Cloning bovine cytokine cDNA fragments and measuring bovine cytokine mRNA using the reverse transcription-polymerase chain reaction

by

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GENERAL INTRODUCTION

Over the past decade numerous studies have focused on the role of cytokines in physiological and pathological processes. Cytokines regulate immune and inflammatory responses and may also be involved in coordinating the immune system with other systems of the body. The rapid expansion of research in cytokine biology has been facilitated by the development of methods to analyze cytokine production. However, obtaining meaningful data from any one technique is difficult because each method is hampered by inherent limitations. Therefore, sufficient knowledge of each method's capabilities and limitations is necessary when choosing between techniques available for studying cytokines. In addition, the continued development of new and improved methods and reagents for evaluating cytokine production is necessary. This is especially true in the area of veterinary research where the study of cytokines in domestic animals has been limited by the lack of species-specific reagents.

Explanation of Thesis Format

This thesis is composed of two papers and a brief summary. The first paper is an examination of the most commonly used methods of analyzing cytokines with a focus on each method's capabilities and limitions. This portion of the thesis is written in the form of a review paper (Paper I). In

the second part of the thesis, I have addressed the need for species-specific reagents for studying cytokines in domestic animals by using the reverse transcription-polymerase chain reaction to develop nucleic acid probes that are specific for cytokines in cattle. This technique was also used to study the expression of bovine cytokine genes in mitogen stimulated peripheral blood mononuclear cells (Paper II). The work reported in the second paper has been submitted for publication.

PAPER I. ADVANTAGES AND DISADVANTAGES OF THE COMMONLY USED METHODS FOR ANALYZING CYTOKINES

INTRODUCTION

Cytokines are a group of small molecular weight proteins that are secreted primarily by cells of the immune system and play a central role in regulating immune and inflammatory responses. These molecules have pleiotropic and often overlapping biological effects on lymphoid and non-lymphoid cells (Benveniste and Merrill, 1986; Thornhill et al., 1990, Last-Barney et al., 1988). Cytokines may also be involved in integrating the immune system with other systems of the body (Frei et al., 1989; Breder et al., 1988). Over the past decade, numerous studies have focused on the role of cytokines in physiological and pathological processes.

Cytokines were first identified by their biological activity that was usually present in complex biological fluids (Gery and Waksman, 1972; Gordon and MacLean, 1965; Howard et al., 1982). This resulted in confusion and redundancy in assigning functions and names to the myriad of cytokines and their activities. In the last ten years advancements in protein purification, recombinant DNA technology and monoclonal antibody production have converted the confusing collection of cytokines into well-defined proteins with known activities.

The technological advances of the last decade have also produced reagents necessary for advancing the study of cytokine biology. The availability of recombinant cytokines

has allowed researchers to definitively determine the biologic role of cytokines and to evaluate their potential as therapeutic agents. Purified cytokines have also lead to the development of cytokine-specific antibodies and cytokine-responsive cell lines. In addition, molecular techniques have produced cytokine-specific nucleic acid probes. These reagents have allowed the development of a wide variety of methods for studying cytokines.

Obtaining meaningful data about cytokine production from any one technique is difficult because each method is hampered by inherent limitations. The selection of appropriate methods of cytokine analysis for a particular research objective is important and requires a working knowledge of each technique. This paper is a review of the capabilities and limitations of the most commonly used techniques of cytokine analysis.

NATURAL AND RECOMBINANT CYTOKINES

A prerequisite for developing cytokine assays is the availability of purified cytokines. These preparations are needed to develop various assay reagents, standards, and radioactive compounds. Ideally, the purified cytokine should be identical to the endogenous cytokine for which the assay is intended to measure, but practical considerations prevent this in many cases (Chard, 1982). Purifying cytokines from natural sources is time-consuming and labor-intensive and is not a practical alternative for most laboratories. Cytokines isolated from humans and laboratory animals have been prepared by several methods and are available commercially. These include cytokines isolated from natural sources and recombinant products expressed in prokaryotic and eukaryotic systems. The type of cytokine necessary for developing and/or conducting an assay depends on the type of assay.

Biologically active cytokines are required for developing cytokine biologic assays. The cytokines are necessary when cloning and maintaining cytokine-responsive target cell lines and they are needed to generate standard curves when conducting the assays. Cytokines isolated from natural sources are ideal for this application but suffer from two limitations. These preparations often contain trace amounts of other proteins that may interfere in the assay (Thurman et al.; 1986; Mier et al., 1985), and the elaborate purification

schemes that are used to obtain these products can affect their activity (Thurman et al., 1986; Smith et al., 1983).

Recombinant cytokines offer an attractive alternative because they are available in highly purified and biologically active forms. Most recombinant cytokines produced in prokaryotic expression systems maintain their biologic activity even though they are not glycosylated (Casagli et al., 1989; Tocci et al., 1987; March et al., 1985). These preparations give satisfactory results in most bioassays. However, an ever increasing number of recombinant cytokines are being expressed in eukaryotic systems because they are glycosylated in a manner comparable to the naturally occurring form. These recombinant products may or may not differ functionally from naturally occurring glycosylated cytokines or non-glycoslyated cytokines that are expressed in prokaryotic systems (Casagli et al., 1989). Information concerning the biologic activity of these products is usually provided by the manufacturer.

