primer anneals ending at base 564; the resulting PCR amplified murine is 540 bp (39).

This project would lay a foundation so that future molecular work can be conducted on canine cytokines. The PCR amplified DNA was used for sequencing and can be used to generate species specific primers and probes (2). Ultimately in vitro expression of the genes would result in proteins that could be used for the development of monoclonal antibodies and pharmaceuticals for the study and treatment of various canine diseases.

MATERIALS

Lymphocytes

Peripheral blood lymphocytes were used for this project. Blood was collected from human and canine subjects using heparinized vacutainers. The lymphocytes were isolated sterilely using a discontinuous ficoll-hypaque gradient (appendix).

RNA isolation

Most of the reagents used for RNA isolation were purchased in kit form from Clontech. An acid guanidinium thiocyanate-phenol-chloroform extraction method was used. The denaturing solution with guanidinium thiocyanate and B-2-mercaptoethanol is a strong protein denaturant and ribonuclease inhibitor. The coextraction with phenol at reduced pH removes protein and DNA. The reagents include:

Denaturing solution	4 M guanidinium isothiocyanate 0.5 mM sodium citrate, pH 7 0.5% sarcosyl 0.1 M B-mercaptoethanol
2 M sodium acetate, pH 4.0	
TE buffer	10 mM Tris-HCl, pH 7.4 0.1 mM EDTA

cDNA synthesis

The reagents used for cDNA synthesis were purchased from Clontech. Random hexamers were used to prime the mRNA for cDNA synthesis. The short primers have all possible nucleotides at each position and are randomly annealed to the mRNA. With this method the entire population of mRNA is reverse transcribed to cDNA; this is the method of choice when location of gene sequences is not known. The reagents include:

diethylpyrocarbonate ddH_2O	
random hexamer primers, 20 uM	
5X reaction buffer	250 mM Tris-HCl, pH 8.3 375 mM KCl 15 mM $MgCl_2$
dNTP mix	10 mM dATP 10 mM dCTP 10 mM dGTP 10 mM dTTP
RNase inhibitor, 40 Units/microliter (ul)	
M-MLV reverse transcriptase, 200 Units/ul	

cDNA

Murine cDNA (20 picograms/ul) used in the PCR was purchased from Clontech. The murine cDNA used was different for each PCR; each murine cDNA was positive control cDNA specific for each primer set.

Primer sets

Human and murine primers (20 uM) used in the PCR were supplied by Clontech. The primer sequences are:

Human B-actin

sense	5' ATGGATGATGATATCGCCGCG 3'
anti-sense	5' CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC 3'

Human CD4

sense	5' GTGAACCTGGTGGTGATGAGAGC 3'
anti-sense	5' GGGGCTACATGTCTTCTGAAACCGGTG 3'

Human CD8

sense	5' CTTACCAGTGACCGCCTTGCTC 3'
anti-sense	5' CGCGATGGTGGGCGCCGGTGTTGGTGGTCG 3'

Human IL-2

sense	5' ATGTACAGGATGCAACTCCTGTCTT 3'
anti-sense	5' GTTAGTGTTGAGATGATGCTTTGAC 3

Human gIFN

sense	5' ATGAAATATACAAGTTATATCTTGGCTTT 3'
anti-sense	5' GATGCTCTTCGACCTCGAAACAGCAT 3'

Human TNF-A

sense	5' ATGAGCACTGAAAGCATGATCCGG 3'
anti-sense	5' GCAATGATCCCAAAGTAGACCTGCCC 3'

Human TNF-B

sense	5' ATGACACCACCTGAACGTCTCTTC 3'
anti-sense	5' CGAAGGCTCCAAAGAAGACAGTACT 3'

Murine B-actin

sense	5' GTGGGCCGCTCTAGGCACCAA 3'
anti-sense	5' CTCTTTGATGTCACGCACGATTTC 3'

Murine CD4

sense	5' TGTGCCGAGCCATCTCTCTTAGG 3'
anti-sense	5' GCACTGAGAGTGTCATGCCGAAC 3

Murine CD8

sense	5' ATGCAGCCATGGCTCTGGCTGG 3'
anti-sense	5' GCATGTCAGGCCCTTCTGGGTC 3'

Murine IL-2

sense	5' ATGTACAGCATGCAGCTCGCATC 3'
anti-sense	5' GGCTTGTTGAGATGATGCTTTCACA 3'

Murine gIFN

sense	5' TGACCGCTACACACTGCATCTTGG 3'
anti-sense	5' CGACTCCTTTTCCGCTTCCTGAG 3'

Murine TNF-A

sense	5' ATGAGCACAGAAAGCATGATCCGC 3'
anti-sense	5' CCAAAGTAGACCTGCCCGGACTC 3'

Murine TNF-B

sense	5' TGACACTGCTCGGCCGTCTCCA 3'
anti-sense	5' GTTGCTCAAAGAGAAGCCATGTCG 3'

Polymerase Chain Reaction

Reagents used for the PCR were purchased from Perkin Elmer Cetus. The reagents include:

10X reaction buffer	500 mM KCl 100 mM Tris-HCl, pH 8.3 15 mM $MgCl_2$ 0.1% gelatin
dNTP mix	125 uM dATP 125 uM dCTP 125 uM dGTP 125 uM dTTP
Taq polymerase, 5 Units/ul, diluted 1:4	

Cycle sequencing

Most of the reagents used for cycle sequencing were purchased in kit form from Stratagene. The reagents include:

10X sequencing buffer	100 mM Tris-HCl, pH 8.8 500 mM KCl 0.01% gelatin 40 mM $MgCl_2$ 20 uM dATP 50 uM dCTP 50 uM dGTP 50 uM dTTP
ddNTP	600 uM ddATP 600 uM ddCTP 100 uM ddGTP 1000 uM ddTTP
Stop dye mix	80% formamide 50 mM Tris, pH 8.3 1 mM EDTA 0.1% bromophenol blue 0.1% xylene cyanol

METHODS

Lymphocyte Isolation

The procedure was performed sterilely to prevent contamination of the cell culture. Blood was diluted 1:3 with phosphate buffered saline (PBS; appendix; Sigma Chemicals; St. Louis, MO) and layered over ficoll-hypaque with a density of 1.08 grams/milliliter (ml; appendix; Type 400; Sigma Chemicals; St. Louis, MO; 75%; Winthrop Pharmaceuticals; New York, NY) in a 3:1 ratio. The blood was centrifuged at 250 g for 20 minutes and the lymphocytes were aspirated off to a clean tube and washed with PBS. Any contaminating red blood cells were removed by hypotonic lysis and the remaining white cells pelleted at 250 g for 15 minutes. Pellets were washed twice in PBS during the last PBS wash a granulocyte decontamination was performed by fluffing the pellets with a pipette and allowing the granulocyte clumps to settle to the bottom. The resulting suspensions were transferred to new tubes and PBS was added. The cells were pelleted slowly at 50 g for 8 minutes to remove any contaminating platelets.

The pelleted lymphocytes were resuspended in 1 ml of the appropriate nutrient medium (appendix) and the number of cells were counted manually using a hemacytometer. The concentrations were adjusted to $1x10^6$ cells per ml of medium

(appendix) and the suspensions were transferred into 25 cm^2 cell culture flasks (Corning Glass Works; Corning, NY).

Lymphocyte Stimulation

Three 100 ul aliquots of each cell suspension were placed in a well of a 96 well round bottom plate (Flow Laboratories, Inc.; McLean, VA) and the cells were incubated at 37°C with 5% CO_2 for 48 hours. Phytohemagglutinin (PHA; Sigma Chemicals; St. Louis, MO) was added to the cells remaining in the culture flask at 25 nanograms (ng) per ml of suspension and the cells were induced for 48 hours at 37°C with 5% CO_2. After induction, three 100 ul aliquots were removed from each flask and each aliquot was placed in a well of the 96 well round bottom plate. 20 ul of tritiated (^3H) thymidine (0.05 uCurie/ml; Amersham; Arlington Heights, IL) was added to the six wells for each cell suspension and ^3H thymidine was incorporated for 24 hours again at 37°C with 5% CO_2. The cells in the culture flask were transferred to sterile 15 ml polypropylene centrifuge tubes (Corning, Incorporated; Corning, NY) and pelleted at 250 g for 15 minutes to begin the RNA isolation procedure. The procedure was repeated in the same manner; however, the cells were induced for 5 hours and ^3H thymidine was incorporated for 3 hours.

The cells were harvested after incorporation of ^3H thymidine using a micro cell harvester (Skatron; Sterling, VA). To each sample 10 ul of soluene 350 (Packard

Instruments Company; Downers Grove, IL) was added and after 30 minutes 1 ml of high flash point cocktail safety solve (Research Products International Corporation; Mount Prospect, IL) was added. The samples were placed in a beta-trac liquid scintillation counter (TM Analytic #F6895; Brandon, FL) and allowed to dark adapt for two hours. After three complete cycles of counting the stimulation index (SI) was determined from the counts per minute (cpm). The following formula was used:

SI=cpm stimulated cells/cpm non-stimulated cells.