The type of cytokine preparation used for making antibodies to be used in cytokine immunoassays depends largely on whether polyclonal or monoclonal antibodies are being produced. Cytokines isolated from natural sources are antigenically analogous to the cytokines that are to be assayed, and therefore they are ideal immunogens for producing antibodies. However, the impurity of these preparations

increases the likelihood of obtaining cross-reacting immunoglobins when producing polyclonal antibodies (Tijssen, 1985, Auron et al., 1984; Mier et al., 1985; Thurman et al., 1986). This is not a concern when producing monoclonal antibodies since cross-reacting antibodies can be eliminated when screening and selecting the antibody-producing clone (Tijssen, 1985).

Recombinant cytokines are highly purified, but there is a potential risk in using these products to generate antibodies. Recombinant cytokines may differ antigenically from the naturally occurring cytokines because of differences in post-translational processing and conformational structure (Rafferty et al., 1991; Peppard et al., 1992; Thurman et al., 1986). Antibodies produced against a recombinant product may not bind to the naturally occurring protein. Therefore, antibodies must be chosen that recognize epitopes shared by the recombinant and natural cytokines (Gaffney et al., 1987; Abrams and Pearce, 1988; Sato et al., 1986).

Purified cytokine preparations for humans and laboratory animals have been widely developed and are available commercially. However, this is not the case for cytokines from other species. This has severely limited the development of cytokine assays for other species including domesticated animals. Several cytokine genes in pigs and cattle have been cloned (Blecha, 1991), although the recombinant products are

not yet commercially available. This situation may improve as the study of cytokines in domestic animals progresses. Fortunately, cytokines exhibit varying degrees of cross-species reactivity and many human and murine cytokines have been found to be biologically active on cells from other species (Fong and Doyle, 1986; Stott et al., 1986; Nagi and Babiuk, 1989). Therefore, murine and human cytokines can sometimes be used to develop and conduct cytokine assays for other species when homologous cytokine preparations are not available.

BIOASSAYS

Bioassays detect cytokines by assessing their biologic affect on an indicator cell population. Because cytokines are potent effector molecules, bioassays are a sensitive method of detecting cytokines and are usually used to measure cytokines that are present in body fluids and culture supernatants. Numerous cytokine bioassays have been described and many are able to detect cytokines in the sub-picomolar range (Eskandari et al., 1990; Orencole and Dinarello, 1989).

Cytokine bioassays take advantage of each cytokine's unique functional properties and therefore differ markedly depending on the cytokine being studied. In addition, some cytokines can be analyzed in a variety of ways because of the pleiotropic nature of their bioactivity (Falk et al., 1987; Conlon, 1983; Gillis and Mizel, 1981). Despite this diversity, many cytokines are assayed by their ability to stimulate cell proliferation. Bioassays have also been developed that measure other cytokine-induced responses. For example, tumor necrosis factor is assayed by its ability to lyse tumor cell lines (Espevik and Nissen-Meyer, 1986; Eskandari et al., 1990). The neutrophil activator, IL-8, can be detected by its affect on specific neutrophil functions (Matsushima and Oppenheim, 1989).

Most biological assays require the measurement of surviving and/or proliferating mammalian cells. This can be

accomplished by several methods including counting viable cells, measuring released ^{51}Cr-labeled protein after cell lysis (Zheng et al., 1990), and measuring the incorporation of radioactive nucleotides (Gillis et al., 1978). Another alternative is to pulse the cells with the tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). This compound is converted by viable cells to a colored product that can be quantitated spectrophotometrically (Mosmann, 1983). When combined with limiting dilution techniques and probit analysis, cytokine biologic assays allow the accurate quantitation of cytokine activity (de St. Groth, 1982; Jordan, 1972).

Clonally-derived cells lines are preferred as indicator cells in bioassays because they provide a homogeneous, well-characterized, population of cells that give a uniform response. The availability of purified cytokines has facilitated the isolation and cloning of numerous cytokine-responsive cell lines (Gillis and Mizel, 1981; Gillis et al., 1978; Ihle et al., 1982; Hu-Li et al., 1989; Lansdorp et al., 1986). Some of these cell lines can be purchased for a minimal price from the American Type Culture Collection (ATCC, Rockville, Maryland).

Although it has been demonstrated that cytokines are a highly conserved group of molecules, they still exhibit varying degrees of species-specificity (Olsen and Stevens,

1993; Rot, 1991; Goodwin et al., 1989). For optimum responses in bioassays, indicator cells from the same species as the cytokine to be assayed should be used. This is rarely a problem when developing bioassays for measuring human or laboratory animal cytokines since indicator cell lines and neutralizing antibodies for these species have been extensively developed and are commercially available. These reagents are not as abundant for other species, and this can pose a problem when developing bioassays to measure cytokines in domestic animals. Generally, investigators wishing to measure cytokines from these species must either use heterologous cell lines and antibodies or assume the laborious task of developing these reagents themselves.

A major disadvantage associated with bioassays is their lack of specificity. Samples that are assayed for a specific cytokine often contain other biologically active molecules that may have stimulatory or inhibitory affects on the indicator cell lines that are used in the assay (Ranges et al., 1988; Helle et al., 1988; Baker et al., 1981). Many assays that were once thought to be specific for a certain cytokine have now been shown to respond to other cytokines. For example, several cells lines that have been used to assay for IL-2, such as CTLL2 and HT-2, have been found to also proliferate to IL-4 (Grabstein et al., 1986; Ho et al., 1987). In some cases, contaminating substances have an antagonistic

effect on the target cell response (Gajewski and Fitch, 1990; Tilden and Balch, 1982). These studies and numerous others make it apparent that bioassays are of limited utility unless steps are taken to demonstrate their specificity.

The most effective way to demonstrate the specificity of a bioassay is to use neutralizing antibodies. These are either monoclonal or polyclonal antibodies raised against a cytokine that bind to the active site on the molecule and inhibit its activity (Brown et al., 1990). The antibody is added to the test sample and the ability of the antibody to negate the activity in the sample demonstrates the cytokine specificity of the response. Similarly, antibodies to cytokines that are known to interfere in the assay can be used to eliminate the interfering activity and allow the cytokine of interest to act on the target cells (Ohara and Paul, 1985). In addition, soluble cytokine receptors, antibodies to cytokine receptors and cytokine receptor antagonists have also been used to block cytokine/receptor binding and thereby neutralize cytokine bioactivity (Sheehan et al., 1988; Fanslow et al., 1991; Estrov et al., 1992).

Another disadvantage of bioassays is that they can only detect extracellular, biologically active cytokines. While most cytokines are secreted proteins, biologically active, membrane-bound forms of some cytokines have been identified (Kriegler et al., 1988; Kurt-Jones et al., 1985; Peck et al.,

1989). In addition, internalization of the cytokine/receptor complex is necessary for the activation of many cytokine responsive cells (Smith, 1988; Lowenthal and MacDonald, 1986). This process removes cytokines from solution and precludes them from being detected in bioassays. Consequently, bioassays may underestimate the amount of cytokines being produced by cells by not detecting cell-associated molecules.

Another characteristic of bioassays that limit their use in many situations is that they require the technical skill, equipment, and reagents that are necessary for maintaining mammalian cell cultures. The indicator cells must be passed to fresh media at regular intervals and they must be grown in sufficient quantities when conducting the assays. Furthermore, some cell lines may require special media additives for optimal growth and/or viability. These culture manipulations and reagents that are required to conduct cytokine bioassays can be time-consuming and expensive.

In summary, bioassays can be a very sensitive and quantitative means of measuring extracellular, biologically-active cytokines. The disadvantages of these assays are that they can be technically difficult and expensive to complete and measures must be taken to demonstrate their specificity.

IMMUNOASSAYS

The production and characterization of antibodies to cytokines has facilitated the development of a variety of antibody-based assay techniques. These methods take advantage of the antigen/antibody bond which allows the accurate identification and discrimination of the antigen (i.e. cytokine) in complex mixtures of proteins. Cytokine immunoassays are sensitive, specific, and can give quantitative results without the technical difficulties that are associated with bioassays.

The most critical parameter in developing a cytokine immunoassay is the development of appropriate antibodies. The sensitivity of immunoassays has been shown to vary dramatically depending on the affinity and specificity of the antibodies used (Brown et al., 1990; Schumacher et al., 1988). All antibodies must be rigorously screened by western blotting for cross-reactivity and non-specific binding to other molecules. Both polyclonal and monoclonal antibodies have been successfully used in cytokine immunoassays (Wadhwa et al., 1990; DeForge and Remick, 1991; Gaffney et al., 1987). However, there are several factors to consider when choosing between these two reagents.

Monoclonal antibodies are produced by a single B cell clone and are therefore highly specific and are free of irrelevant immunoglobins. In addition, they can be produced in

virtually unlimited quantities with predictable and reproducible specificities and affinities (Campbell, 1984). As mentioned earlier, purity of the immunizing cytokine preparation is not critical because non-specific antibodies are eliminated during selection and cloning. The disadvantages associated with monoclonal antibodies are that they are time-consuming and expensive to produce and their affinity may be too low for successful use in binding assays (Campbell, 1984).

The generation of polyclonal antibodies requires a highly purified immunogen since antibodies raised against the impurities are not eliminated and they may interfere in the assay (Tijssen, 1985). Even when a pure immunogen is used, cross-reacting antibodies can still be a problem if the cytokine shares structural homology with other proteins (Auron et al., 1984; Dinarello et al, 1977). Furthermore, obtaining reproducible reagents is difficult because of the variation in immune responses between animals. (Tijssen, 1985). Despite these problems, there are some advantages to using polyclonal antibodies in cytokine immunoassays. The many different epitopes involved in cytokine/antibody binding creates an additive effect and results in a high affinity reaction. In addition, polyclonal antibodies are easy and inexpensive to develop (Campbell, 1984).