RNA Isolation

The RNA isolation procedure was performed using sterilized plasticware and diethylpyrocarbonate (DEPC) treated reagents to prevent RNase contamination. The RNA was isolated using a modified acid guanidinium thiocyanate method (41). To each of the lymphocyte pellets 100 ul of the denaturing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M B-mercaptoethanol) was added per $1x10^6$ cells. After shaking the tubes for 30 seconds, 100 ul 2 M sodium acetate, pH 4 (Baker; Philipsburg, NJ), 1 ml water saturated phenol (Sigma Chemicals; St. Louis, MO), and 200 ul chloroform:isoamyl alcohol (19:1; Baker; Philipsburg, NJ; Sigma Chemicals; St. Louis, MO) were added. The tubes were inverted between each addition and after the addition of the chloroform:isoamyl

alcohol, the tubes were shaken and the samples were split into 1.5 ml Eppendorf tubes (Bio-rad; Hercules, CA) and chilled on ice for 15 minutes. The tubes were centrifuged at 9600 g for 20 minutes at 4°C, the aqueous phases were transferred to clean Eppendorf tubes and 1 ml room temperature 100% isopropanol (Baker; Philipsburg, NJ) was added.

The RNA was precipitated overnight at -20°C after which the RNA was then pelleted by centrifuging at 9600 g for 20 minutes at 4°C. The supernatants were poured off and the pellets were redissolved in 300 ul of the denaturing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). The RNA was again precipitated by adding 1 ml of room temperature 100% isopropanol and leaving overnight at -20°C.

The RNA was again pelleted at 9600 g, 4°C for 20 minutes and the supernatants were poured off. Twice, 500 ul of cold 75% DEPC treated ethanol was added, the RNA was pelleted at 9600 g, 4°C for 20 minutes, and the supernatants were poured off. The RNA was dried for ten minutes using a speed vac at 43°C (Savant SC1000/VG 5/24903; Farmingdale, NY). To store the RNA, the pellet was resuspended in 500 ul of the Tris-ethylene diamine tetraacetate (EDTA; TE) buffer, pH 7.4, (10 mM Tris-HCl, 0.1 mM DEPC treated EDTA), 50 ul 3 M sodium acetate, pH 5.4 and 150 ul 100% ethanol (Baker; Philipsburg,NJ). The RNA was stored at -70°C.

The concentration and purity of the RNA were determined by UV spectrometry using a UV/VIS Lambda 2 spectrometer (Perkin Elmer Cetus; Norwalk, CT). The RNA was diluted 1:500 with distilled deionized water (ddH_2O) and the absorbence was measured at 260 nanometers (nm) and 280 nm. The A260/A280 ratio was used to determine purity and the concentration (ug/ul) was determined by (A260)(500/1)(40ug)/1000ul. A purity ratio of 1.8-2.0 is optimal; however, samples with purity ratios less than 1.8 may be used.

Polyacrylamide gel electrophoresis (PAGE) was used to confirm the integrity of the RNA (42). To 1.0 ul RNA was added 9.0 ul ddH_2O and 5.0 ul of the loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll in ddH_2O; Sigma Chemicals; St. Louis, MO). The RNA, along with 5 ul DNA molecular weight marker VIII (250 ug/ml; Boehringer Mannheim, Indianapolis, IN), were electrophoresed in separate lanes on a 6% polyacrylamide gel (acrylamide:N,N'-methylene bisacrylamide, 29:1; Bio-rad; Hercules, CA) with 1X Tris Acetate EDTA (TAE; appendix) buffer using a mini-protean II gel system (Bio-rad; Hercules, CA) for 15-20 minutes by applying 150 volts (V). The gel was stained with ethidium bromide in ddH_2O (0.5 ug/ml) for 15 minutes and then washed with ddH_2O. The RNA bands were seen using a Ultra Violet (UV) transillumination box (UVP, Inc. ts36; San Gabriel, CA) at 254 nm. A picture was taken using an FCR-10 camera (Fotodyne), Inc.; New Berlin, WI) and type 667

Polaroid film for documentation (Polaroid Corporation; Cambridge, MA).

cDNA Synthesis

The cDNA synthesis procedure was performed as described (18,42). For each cDNA synthesis 2 micrograms (ug) of RNA was used and DEPC treated ddH_2O was added for a final volume of 18.75 ul. The RNA was heated to 95°C for 5 minutes followed by a quick chilling on ice. To the tube was added 1.0 ul random hexamer primers (20 uM), 5.0 ul 5X reaction buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$), 1.5 ul dNTP mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP), 0.75 ul recombinant RNase inhibitor (40 Units/ul) and 2.0 ul Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (200 Units/ul). The tube was spun briefly and incubated for 1 hour at 42°C, followed by heating to 70°C for 5 minutes to terminate the synthesis. All incubation were performed using the thermal cycler (Perkin Elmer Cetus; Norwalk, CT).

PAGE was used to confirm the integrity or the cDNA (42). To 10.0 ul cDNA was added 5.0 ul of the loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll in ddH_2O; Sigma Chemicals ;St. Louis, MO). The cDNA, along with 5 ul DNA molecular weight marker VIII (250 ug/ml; Boehringer Mannheim, Indianapolis, IN), were electrophoresed in separate lanes on a 6% polyacrylamide gel (acrylamide:N,N'-methylene bisacrylamide, 29:1; Bio-rad; Hercules, CA) with

1X TAE (appendix) buffer using a mini-protean II gel system (Bio-rad; Hercules, CA) for 15-20 minutes by applying 150 V. The gel was stained with ethidium bromide in ddH_2O (0.5 ug/ml) for 15 minutes and then washed with ddH_2O. The cDNA bands were seen using a UV transillumination box (UVP, Inc. ts36; San Gabriel, CA) at 254 nm. A picture was taken using an FCR-10 camera (Fotodyne), Inc.; New Berlin, WI) and type 667 Polaroid film for documentation (Polaroid Corporation; Cambridge, MA).

Polymerase Chain Reaction

For all Polymerase Chain Reactions (PCR) standard amounts of deoxy nucleotide triphosphate (dNTP) mix (125 uM dATP, 125 uM dCTP, 125 uM dGTP, 125 uM dTTP), 10X reaction buffer (500 mM KCl; 100 mM Tris-HCl, pH 8.3; 15 mM $MgCl_2$; 0.1% gelatin) and recombinant Taq DNA polymerase (5 Units/ul) were used, 16 ul, 10 ul and 2 ul, respectively; and ddH_2O was added to give a final volume of 100 ul (18,43). For human reactions 5 ul human cDNA (0.01 ug/ul) and 2.5 ul of the human sense and anti-sense primers (20 uM) were used. For murine reactions 5 ul of a 1:10 dilution of the murine positive control cDNA (50 picograms/ul) specific for the primers, and 2.5 ul of the murine sense and anti-sense primers (20 uM) were used. For canine reactions 5 ul canine cDNA (0.01 ug/ul) and 2.5 ul of the sense and anti-sense primers were used. Initially, human primers were used in attempt to amplify canine cDNA; where human primers failed

to amplify canine cDNA, murine primers were used in attempt to amplify canine cDNA. For all canine PCR's either a human or a murine reaction was included as a positive control and a blank reaction was included as a negative control. Blank reactions contain all of the PCR components except DNA; DNA is replaced with ddH_2O. For all PCR's the denaturation step was 95°C for 1 minute and the extension step was 72°C for 1 minute. The annealing time was held constant at 1 minute for all PCR's. The optimal annealing temperature for each primer set is 61°C for the appropriate species, however the annealing temperature was empirically determined for each primer set for the canine. Each reaction mixture was overlaid with 4 drops of sterile mineral oil to prevent evaporation and condensation (Sigma Chemicals; St. Louis, MO). All amplifications were done using a thermal cycler (Perkin Elmer Cetus; Norwalk, CT).

Gel Electrophoresis

The PCR fragments were separated using PAGE to determine the fidelity of the products (19,42). To 15 ul of each PCR product was added 5 ul of the loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll in ddH_2O; Sigma Chemicals; St. Louis, MO). The products, along with 5 ul DNA molecular weight marker VIII (250 ug/ml; Boehringer Mannheim, Indianapolis, IN), were electrophoresed in separate lanes on a 6% polyacrylamide gel (acrylamide:N,N'-methylene bisacrylamide, 29:1; Bio-rad; Hercules, CA) with

1X TAE (appendix) buffer using a mini-protean II gel system (Bio-rad; Hercules, CA) for 15-20 minutes by applying 150 V. The gel was stained with ethidium bromide in ddH_2O (0.5 ug/ml) for 15 minutes and then washed with ddH_2O. The DNA bands were seen using a UV transillumination box (UVP, Inc. ts36; San Gabriel, CA) at 254 nm. A picture was taken using an FCR-10 camera (Fotodyne), Inc.; New Berlin, WI) and type 667 Polaroid film for documentation (Polaroid Corporation; Cambridge, MA).

DNA Electroelution

The amplified DNA fragments were excised from the gel and the DNA was electroeluted with 1X TAE (appendix; Sigma Chemicals; St. Louis, MO) into centrifugal dialysis units (Amicon; Danvers, MA) using a micro-electroeluter system (Amicon; Danvers, MA) by applying 300 V over several hours. The DNA was concentrated by centrifuging with a fixed angle rotor for 20 minutes at 2775 g. The DNA was then washed twice using sterile ddH_2O and centrifuging for 20 minutes at 2775 g. The DNA was retrieved by inverting the centrifugal dialysis unit and centrifuging for 2 minutes at 1100 g.

Cycle Sequencing

For each human product the appropriate human primer sets were used and for each murine product the appropriate murine primer sets were also used. For each canine product, the primer sets used were those which had generated the product.

The total reaction volume for the sequencing mixture was 22.0 ul, when necessary, ddH_20 was added to achieve the total volume. The components of the reaction mixture were added in the following order: ddH_20, 4.0 ul of 10X sequencing buffer (100 mM Tris-HCl, pH 8.8; 500 mM KCl; 0.01% gelatin; 40 mM $MgCl_2$; 20 uM dATP; 50 uM dCTP; 50 uM dGTP; 50 uM dTTP), 12.0 ul DNA, sense or anti-sense primer (0.0588 ul/base; 20 uM), 1.0 ul 33P-dATP (10 mCurie/ml; New England Nuclear; Wilmington, DE), 1.0 ul Taq polymerase (5 Units/ul; Perkin Elmer Cetus; Norwalk, CT). The reaction components were mixed and then 5.0 ul was added to 5.0 ul of each dideoxy nucleotide triphosphate (ddNTP)-ddATP, ddCTP, ddGTP, and ddTTP. For each human, murine and canine cytokines, separate reactions for both the sense and anti-sense primers were performed in order to produce single stranded DNA and to sequence the DNA fragment from both directions. The sequencing reaction mixtures were heated to 95C for 5 minutes and then subjected to the same cycling temperatures which resulted in optimal amplification of the DNA. The Gene-Amp PCR system 9600 (Perkin Elmer Cetus; Norwalk, CT) was used for sequencing reactions; the denaturation time was 10 seconds, the annealing time was 20 seconds, and the extension time was 30 seconds. Upon completion of the cycles 5.0 ul of the stop mix (80% formamide; 50 mM Tris, pH 8.3; 1 mM EDTA; 0.1% bromophenol blue; 0.1% xylene cyanol) was added to each tube and the reaction mixtures were stored at -20C (44).

Sequencing Gel Electrophoresis

The DNA sequences were separated on a 6% polyacrylamide gel (acrylamide:N,N'-methylene bisacrylamide, 29:1; Bio-rad) with 7 M urea (Baker; Philipsburg, NJ) with Tris Borate EDTA (TBE; appendix) using a Sequi-gen sequencing cell (Bio-rad; Hercules, CA). The gel was pre-heated to 50^{o}C by applying 1700 volts, the sequencing reaction mixtures were heated in boiling water for two minutes and 2.0 ul of the four ddNTP sequencing mixtures were loaded into four consecutive wells. The DNA fragments were separated by electrophoresis at 50^{o}C (45). The voltage was adjusted from 1500 to 2000 V to keep a constant temperature. Once the first dye front had run off the bottom of the plate, the sequencing reaction mixtures were again heated in boiling water for 2 minutes. 2.0 ul of each of the four mixtures was loaded into the next 4 wells and the electrophoresis was continued at 50^{o}C. This allowed for separation of the larger DNA fragments in addition to the smaller DNA fragments. The smaller fragments closer to the primer can be read in the last four lanes while the larger fragments further from the primer can be read in the first four lanes. The electrophoresis was continued until the first dye front from the second loading was near the bottom of the plates. The gel was removed from the plates, transferred and dried onto a filter paper under vacuum using a slab dryer (Fisher-Biotech FB-GDS-4050; Springfield, NJ) at 80C. The incorporated radiolabeled

nucleotide allows for detection of DNA fragments using audoradiography. Audoradiograph film (X-OMAT AR; Eastman Kodak Company; Rochester, NY) was placed over the gel for several days after which time the film was developed. The sequence was read using a Graf/Bar mark II gel reader and light box (Science Accessories Corporation; Stratford, CT) and the fidelity of the sequence was determined by comparison to published sequences in GenBank using the FastA program of the Genetics Computer Group Sequence Analysis Software Package (GCG; Madison, WI).

RESULTS

Initially, non-stimulated lymphocytes were used for RNA isolation. The messages for B-actin should be isolated without stimulation because B-actin is a "housekeeping" gene which is constantly present in cells. The messages for CD4 and CD8 should also be isolated without stimulation because they are surface markers which are present continually. Three successful human non-stimulated lymphocyte isolations were performed with numbers of cells isolated between 11 million and 13.85 million. From each of these RNA was isolated with purity ratios of 1.34, 1.47 and 1.93; and concentrations of 1.66 ug/ul, 1.0 ug/ul and 7.46 ug/ul, respectively. Two successful canine non-stimulated lymphocyte isolations were performed with numbers of cells isolated approximately 11 million. From both of these RNA was isolated with purity ratios of 1.3 and 1.83 and concentration of 2.04 ug/ul and 0.66 ug/ul. One canine non-stimulated lymphocyte RNA isolation was performed which was not used in any PCR amplifications. Figure 1 is a representative photograph depicting the integrity of the RNA isolated and the cDNA synthesized. The isolation of messages from non-stimulated lymphocytes was detected by PCR amplification.

PCR amplification of canine cDNA with human B-actin primers resulted in no product, however PCR amplification of

canine cDNA with murine B-actin primers resulted in two products of incorrect size, approximately 281 bp and 216 bp. The correct size of the murine B-actin PCR product was obtained, 540 bp. Results of the B-actin PCR with murine primers are seen in Figure 1.

PCR amplification of canine cDNA with human CD4 primers resulted in one product of correct size, 437 bp. The correct size of the human CD4 PCR product was also obtained, 437 bp. PCR amplification of canine cDNA with murine CD4 primers resulted in no product. Results of the CD4 PCR with human primers are seen in Figure 2. For confirmation of the products obtained, the human and canine CD4 PCR products were excised, electroeluted and sequenced. Sequencing of the human CD4 product resulted in an accurate sequence and sequencing of the canine CD4 PCR product resulted in a 395 bp sequence which is 75% homologous to human CD4. The complete canine CD4 sequence is shown in Figure 3 and the overlap of canine CD4 with human CD4 is shown in Figure 4. PCR amplifications of canine cDNA with human CD8 primers and with murine CD8 primers resulted in no products.

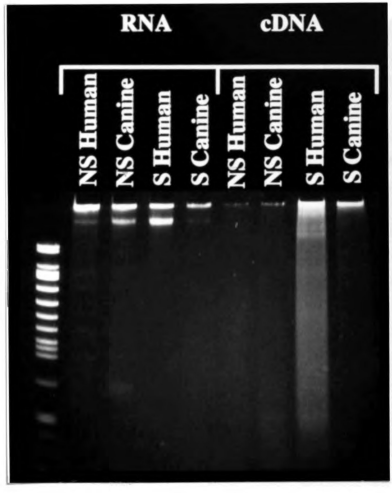

Figure 1. Polyacrylamide gel electrophoresis of RNA and cDNA. PAGE was performed to determine the integrity of the isolated RNA and the synthesized cDNA. S refers to stimulated and NS refers to non-stimulated.

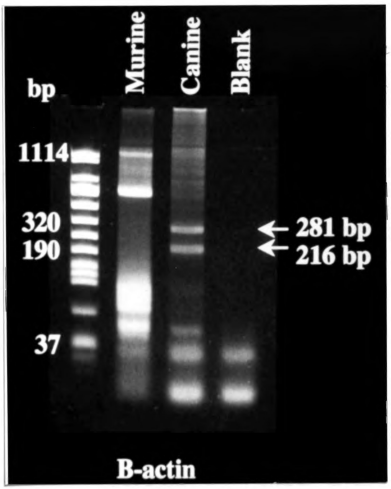

Figure 2. B-actin Polymerase Chain Reaction. Results from amplification of murine and canine cDNA with a murine primer set for B-Actin. The arrows indicate the 281 bp and the 216 bp canine B-actin PCR products.