Like bioassays, cytokine immunoassays only detect secreted cytokines. However, unlike bioassays, immunoassays do

not require the cytokine to be functional in order to be detected. Consequently, immunoassays may detect inactive cytokines, cytokine precursors or cytokine break-down products that still maintain their structural epitopes but are not biologically active (Jaattela et al., 1988). This may result in overestimating the amount of biologically active cytokines that are present in the assayed sample. Running concurrent bioassays is one way of ensuring that the immunoassays are detecting biologically active cytokines (Rampart et al., 1992; van der Meer et al., 1988; Jaattela et al., 1988; Wadhwa et al., 1990).

The most common immunoassays that are used in cytokine research are the enzyme-linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA).

Enzyme-linked Immunosorbent Assay

This technique, originally described by Engvall and Perlmann (1971), has proven invaluable for detecting and quantifying antibodies in serum, hybridoma supernatants, and ascites fluids. Modifications of the original format have allowed this method to be used for measuring antigens that are present in biological fluids (Tijssen, 1985). ELISA's are rapidly replacing most other immunoassays in both clinical and research laboratories because they are fast, easy to conduct, give quantitative results, and require a minimum of reagents

and equipment.

The ELISA techniques that are used in most cytokine assays rely on the use of a cytokine specific-antibody that selectively binds to the cytokine of interest. A direct or indirect method of detecting the cytokine can be used. In the direct method, the cytokine-specific antibody is conjugated to an enzyme. In the indirect method, the cytokine-specific antibody is unlabelled and a second labelled antibody is used to bind to the first antibody (Carson, 1990).

After antigen/antibody binding the conjugated enzyme cleaves a substrate to generate a colored reaction product that is soluble and can be detected spectrophotometrically. The absorbance of the colored solution is proportional to the amount of cytokine present in the test sample. ELISA's are routinely conducted in 96-well microtiter plates using serial dilutions of the test sample. Data is collected by using a microtiter plate reader that measures the absorbance of the solutions contained in the individual microtiter plate wells (Tijssen, 1985).

ELISA's are termed a solid phase immunoassay because either the antigen or antibody is attached to the microtiter plate wells. The two types of ELISA techniques that are used most frequently in measuring cytokines are the direct and capture or sandwich ELISA. These two methods differ in how the antigen is immobilized onto the microtiter plates. The direct

ELISA involves binding serial dilutions of the cytokine-containing sample directly to microtiter wells. The capture ELISA binds the cytokine to the plates by "capturing" it with a cytokine-specific antibody that is bound to the microtiter well.

The capture ELISA has been shown to be more sensitive because this method selectively absorbs the cytokine to the plates; thus eliminating contaminating and possibly cross-reacting material (DeForge and Remick, 1991). The capture ELISA requires two antibodies that bind two separate epitopes so that the capture antibody and the enzyme-linked antibody can bind the cytokine simultaneously. These requirements can be satisfied with one or two polyclonal (Gaffney et al., 1987), two monoclonal (Schumacher et al., 1988; Andersson et al., 1989) or a combination of polyclonal and monoclonal antibodies (Wadhwa et al., 1990; Ko et al., 1992). Most commercially available cytokine ELISA "kits" use the capture or sandwich format and they have a detection limit of approximately 10 picograms. However, the sensitivity of the assays may vary depending on the complexity of the assayed samples (Andersson et al., 1989).

ELISA test kits and reagents for detecting human and laboratory animal cytokines have been extensively developed and many are commercially available. Similar kits and reagents for detecting cytokines in domestic animals are not as readily

available except for one commercially-produced kit for detecting bovine interferon-γ. ELISA's developed for the detection of cytokines in one species may or may not function in other species since cytokine-specific antibodies have been shown to exhibit species-specificity (Schumacher et al., 1988). Manufacturers often provide information on whether or not their reagents and/or kits can be used to measure cytokines in other species.

Radioimmunoassay

Radioimmunoassays were first developed for the quantitation of insulin in serum (Yallow and Berson, 1960) and historically have been used in the field of endocrinology for the sensitive detection of peptide hormones. In the last decade, with the availability of purified cytokines and cytokine specific-antibodies, sensitive radioimmunoassays have been developed for use in cytokine research.

RIA's are liquid-phase antibody-based assays that belong to a series of related techniques called binding assays. Binding assays involve quantitating a ligand in solution by allowing the ligand to saturate a specific binder and then determining the distribution of the ligand between the bound and free phases (Chard, 1982). In the case of the RIA's for cytokines, the cytokine acts as the ligand, or substance to be measured, and an antibody specific for the cytokine serves as

the binder. Also included in the assay is a tracer or known amount of purified radio-labelled cytokine which allows quantitating the amount of cytokine between the bound and free states (Poole et al., 1989; Rampart et al., 1992). The amount of radioactivity in the bound state is inversely proportional to the amount of cytokine in the test sample. A standard curve of purified unlabelled cytokine is included in the assay and unknown samples are quantified by regression analysis (Chard, 1982).

RIA's are a very sensitive and quantitative method of analyzing cytokines in biological fluids. The reported sensitivities for RIA's vary, but most systems can detect cytokines in the nanomolar range (Poole et al, 1989; Rampart et al., 1992; van der Meer et al, 1988). Some commercially available cytokine RIA kits are able to detect cytokines in the picogram range, although the sensitivity may vary depending on the nature and complexity of the test solution. Some companies report that RIA's are less sensitive when assaying serum as opposed to culture supernatants.

The one major disadvantage of RIA's is that it requires the use of radioisotopes which necessitates the safe handling of reagents and the proper disposal of radioactive waste. In addition, radio-labelled tracers must be frequently prepared because of the short half-life of some of the commonly used isotopes. These precautions and considerations associated with

the use of radioisotopes can make RIA's inconvenient to conduct.

In summary, obtaining antibody preparations that are appropriate for use in immunoassays is the major limiting factor in developing these techniques for cytokine analysis. These methods do not discriminate between biologically active and inactive cytokines and they detect only extra-cellular cytokines. However, they do offer a convenient, reliable method of cytokine analysis that can give sensitive, specific and quantitative results.

TRANSCRIPT ANALYSIS

Transcript analysis monitors cytokine production by using nucleic acid hybridization methodology to detect cytokine-specific mRNA molecules. Most cytokine genes are not constitutively expressed and have been shown to be regulated at the transcriptional (Fenton et al., 1987; Servan-Chiara et al., 1986) and post-transcriptional levels (Sariban et al., 1988; Zuckerman et al., 1991; Bickel et al., 1990). The presence of cytokine-specific mRNA molecules in a cell is indicative of the level of cytokine gene expression and can provide information about the regulation of cytokine production.

Transcript analysis uses sequence-specific nucleic acid probes that recognize and bind to mRNA molecules that bear a complementary nucleotide sequence (Sambrook et al., 1989). Effective nucleic acid probes can be single or double stranded, RNA or DNA. The probes vary in length depending on the technique being used (Keller and Manak, 1989). The unique nucleotide sequence of the probe confers its specificity to the target mRNA molecules and allows the detection of minute amounts of target mRNA molecules in complex mixtures of macromolecules.

Analysis of cytokine mRNA is usually performed with the assumption that the defined levels of cytokine mRNA expression correspond to certain degrees of cytokine production. This

correlation has been proven in many instances. However, cytokine production can also be regulated post-transcriptionally and in these instances the presence of cytokine mRNA may not be correlated with cytokine production and secretion (Chantry et al., 1989; Nguyen et al., 1990; Descoteaux and Matlashewski, 1990). Therefore, evaluating cytokine production solely on the basis of cytokine-specific mRNA expression may give erroneous results concerning the amount of biologically active cytokine that is being secreted by the cells.

The most extensive cloning of cytokine genes has been done in humans and laboratory animals and this has resulted in a wide range of probes that are available for cytokine transcript analysis in these species. The cloning of cytokine genes in domestic animals has been relatively limited. Few cytokine genes have been cloned in cattle, pigs, and sheep (reviewed in Blecha, 1991), and the unavailability of species-specific probes has hampered progress in studying the transcription of cytokine genes in these domestic animals. Sufficient homology often exists between cytokine genes of different species to allow the use of heterologous probes in detecting cytokine transcripts (Jensen and Schultz, 1991; Stevens et al., 1992). However, the imperfect homology detracts from the specificity and sensitivity of the probes (Sambrook et al., 1989).

Most methods of transcript analysis require RNA to be isolated from cells. The unique characteristics of cytokine gene transcripts and gene expression pose several problems in accomplishing this task. First of all, cytokine gene expression is often a transient event which requires cells or tissue samples to be taken at frequent intervals (Weinberg et al., 1990; Shaw et al., 1988). Secondly, while all RNA requires special handling because of its susceptibility to degradation by nucleases, many cytokine mRNA's contain highly conserved AU-rich regions that make these molecules extremely unstable (Shaw and Kamen, 1986; Caput, 1986). Thirdly, many cytokines are expressed at low levels and often by only a few cells (Hutchings et al., 1989; Powers et al., 1988). Therefore, cytokine transcripts can be lost in the noise of the many times more numerous background cells that are not expressing the target gene. Consequently, the analysis of these transcripts requires isolating large quantities of RNA and using highly sensitive detection methods.

Methods of transcript analysis can be divided into two general categories based on whether the analyzed RNA is fixed to a solid support or is in solution. Northern and dot-blot hybridization require that the RNA is fixed to a membrane while the nuclease protection assay and RT-PCR are solution hybridization techniques.

Northern Blots

Northern blotting is widely used for the analysis of gene expression and has been extensively used in cytokine research. This technique can provide qualitative and semi-quantitative information about the relative abundance of specific mRNA molecules. In addition, it is also useful in demonstrating the transcript specificity of nucleic acid probes.

Northern blotting involves the transfer of RNA that has been size fractionated by electrophoresis to a solid support membrane. The RNA is permanently fixed to the membrane and the membrane is incubated in a solution containing a labelled probe that is specific for the target mRNA molecule. After the probe is allowed to bind to the RNA, the support membrane is washed extensively to remove non-specific probe binding, and a detection system is used to visualize the probe/transcript complex. The approximate size of the detected transcript can be determined by including RNA molecular weight size standards in the assay (Sambrook et al., 1989).