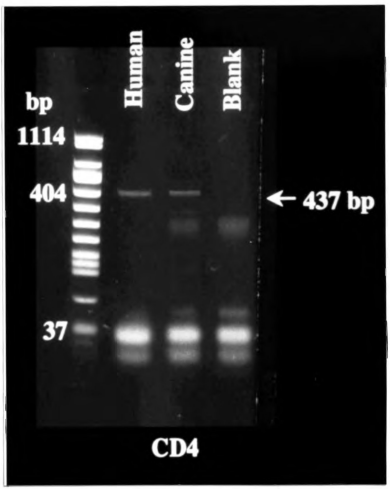

Figure 3. CD4 Polymerase Chain Reaction. Results from amplification of human and canine cDNA with the human primer set for CD4. The arrow indicates the 437 bp canine CD4 PCR product.

```
  1  AGGTCTGGGA CCCACCTCCC CTGAGCTGAC ACTGAGCTTG ATCTGAAAG
 51  AGCAGGCTGC CAAGGTCTAA AGCAGCAGAA GCTGGTATGG GTGGTGGATC
101  CTGAGGGGGG AACGGTTGCA GTGTCTCTGA GTGACAAGGA AAAGTCTGC
151  TGCATCCAGC CTCAATGTTT CATCCCCAGT GGTCATCAAG TCCTGCCAAA
201  GTTCTTGCAT TCACGCTGGG CGGGATCTTA GGCCTTCTGC TTCTAATTGG
251  CTTTGCGTCT TCTGCTGTGT TAAGACTCTG CCCAAGGCCC AGCAGCAGCG
301  GATCT
```

Figure 4. Canine CD-4 sequence.
71% homology and 317 base pair overlap with human CD-4; length 305 base pairs. The numbers indicate the bases.

```
                                          10        20
                                        AGGTCT-GGGACCCACCTCCCCTGAGCTGA
                                        |||| | |||||||||||||||| ||||||
CCACTCAGCTCCAGAAAAATTTGACCTGTGAGGTGTGGGGACCCACCTCCCCTAAGCTGA
      1040      1050      1060      1070      1080      1090

30        40        50        60        70        80
CACTGAGCTTGAATCTGAAAGAGCAGGCTGCCAAGGTCT-AAAGCAGCAGAAGCTGGTAT
  ||||||||||| ||| |  |  |||  || ||||||||  |||| | |||||  ||| |
TGCTGAGCTTGAAACTGGAGAACAAGGAGGCAAAGGTCTCGAAGCGGGAGAAGGCGGTGT
      1100      1110      1120      1130      1140      1150

90        100       110       120       130       140
GGGTGGTGGATCCTGAGGGGGGAACGGTTGCAGTGTCT-CTGAGTGAC-AAGGACAAAGT
|||||  ||  | ||||||| |||  |  || |||||||||  ||||||||||   |||||
GGGTGCTGAACCCTGAGGCGGGGA-TGTGGCAGTGTCTGCTGAGTGACTCGGGACAGGTC
      1160      1170       1180      1190      1200

 150       160       170       180       190       200
CTGCT-GCATCCAGCCTCAATGTTTCATCCCCAGTGGTC--ATCAAGTCCTGCCAAAGTT
|||||  | |||||  | ||||  | |||  |||   |||||    |  ||  | |||||  |
CTGCTGGAATCCAACATCAA-GGTTCTGCCCACATGGTCCACCCCGGTGCAGCCAATGGC
1210      1220       1230      1240      1250      1260

    210       220       230       240       250       260
CTTGCATTCACGCTGGGCGGGATCTTAGGCCTTCTGCTTCTAATT-GGCTTTGCGTCTTC
|  ||  |||   |||||| ||   ||   ||||| |||||| |  |||  ||||   ||  ||||||
CCTG-ATT-GTGCTGGGGGGCGTCGCCGGCCTCCTGCTTTTCATTGGGCTAGGCATCTTC
1270       1280      1290      1300      1310      1320

     270       280            290       300
TGCTGTGTTAAGACTCTG--CCCAAG--GCCCAGCAGCAGCGGATCT
|  |||||| |  |   |  |   ||  |||   |||  |||||  |||||||  |
TTCTGTGTCAGGTGCCGGCACCGAAGGCGCCAAGCAG-AGCGGATGTCTCAGATCAAGAG
1330      1340      1350      1360       1370      1380
```

Figure 5. Comparison of human and canine CD4 sequences. 71% homology. The top sequence is canine CD4 and the bottom sequence is human CD4. The numbers indicate the bases. Base number 1 of the canine CD4 sequence begins matching at base number 1064 of the human CD4 sequence and the overlap is 317 bp in length.

Because cytokines are produced only after activation, isolated lymphocytes were stimulated for isolation of RNA for IL-2, gIFN, TNF-A, and TNF-B. PHA was used for stimulation because as a mitogen it non-specifically stimulates peripheral blood lymphocytes without the MHC involvement and without previous exposure. PHA was used also because it is a mitogen used in standard mitogen lymphoblastogenesis assays to stimulate T lymphocytes in particular. The lymphocytes were stimulated for 72 hours because this is the standard time for mitogen lymphoblastogenesis assays; stimulation for 72 hours allows the lymphocytes to reach peak cell division. When cells are stimulated to divide, RNA appears within the cells before cell division occurs; however, whether a peak cytokine message is or is not present at 72 hours depends upon the cytokine. One human lymphocyte isolation was performed for stimulation; 8.25 million cells were isolated. The cells were stimulated for 72 hours and the stimulation index was 14.30. The RNA isolated from these cells had a purity ratio of 1.74 and a concentration of 1.32 ug/ul. Four canine lymphocyte isolations were performed for stimulation; approximately 10 million cells were isolated from each. Cells from all four isolations were stimulated for 72 hours and the stimulation indices were 20.96, 30.06, 50.93 and 68.44. The RNA isolated from these cells had purity ratios of 2.0, 1.5, 2.08 and 1.8, respectively. The concentrations were 1.52 ug/ul, 0.72 ug/ul, 3.24 ug/ul and 1.87 ug/ul,

respectively. Figure 1 is a representative photograph depicting the integrity of the RNA isolated and the cDNA synthesized. The isolation of messages from lymphocytes stimulated for 72 hours was detected by PCR amplification.

Because no products were obtained for CD8 previously, human CD8 primers and murine CD8 primers were used again in attempt to detect canine CD8 messages. PCR amplification of canine cDNA with human CD8 primers resulted in one product of incorrect size, approximately 362 bp. The correct size of the human CD8 PCR product is 454 bp; however the human CD8 amplified product obtained was also of incorrect size, 404 bp. PCR amplification of canine cDNA with murine CD8 primers resulted in no product. Results of the CD8 PCR with human primers are seen in Figure 5. For confirmation of the products obtained, the human and canine CD8 PCR products were excised, electroeluted and sequenced. Sequencing of the human CD8 and the canine CD8 PCR products resulted in inaccurate sequences; the FastA program did not match them to any CD8 sequences in GenBank. The human CD8 and canine CD8 sequences were, however, 94% homologous with each other as determined using the Bestfit program of the GCG Sequence Analysis Software Package.

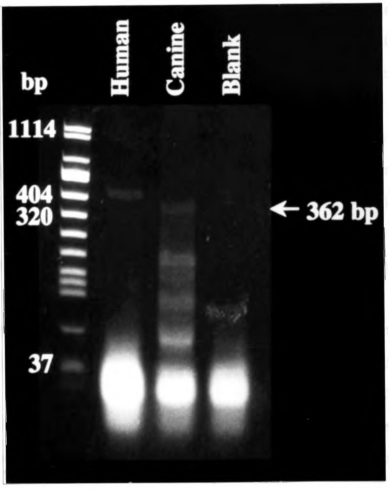

Figure 6. CD8 Polymerase Chain Reaction. Results from amplification of human and canine cDNA with the human primer set for CD8. The arrow indicates the 362 bp canine CD8 PCR product.

PCR amplification of canine cDNA with human IL-2 primers resulted in no product, however PCR amplification of canine cDNA with murine IL-2 primers resulted in two products of incorrect size, approximately 320 bp and 216 bp. The correct size of the murine IL-2 PCR product was obtained, 502 bp. For confirmation of the products obtained, the murine and canine IL-2 PCR products were excised, electroeluted and sequenced. Sequencing of the murine IL-2 PCR product resulted in an accurate sequence; however, sequencing of the canine IL-2 PCR products resulted in no sequences.

PCR amplification of canine cDNA with human gIFN primers resulted in no product, however PCR amplification of canine cDNA with murine gIFN resulted in two products of incorrect size, approximately 320 bp and 242 bp, and one product of correct size. The correct size of the murine gIFN PCR product was obtained, 460 bp. For confirmation of the products obtained, the murine and canine gIFN PCR products were excised, electroeluted and sequenced. Sequencing of the murine gIFN PCR product resulted in an accurate sequence; however, sequencing of the canine gIFN PCR products resulted in no sequences.

PCR amplification of canine cDNA with human TNF-A primers resulted in no product, however PCR amplification of canine cDNA with murine TNF-A primers resulted in one product of incorrect size, approximately 216 bp. The correct size of murine TNF-A PCR product was obtained, 692 bp. For

confirmation of the products obtained, the murine and canine TNF-A PCR products were excised, electroeluted and sequenced. Sequencing of the murine TNF-A PCR product resulted in an accurate sequence; however, sequencing of the canine TNF-A PCR products resulted in an inaccurate 59 bp sequence; the FastA program did not match it to any TNF-A sequences in GenBank.

PCR amplification of canine cDNA with human TNF-B primers resulted in two products of incorrect size, 281 bp and 147 bp. The correct size of human TNF-B PCR product was obtained, 610 bp. PCR amplification of canine cDNA with murine TNF-B resulted in one product of correct size, 278 bp. The correct size of murine TNF-B PCR product was also obtained, 278 bp. Results of the TNF-B PCR with murine primers are seen in Figure 6. For confirmation of the products obtained, the murine and canine PCR products were excised, electroeluted and sequenced. Sequencing of the murine TNF-B PCR product resulted in an accurate sequence and sequencing of the canine TNF-B PCR product resulted in a 250 bp sequence which was 92% homologous to murine TNF-B mRNA. The complete canine TNF-B sequence is shown in Figure 7 and the overlap of canine TNF-B with murine TNF-B is shown in Figure 8.

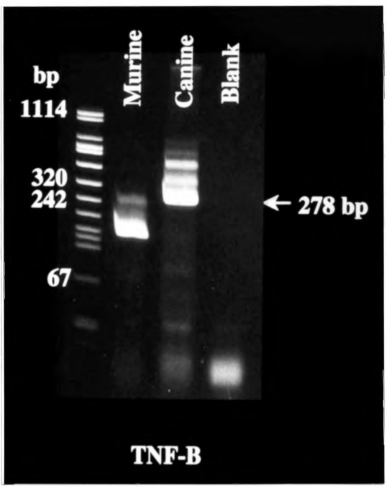

Figure 7. TNF-B Polymerase Chain Reaction. Results of amplification of murine and canine cDNA with the primer set for murine TNF-B. The arrow indicates the 278 bp canine TNF-B PCR product.

```
  1  TGCTCGGCGT CTCACCTGCT TGAGGTTTGC TTGGACCCCT CCTGTCTTCC

 51  TCCTGGGGCT GCTGCTGGCC CTGCCTCTAG GGGCCCAGGG ACTCTCTGGT

101  GTCCGCTTAT CCGCTGCCAG GACAGCCCAT CCACTCCCAT CAGAAGCACT

151  TTAGACCCAT GGCATCCGTA AACCTGCTGC TCACCTTGTT GGGTACCCCA

201  GTCTAAGCAG AACTACACTA GCTCTAGTAG AGCAAGCACG ATCGTGCCT

251  TT
```

Figure 8. Canine TNF-B sequence.
92% sequence homology and 252 base pair overlap with murine TNF-B; length 252 base pairs. The numbers indicate the bases.

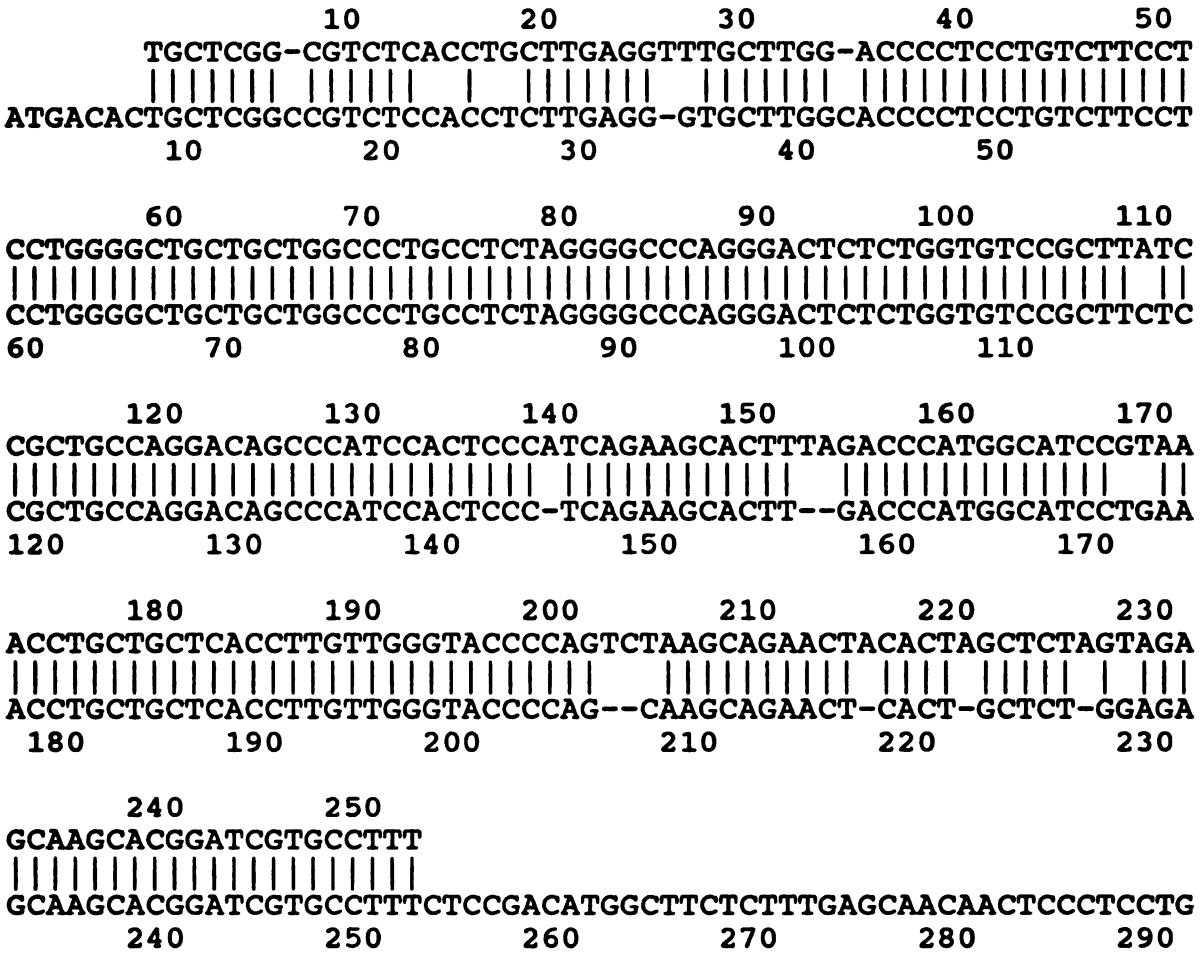

Figure 9. Comparison of murine and canine TNF-B sequences. 92% homology. The top sequence is canine TNF-B and the bottom sequence is human TNF-B. The numbers indicate the based. Base number 1 of the canine TNF-B sequence begins matching at base number 9 of the human TNF-B sequence and the overlap is 252 bp in length.

Because no sequences were obtained for canine IL-2, canine gIFN and canine TNF-A after many trials, it was suspected that the canine PCR products obtained for each of the messages were not correct products. This suggests that when products of incorrect size are obtained, no sequences or incorrect sequences will be obtained. After reviewing the literature again, it was found that peak expression of IL-2 is seen 6 hours after induction, that peak expression of gIFN is also seen 6 hours after induction and that peak levels of TNF-A are seen after 4 hours of induction (5,13,20,31,32). Peak levels of TNF-B are seen 24-48 hours after induction, which is one reason that correct canine PCR products for TNF-B were obtained (13,29). Therefore, lymphocytes were then stimulated for 8 hours. Stimulation for 8 hours would allow the messages for IL-2, gIFN and TNF-A to be isolated even though it does not allow the lymphocytes to reach peak cell division. One additional canine lymphocyte isolation was performed for stimulation; 4.15 million cells were isolated. These cells were stimulated for 8 hours and the stimulation index was 2.56. The RNA isolated from these cells had a purity value of 1.0 and a concentration of 2.22 ug/ul. The isolation of messages from lymphocytes stimulate for 8 hours was detected by PCR amplification.

PCR amplification of canine cDNA with human IL-2 primers resulted in one product of correct size, 458 bp. The correct size of the human IL-2 PCR product was also

obtained, 458 bp. Results of the IL-2 PCR with human primers are seen in Figure 9. For confirmation of the product obtained, the canine IL-2 PCR product was excised, electroeluted and sequenced. Sequencing of the canine IL-2 PCR product resulted in a 324 bp sequence which was 81% homologous to human IL-2. The complete canine IL-2 sequence is shown in Figure 10 and the overlap of canine IL-2 with human IL-2 is shown in Figure 11.

PCR amplification of canine cDNA with human gIFN primer resulted in one product of correct size, 494 bp. The correct size of the human gIFN PCR product was also obtained, 494 bp. Results of the gIFN PCR with human primers are seen in Figure 12. For confirmation of the product obtained, the canine gIFN PCR product was excised, electroeluted and sequenced. Sequencing of the canine gIFN PCR product resulted in a 268 bp sequence which was 97% homologous to canine gIFN and 79% homologous to human gIFN. The complete canine gIFN sequence is shown in Figure 13 and the overlap of canine gIFN with published canine gIFN is shown in Figure 14.