The probes used in northern blots can be labelled in a variety of ways. The most commonly used method involves labelling the probes with a radioisotope such as ^{32}phosphorus (Keller and Manak, 1989). The probe is detected by exposing the membrane to X-ray film. In addition to being very sensitive, the use of radioactive probes allows the membrane to be stripped of one probe and re-hybridized to a different

probe (Sambrook et al., 1989). Several non-radioactive methods of labelling and detecting nucleic acid probes are also available. These include biotin-avidin linked enzyme detection systems, and enzyme-linked antibody detection systems (Langer et al., 1981; Raap et al., 1989). These methods are safer and more convenient than radioactive labelling methods, but they are less sensitive and do not allow the membrane to be stripped and re-probed with a different probe (Ambros and Karlic, 1987).

Detecting low abundance cytokine mRNA can be a problem when using northern blots, even when using highly sensitive probes. In order to provide sufficient cytokine mRNA for detection using this method large amounts of RNA must be electrophoresed and transferred to the membrane. As much as 10 to 20 μg of total RNA is often required for detection of some cytokine mRNAs (Stabel et al., 1993; Weinberg et al., 1990). Extractions on this scale require large quantities of starting material, which may preclude the use of this technique in situations where tissue samples are small.

The sensitivity of northern blots can be improved by isolating mRNA from the total RNA preparations (Sambrook et al, 1989). This procedure eliminates other RNA species from the preparation and makes the mRNA more accessible for hybridization. mRNA can be isolated from other RNA species by oligo(dt) cellulose chromatography (Aviv and Leber, 1972).

mRNA isolation may increase the sensitivity of northern blots, but the extra purification step also significantly reduces the amount of usable RNA that is obtained from a given amount of cells or tissue. Several papers have reported greater sensitivity in blot-hybridization analyses when single-stranded RNA probes are used instead of the commonly used double-stranded DNA probes (Cerretti et al., 1986; Melton et al., 1984).

In addition to problems with sensitivity, another disadvantage of northern blot analysis is that quantitative data is difficult to obtain. This is partially due to the problems associated with accurately quantifying RNA and achieving uniform loading of agarose gels. One method that has been used to address this problem is to compare the transcript levels for the cytokine mRNA to the transcript level of a "house keeping" or control gene on the same blot. Probes specific for ß-Actin (Stabel et al., 1993), glyceraldehyde 3-phosphate dehydrogenase (Zentella et al., 1991), and ribosomal RNA species (Chantry et al., 1989) are commonly used in northern blots as control genes. The control gene is assumed to be expressed at a constant level and the data obtained for the cytokine transcript is normalized to that of the control transcript.

The major criticism of using this approach is that the control gene must be expressed at a constant level under all

conditions that are used in the study. This is difficult to prove since quantitatively measuring the expression of control genes is inherently difficult. Indeed, the expression of the commonly used control gene, ß-actin, has been found to fluctuate in lymphoid cells under a variety of stimuli (Siebert and Fukuda, 1985; Degen et al., 1983). Therefore, if data for a cytokine transcript is to be normalized to a control gene, the expression of the control gene must be rigorously checked for consistent expression under all conditions that are used in the study.

Two methods are available for assigning numerical values to hybridization signals that are obtained from northern blots. The most sensitive technique involves excising the portion of the membrane containing the hybridized probe and counting it in a scintillation counter (Stabel et al., 1993). A second method uses a densitometer to quantify the hybridization signals on autoradiographs or directly on membranes if an enzyme-based detection system is used (Nguyen et al., 1990). Both methods assign numeric values to the hybridization signals based on the intensity of the bands and they allow comparisons between the relative amounts of specific mRNA molecules that are present in the samples.

Dot Blots

This method of transcript analysis is similar to northern blots in that RNA is attached to a solid support for hybridization, but differs in that the electrophoretic separation and transfer steps are omitted. The RNA is spotted directly onto the membrane usually with the aid of a vacuum manifold that has wells to contain each sample (Sambrook et al., 1989). There are several manifolds commercially available with different sizes, shapes and arrangements of the wells. This technique may also be referred to as spot or slot hybridization depending on the shape of the wells in the manifold.

While similar in principle to northern blots, dot blots offer some advantages over northern blots. The obvious advantage is the time savings involved in eliminating the RNA electrophoresis step. RNA losses that occur during gel blotting can also be avoided. Moreover, the discrete dot (or slot) hybridization signals obtained with the dot-blot method are more conducive to quantitation using either densitometry or scintillation counting than the broad and often diffuse bands that are obtained with northern blots (Shaw et al., 1988; Kronke et al., 1985).

The major disadvantage associated with the dot-blot technique is the occurrence of non-specific binding of the probe to other molecules that are contained in the spotted

sample. Non-specific binding of the probe is not easily detected, because the sample is not size-fractionated by electrophoresis as is done in northern blots. Therefore, probes used in dot-blot analysis must be carefully checked for specificity using northern blots prior to use in dot blots. Furthermore, RNA samples used in dot-blots must be completely void of contaminating DNA. Since electrophoresis is not performed in dot-blots, any contaminating DNA will not be separated from RNA and the DNA will also bind to the probe.