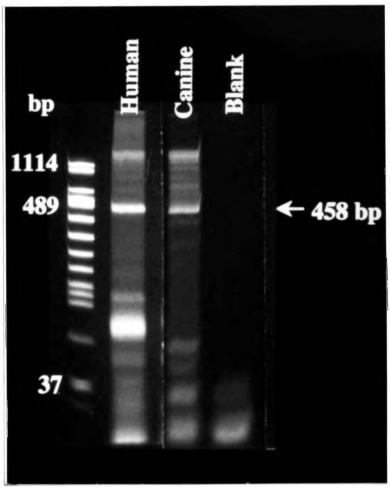

Figure 10. IL-2 Polymerase Chain Reaction. Results of amplification of human and canine cDNA with the primer set for human IL-2. The arrow indicates the 458 bp canine IL-2 PCR product.

```
  1  CTCAAGCTCT ACAGGACAGA GCAACAGATG GAGCAATTAC GCTGGATTT
 51  ACAGTTGCTT TTGAATGGAG TTAATAATTA TGAGAACCCC AACTGCTCCA
101  GGATGCTCAC ATTTAAGTTT TACACGCCCA AGAAGGCCAC AGAATGTTAC
151  ACGACCTTCA ATGTCTAGCA GAAGAACTCA AAAACCTGGA GAAGTGCTA
201  GGTTTACCTC AAAGCAAAAA CGTTCACTTG ACAGACACCA AGGAATTAAT
251  CAGCAATATG ATGTACACAC TTCTGAAACT AAAGGGATCT GAACAGTTAC
301  ACTGTGAATA TGATGACGAG AAGC
```

Figure 11. Canine IL-2 sequence.
81% homology and 330 base pair overlap with human IL-2; length 324 base pairs. The numbers indicate the bases.

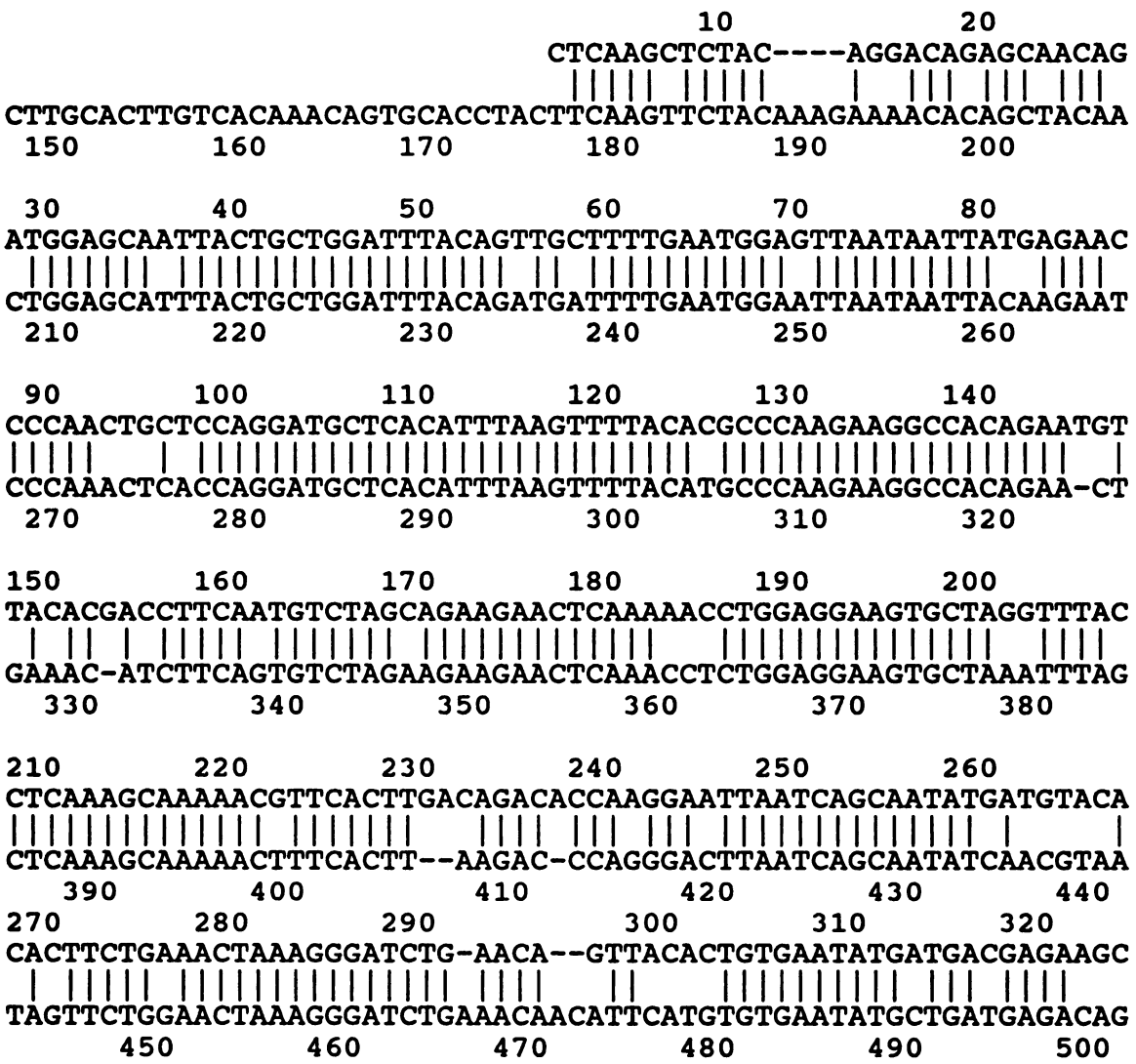

Figure 12. Comparison of human and canine IL-2 sequences. 81% homology. The top sequence is canine IL-2 and the bottom sequence is human IL-2. The numbers indicate the bases. Base number 1 of the canine IL-2 sequence begins matching at base number 178 of the human IL-2 sequence and the overlap is 330 bp in length.

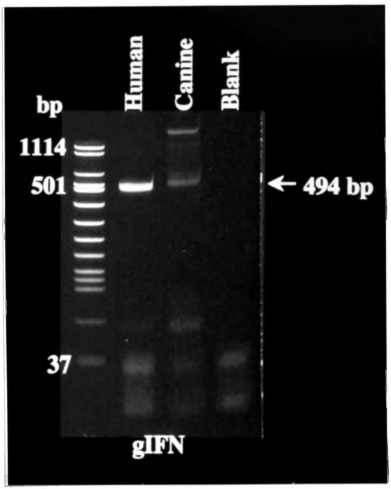

Figure 13. gIFN Polymerase Chain Reaction. Results of amplification of human and canine cDNA with the primer set for human gIFN. The arrow indicates the 494 bp canine PCR product.

```
  1  AATGCAAGTA ATCCAGATGT ATCGGACGGT GGGTCTCTTT TCGTAGATAT

 51  TTTGAAGAAA TGGAGAGAGG AGAGTGACAA AAACATCATT CAGAGCCAAA

101  TTGTCTCTTT CTACTTGAAA CTCTTTGACA ACTTTAAAGA TAACCAGATC

151  ATTCAAAGGA GCATGGATAC CATCAAGGAA GACATGCTTG GCACAGTTCT

201  TAAATAGCAG CACCAGTAAG AGGGAGGACT TCCTTAAGCT GATTCAAATC

251  CTTGGAACGA TCTGCAGG
```

Figure 14. Canine gIFN sequence.
97% homology and 268 base pair overlap with canine gIFN; length 269 base pairs. The numbers indicate the bases.

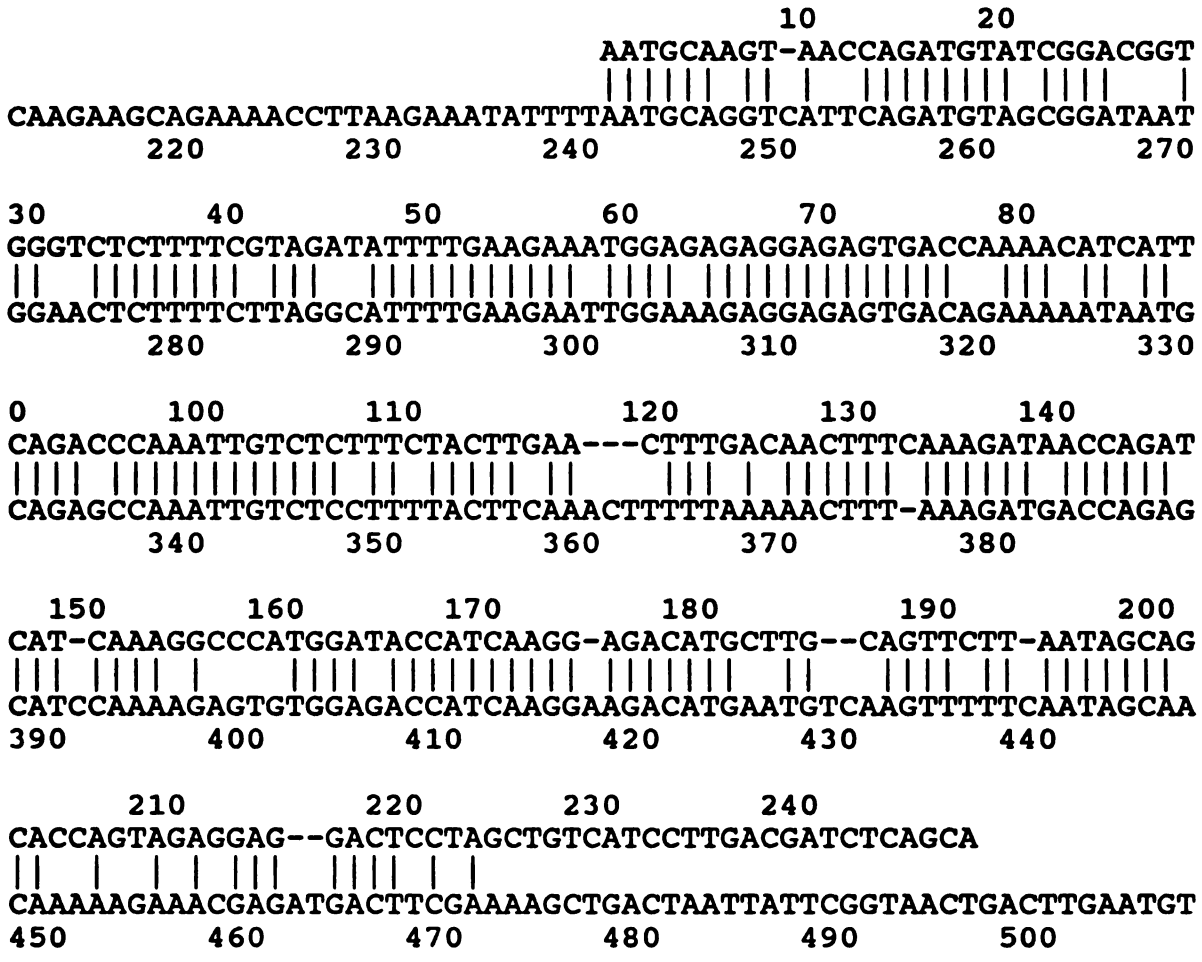

Figure 15. Comparison of human and canine gIFN sequences. 75% homology. The top sequence is canine gIFN and the bottom sequence is human gIFN. The numbers indicate the bases. Base number 1 of the canine gIFN sequence begins matching at base number 242 of the human gIFN sequence and the overlap is 249 bp in length.

PCR amplification of canine cDNA with human TNF-A primers still resulted in no products while PCR amplification of canine cDNA with murine TNF-A primers resulted in an additional product that was not obtained previously, approximately 447 bp. Results of the TNF-A PCR with murine primers are seen in Figure 15. For confirmation of the product obtained, the canine PCR product was excised, electroeluted and sequenced. No sequence was obtained for the canine TNF-A PCR product, again indicating that an incorrect PCR product size will probably result in no sequence or inaccurate sequence.

The optimal parameters for PCR amplification of the canine messages as well as the primer set origins are given in Table 1. The sizes of human or murine and canine PCR products are given in Table 2. As shown, canine CD4, IL-2 and gIFN are the same sizes as human CD4, IL-2 and gIFN; and canine TNF-B is the same size as murine TNF-B. Sequencing was successful for these canine PCR products and the parameters and amounts of primers for sequencing are given in Table 3.

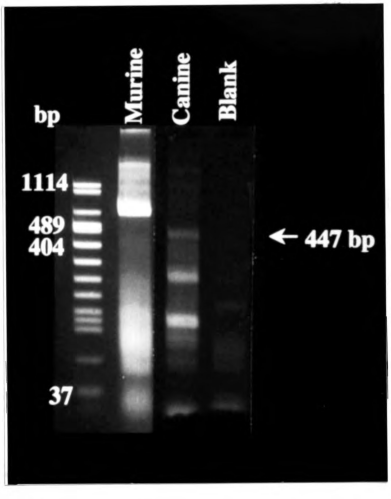

Figure 16. TNF-A Polymerase Chain Reaction. Results of amplification of murine and canine cDNA with the primer set for murine TNF-A. The arrow indicates the 447 bp canine PCR product.

Table 1. Optimal parameters for PCR amplification.

MESSAGE SOUGHT	ORIGIN OF PRIMERS	ANNEALING TEMPERATURE	CYCLE NUMBER
B-actin	Murine	55°C	30
CD4	Human	58°C	30
CD8	Human	55°C	40
IL-2	Human	58°C	35
gIFN	Human	58°C	35
TNF-A	Murine	61°C	35
TNF-B	Murine	61°C	45

Table 2. Sizes of amplified products.

MESSAGE SOUGHT	CONTROL cDNA CORRECT SIZE	CONTROL cDNA SIZE OBTAINED	CANINE cDNA
B-actin	540 bp	540 bp	281,216 bp
CD4	437 bp	437 bp	437 bp
CD8	454 bp	404 bp	362 bp
IL-2	458 bp	458 bp	458 bp
gIFN	494 bp	494 bp	494 bp
TNF-A	692 bp	692 bp	447,320,216 bp
TNF-B	278 bp	278 bp	278 bp

Table 3. Parameters and primer amounts for cycle sequencing.

MESSAGE SOUGHT	SENSE PRIMER	ANTI-SENSE PRIMER	ANNEALING TEMPERATURE	CYCLE NUMBER
CD4	1.3 ul	2.2 ul	58°C	30
IL-2	2.0 ul	2.0 ul	58°C	35
gIFN	1.7 ul	1.5 ul	55°C	35
TNF-B	1.2 ul	1.4 ul	61°C	45

DISCUSSION

Lymphocytes were isolated from peripheral blood by density gradient centrifugation and purified to remove other cell types present. Because 30-50% of the peripheral blood white blood cells are lymphocytes, lymphocytes were successfully isolated from peripheral blood. The lymphocytes were observed using a microscope and counted manually. Cell counts ranged from 4.15 million cells to 13.85 million cells for the different cell isolations performed.

The RNA isolation procedure used in this project allowed for the successful isolation of intact RNA which could be used for the synthesis of cDNA. The purity ratios of RNA ranged from 1.0 to 2.08 and the concentrations ranged form 0.66 ug/ul to 7.46 ug/ul. Many of the purity ratios were below the 1.8 minimum; however, the methods used in this project were not hindered by lower purity ratios. The integrity of both the RNA and the cDNA has been documented in Figure 1.

The presence or absence of the particular messages was detected by the PCR. Stimulation of lymphocytes was not necessary to isolate RNA and amplify B-actin and CD4 as is seen in Figures 1 and 2. T lymphocytes, in particular, were successfully stimulated for 72 hours because PHA stimulates T lymphocytes. The stimulation indices ranged from 14.30 to 50.93. Stimulation for 72 hours was necessary to isolate

RNA and amplify TNF-B as is seen in Figures 6. T lymphocytes were also successfully stimulated for 8 hours with PHA. The stimulation index, 2.56, was much less than the indices for 72 hours. Stimulation for 8 hours does not allow the lymphocytes to reach peak stimulation; however, stimulation for 8 hours was necessary to isolate RNA and amplify IL-2 and gIFN as is seen by Figures 9 and 12.

These results indicate that human and murine cytokine primer sets can be used to amplify canine messages. The fact that stimulation was not necessary to isolate RNA and amplify B-actin and CD4 is because these messages are actively being produced at all times. The fact that stimulation is necessary to isolate RNA and amplify IL-2, gIFN and TNF-B is because cytokines are not actively being produced at all times. The stimulation times necessary to isolate RNA and amplify for IL-2, gIFN, and TNF-B coincide with the stimulation times necessary to obtain peak levels for each of these cytokines. This demonstrates the transiency of cytokines and shows the importance of isolating RNA at specific times after stimulation for the different cytokines. This is also supported by the fact that stimulation for too long, 72 hours, before RNA isolation resulted in failure to isolate canine IL-2 and gIFN RNA and subsequent failure to amplify these messages in the PCR with human IL-2 and human gIFN primers. These PCR results were negative as were those for amplification of canine RNA from non-stimulated cells. As state previously,

however, isolation of IL-2 and gIFN RNA is not expected from non-stimulated cells. Stimulation for the correct time, 8 hours, before isolation allowed for isolation of canine IL-2 and gIFN RNA and amplification of these messages in the PCR with human IL-2 and human gIFN primers.

The necessity for stimulation to isolate RNA and amplify CD8 and the inaccurate sequence data obtained from human and canine CD8 PCR products is related to the fact that the human CD8 primers supplied by Clontech do not amplify CD8. This was discovered after some follow-up amplifications and restriction enzyme digests performed by Clontech after reviewing the results presented in this project. Stimulation should not be necessary to isolate RNA and amplify CD8 because this message is actively being produced at all times.