Nuclease Protection Assay

Nuclease protection assays are a type of solution hybridization technique that was first described by Berk and Sharp (1978) for studying RNA splicing in viruses. Other names for this technique include S1 mapping, and RNase protection and S1 nuclease protection assay. The variety of different names for this method are derived from the type of nuclease (S1 nuclease or RNase) that is used and because this technique was originally developed to study or "map" gene and mRNA structure.

This technique is called a solution hybridization technique because the probe and mRNA are allowed to hybridize while in solution. After hybridization, the reaction is digested with a nuclease that specifically degrades single-stranded nucleic acids. Being double stranded, the probe/mRNA

complex is left intact and is detected by gel electrophoresis and/or autoradiography (Sambrook et al., 1989).

Nuclease protection assays were first developed using double stranded DNA probes, but great improvements have been made with the use of single-stranded RNA probes (Williams et al., 1986). Single-stranded RNA probes eliminate the competing re-annealing reaction that occurs between the two strands of a double-stranded probe (Melton et al., 1984). Moreover, RNA probes form a RNA:RNA duplex with the mRNA that is more stable and less prone to artifacts when digested with RNase (Casey and Davidson, 1977). With the development of cloning vectors that facilitate the production of single-strand, labelled RNA probes (Melton et al., 1984), the "RNase protection assay" has become the nuclease protection assay of choice in detecting specific mRNA molecules.

Semi-quantitative information can be obtained from RNase protection assays, although this technique is subject to the same limitations as northern blots. Accurately quantitating and aliquoting RNA samples is crucial to making sample to sample comparisons, and as with northern blots a control transcript is often used to normalize the data for sample variations. Quantitation of the hybridization signals can be accomplished by scanning autoradiographs with a densitometer or by excising the probe/transcript band from the gel and counting it in a scintillation counter. A unique method of

obtaining quantitative data using this technique was described by Durnan and Palmiter (1983) and involves applying the RNase digested reaction directly to glass filters which omits the electrophoresis step. The degraded RNA is washed away with trichloroacetic acid and the RNA:RNA duplex remains on the filter and is counted using a scintillation counter.

RNase protection assays are more sensitive than membrane hybridization techniques. Several assays have been able to detect as little as 1 picogram of mRNA in a sample containing total RNA (Melton et al., 1984). With this sensitivity, extracting mRNA from total RNA preparations is usually not necessary. In addition, RNase protection assays do not require the time-consuming pre-hybridization and washing steps that are associated with membrane hybridization techniques.

Despite being more sensitive and convenient, RNase protection assays can be difficult to develop. The hybridization probes must be rigorously tested for specificity on northern blots and effective concentrations of the probe and sample RNA must be titered. In addition, the hybridization and nuclease digestion conditions that are used in the assay must be determined empirically, and at present this technique is limited to the use of radioactive probes (Sambrook et al, 1989). Nevertheless, RNase protection assays offer superior sensitivity over northern or dot blot techniques and they have been extensively used to detect cytokine mRNA molecules

(Hagiwara et al, 1987; Hagiwara et al., 1988)

Reverse Transcription-Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a powerful technique for detecting the presence of specific DNA sequences and amplifying specific DNA fragments for subsequent analytical manipulations (Saiki et al., 1985). Reverse transcription-PCR (RT-PCR) involves reverse transcribing mRNA into cDNA and using this molecule as a template in the PCR (Kawasaki et al., 1988). RT-PCR is a sensitive method of detecting, subcloning, and characterizing specific gene transcripts.

The specificity of the RT-PCR technique is achieved by the use of paired sequence-specific oligonucleotide primers in the PCR. The primers range in size from 15 to 30 bases and are specific for sequences that are present on the transcript to be analyzed. The primers bind to opposing strands of the DNA template and allow the amplification of the intervening region. Amplification is accomplished by rapidly changing the reaction temperature so that template melting, primer annealing and DNA polymerization occurs repeatedly. A thermostable polymerase is used in the reaction so that the activity of the enzyme is not destroyed by the high melting temperatures. The results of RT-PCR analysis are obtained by electrophoresing an aliquot of the PCR on a gel and detecting

the presence or absence of an amplified product by ethidium bromide staining (Erlich, 1989).

The RT-PCR is an extremely sensitive technique and is often the method of choice when only small amounts of sample are available or when analyzing low abundance transcripts (Rappolee et al., 1989). Successful analysis of cytokine mRNA isolated from as few as ten cells has been reported (Carding et al., 1992). In addition, RT-PCR is a rapid method and avoids the time-consuming and cumbersome steps associated with membrane hybridization procedures and RNase protection assays. Generally, results from RT-PCR can be obtained in one day.

Because of the exquisite sensitivity of RT-PCR, rigorous measures must be taken to avoid contamination of samples and reaction mixtures. Sterile and clean reagents, pipette tips and reaction tubes must be used, and gloves should be worn during all steps of the procedure. Moreover, the PCR should be conducted in a room separate from that in which RNA is isolated and reverse transcription reactions are conducted. To ensure that no contamination has occurred, a negative control that does not contain a template should be included in each set of reactions (Saiki, 1989).