The failure to obtain sequence data on canine TNF-A may be due to several reasons. The first is that in all of the sequencing trials, the correct concentrations of all reaction components may not have been found, making it difficult to obtain sequence data. The second is that although PCR products were obtained, none may have been TNF-A, possibly because neither human nor murine primer sets may be capable of binding to the flanking regions of the canine TNF-A DNA sequence. The third is that TNF-A RNA may not have been isolated; stimulation of lymphocytes for 8 hours, however, should allow for the isolation of TNF-A RNA. Macrophages are, however, the main source of TNF-A, with

cytotoxic T lymphocytes being a very minor source. Few, if any, macrophages were observed in the cell preparations. Perhaps because the major cell source TNF-A was not present, TNF-A was not isolated in this project.

CONCLUSION

It is concluded that human and/or murine primers can be used to MAPP canine cytokines and surface markers. Canine CD4 mRNA can be isolated from non-stimulated canine lymphocytes and human CD4 primers can be used to amplify canine CD4 cDNA. Canine IL-2 and canine gIFN mRNA can be isolated from canine lymphocytes stimulated for 8 hours and human IL-2 and human gIFN primers can be used to amplify canine IL-2 cDNA and canine gIFN cDNA, respectively. Canine TNF-B mRNA can be isolated from canine lymphocytes stimulated for 72 hours and murine TNF-B primers can be used to amplify canine TNF-B cDNA. The MAPPing technique has not been established in the literature for the canine system; however the technique was successfully established in this project for the canine cytokines IL-2, gIFN and TNF-B and for the surface marker CD4.

It is also concluded that canine cytokine and surface marker cDNA sequences can be very homologous to human or murine sequences. Canine CD4 is 71% homologous to human CD4; canine IL-2 is 81% homologous to human IL-2; canine gIFN is 75% homologous to human gIFN; and canine TNF-B is 92% homologous to murine TNF-B. Except for canine gIFN, none of these canine sequences have been sequenced and documented in the literature.

FUTURE RECOMMENDATIONS

There are several suggestions for immediate research on the MAPPing technique. Because of the inability to establish the technique for CD8 due to the human CD8 primer set used in this project, the first recommendation is that the cDNA sequence data for human CD8, as published by Sukhatme, et al., be used to synthesize a primer set for human CD8, that the parameters for amplification of canine CD8 be determined and that the fidelity of the canine CD8 amplification product be confirmed.

Because of the inability to establish the technique for TNF-A, the second recommendation is that macrophages be isolated, purified and stimulated for TNF-A RNA isolation, that the parameters for amplification of canine TNF-A be determined using the murine TNF-A primer set and that the fidelity of the canine TNF-A amplification product be confirmed.

Because of the length of time required for the particular RNA isolation procedure used in this project, the third recommendation is that the quality of RNA isolated with newly developed RNA isolation techniques, such as those which isolate RNA from whole blood, be compared to the quality of RNA isolated with the procedure used in this project. It is also suggested that these various techniques be compared for the ease and speed of RNA isolation.

Because of the reason for developing the MAPPing technique for the canine system, the final recommendation is that the technique be used to obtain information pertaining to various canine diseases and evaluate the effectiveness of treatment for the diseases. PCR products obtained by using this project can also be used to develop specific reagents for the treatment of diseases. Each product can be used to make probes for the different cytokine DNA sequences; the probes can be used for detection of the DNA sequences in a canine cDNA library. Each product can also be used to transfect canine cells to replace missing or defective genes for the treatment of these types of diseases. A clinical example a dog that was brought into a veterinary hospital for severe vomiting and diarrhea. The dog was being treated for several different bacterial infections, but during the hospital stay, the dog also developed severe lymphopenia. Because the dog did not respond to intensive care therapy, the veterinarians suspected an immunodeficiency. The dog's peripheral blood lymphocytes were then tested for the ability to respond to PHA stimulation and produce IL-2. The dog did not proliferate in response to PHA and she did not produce IL-2 nor did the dog respond to exogenously added IL-2. The veterinarians felt that this was most likely due to defects in IL-2 production. It was suspected that the dog also had a retroviral infection. Obviously, the retroviral infection needed to be treated and it needed to be determined whether the immunosuppression was due to a

helper T cell defect (46). Part of the treatment, however, could have been transfection of the dogs helper T cells with the genetic information for canine IL-2 to replace the defective IL-2 production. The TNF product can also be transfected into canine tumor infiltrating lymphocytes for treatment of cancers. Each product can also be inserted into an expression vector for the production of proteins which can be used to develop monoclonal antibodies for the treatment of immune-mediated diseases. IL-2 is such a critical mediator of the cellular immune response because it is responsible for T cell proliferation and differentiation. Therefore, monoclonal antibodies against IL-2 would decrease the quantity of IL-2 and its effects on T cells (47).

APPENDIX

APPENDIX

REAGENTS:

A. Lymphocyte Isolation

1) Phosphate buffered saline (PBS), pH 7.4
per 4 liters

32 g	NaCl
0.8 g	KCl
4.6 g	NaH_2PO_4
0.8 g	KH_2PO_4

2) Ficoll-hypaque
per liter

108 g	Ficoll
27.3 ml	75% hypaque

3) 2X EDTA buffered saline (2X EPS), pH 7.4
per 4 liters

72 g	NaCl
28.64 g	$EDTA\text{-}Na_3$
8.64 g	KH_2PO_4

B. RPMI (Roswell Park Memorial Institute) Media
per 500 ml

3 ml	fungizone
0.16 ml	gentamycin sulfate
10 ml	sodium bicarbonate
6 ml	HEPES
2.25 ml	sodium pyruvate

C. Nutrient Media

1) Human Nutrient Media — 90% RPMI media, 10% fetal calf serum

2) Canine Culture Media — 90% RPMI media, 10% canine pooled serum

D. RNA isolation

1) Denaturing solution

4 M	guanidinium isothiocyanate
0.5 mM	sodium citrate, pH 7.0
0.5%	sarcosyl
0.1 M	B-mercaptoethanol

2) 2 M sodium acetate, pH 4.0

3) Phenol, water unsaturated

4) Chloroform:isoamyl alcohol — 19 ml chloroform, 1 ml isoamyl alcohol

5) 100% isopropanol

6) 75% ethanol, DEPC treated
7) TE buffer — 10 mM Tris-HCl, pH 7.4; 0.1 mM EDTA
8) 3 M sodium acetate, pH 5.4

E. cDNA synthesis
1) DEPC ddH_2O
2) random hexamer primers, 20 uM
3) 5X reaction buffer — 250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$
4) dNTP mix — 10 mM dATP; 10 mM dCTP; 10 mM dGTP; 10 mM dTTP
5) RNase inhibitor, 40 Units/ul
6) M-MLV reverse transcriptase, 200 Units/ul

F. Polymerase Chain Reaction
1) 10X reaction buffer — 500 mM KCl; 100 mM Tris-HCl, pH 8.3; 15 mM $MgCl_2$; 0.1% gelatin
2) dNTP mix — 125 uM dATP; 125 uM dCTP; 125 uM dGTP; 125 uM dTTP
3) Taq polymerase, 5 Units/ul, diluted 1:4
4) Primers — Human CD4 primers, 20 uM; Human CD8 primers, 20 uM; Murine IL-2 primers, 20 uM; Murine gIFN primers, 20 uM; Murine TNF-B primers, 20 uM; Murine TNF-A primers, 20 uM

G. Gel electrophoresis
1) 6% polyacrylamide gel
A) 1 ml 30% acrylamide — 29 g acrylamide; 1 g N,N'-methylene bisacrylamide
B) 3.9 ml deionized distilled H_2O
C) 100 ul 50X TAE — per liter: 242 g Tris base; 57.1 ml glacial acetic acid; 100 ml 0.5 M EDTA, pH 8.0
D) 5 ul TEMED
E) 10 ul 40 % ammonium persulfate
2) 5X TAE

H. Cycle sequencing reaction

1) 10X sequencing buffer
   - 100 mM Tris-HCl, pH 8.8
   - 500 mM KCl
   - 0.01% gelatin
   - 40 mM $MgCl_2$
   - 20 uM dATP
   - 50 uM dCTP
   - 50 uM dGTP
   - 50 uM dTTP

2) ddNTP
   - 600 uM ddATP
   - 600 uM ddCTP
   - 100 uM ddGTP
   - 1000 uM ddTTP

3) 33P-dATP, 10 mCurie/ml
4) Taq polymerase, 5 Units/ml
5) Stop dye mix
   - 80% formamide
   - 50 mM Tris, pH 8.3
   - 1 mM EDTA
   - 0.1% bromophenol blue
   - 0.1% xylene cyanol

I. Sequencing gel electrophoresis

1) 50 ml 6% polyacrylamide, per liter:
   - A) 57 g acrylamide
   - B) 3 g N,N'-methylene bis acrylamide
   - C) 100 ml 10X TBE per liter
     - 108 g Tris base
     - 55 g boric acid
     - 40 ml 0.5M EDTA, pH 8.0
   - D) 500 g urea
   - E) 50 ul TEMED
   - F) 100 ul 40% ammonium persulfate

2) 1X TBE

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