Like most other methods of transcript analysis, quantitative information is difficult to obtain using RT-PCR. In addition to the usual problems of quantifying and accurately aliquoting the RNA samples, RT-PCR is subject to

tube-to-tube variability in the reverse transcription and polymerase chain reactions. Because amplification occurs initially at an exponential rate, the slightest differences in reaction efficiencies can result in dramatic differences in the amount of the final product (Wang et al., 1989). Despite this limitation, qualitative and semi-quantitative information can be obtained using the RT-PCR, and the convenience and sensitivity of this technique still make it an attractive method of transcript analysis.

As with northern blots, the co-amplification of a reverse transcribed control mRNA such as ß-actin is often used to ensure the RNA samples have been accurately aliquoted and reverse-transcribed. As with the use of control genes in northern blots, the transcription of the control gene in RT-PCR must be constant under all conditions that are encountered in the study. In addition, several methods have been developed for quantitating the amount of amplified product that is produced in the RT-PCR (Nakayama et al., 1992; Melby et al., 1993). However, it is difficult to demonstrate that the amount of the amplified product is a direct reflection of the amount of starting RNA.

Several investigators have developed modifications of the RT-PCR to allow the accurate quantitation of specific cytokine mRNA molecules that are contained in RNA samples. All of these modifications involve adding to the reverse transcription

reaction or PCR a known amount of an internal standard that competes for the same primers as the cytokine transcript. The product amplified from the standard is a different size than the product amplified from the cytokine transcript. The standard is diluted and added to a constant amount of test RNA. After amplification and electrophoresis, the amount of transcript contained in the test sample is determined by comparing the amounts of the test transcript product and standard transcript product contained on the gel (Kanangat et al., 1992; Siebert and Larrick, 1992; Wang et al., 1989; Becker-Andre et al., 1989). Although this approach is time-consuming and difficult to develop, it does result in an extremely sensitive and quantitative method of transcript analysis.

IN SITU ANALYSIS

All of the methods of cytokine analysis discussed thus far have involved assaying materials that have either been secreted or extracted from tissue samples or cells. Consequently, these methods do not provide any information concerning the identity or frequency of the cytokine-producing cells. To address these issues, several methods of analyzing cytokine production at the single cell level have been developed that allow the characterization, enumeration and in some cases the anatomical localization of cytokine-producing cells.

Enzyme-linked Immunospot Assay

The enzyme-linked immunospot assay (ELLISPOT) is a modification of the ELISA technique and was first developed for enumerating specific antibody-secreting cells (Czerkinsky et al., 1983). Modifications of this original protocol resulted in a technique that can be used to detect and enumerate a variety of antigen-secreting cells (Hutchings et al., 1989; Versteegan et al., 1988; Czerkinsky et al., 1988).

The ELLISPOT technique involves incubating a single-cell suspension in a plastic dish or on a nitrocellulose membrane that has been coated with the pertinent cytokine-specific antibody. The cells are then removed and the cytokines that were secreted from the cells during the incubation remain

bound to the immobilized antibody. The step-wise addition of enzyme-labelled anti-cytokine antibody and enzyme substrate results in the appearance of colored spots at the location of the formerly plated cytokine-secreting cells. These spots can be enumerated under low magnification and the number of cytokine-secreting cells contained in the cell suspension can be determined (Czerkinsky et al., 1991).

The ELLISPOT technique has been used to detect and enumerate cells producing IFN-γ, IFN-α, tumor-necrosis factor and IL-1ß (Czerkinsky et al., 1991). This method is both rapid and easy to perform and uses reagents and techniques that are identical to those employed in the conventional ELISA. By combining the ELLISPOT with a conventional ELISA Versteegan et al., (1988) demonstrated that one spot on the ELLISPOT membrane represented approximately 0.01 U of IFN-γ activity. The ELLISPOT technique has proven especially useful in detecting cytokine-producing cells that have been cultured in vitro, but it has also been used to analyze cells from biopsy specimens (Hutchings et al., 1989; Czerkinsky et al., 1991).

Two major drawbacks are encountered when using this method. The first is that the assay requires long incubation times. The conditions encountered by the cells during these incubations may modulate their cytokine secretion. The second disadvantage is that this technique does not yield any information concerning the nature of the cytokine-producing

cells since they are removed from the assay before spot development (Czerkinsky et al., 1991). Despite these limitations, the reverse ELLISPOT offers a sensitive and convenient method of detecting and enumerating cytokine-producing cells in a heterogenous cell population.

Immunohistochemistry

Immunohistochemistry is a powerful and widely used histologic technique that has found application in research and clinical medicine. This technique uses antibodies to detect antigens that are located on histologically prepared cells or tissues sections. Immunohistochemistry has been most extensively used in pathology to localize pathogenic agents in tissue samples and to phenotypically and functionally characterize cells. The development of cytokine-specific antibodies has made this technique easily adaptable to studying cytokines that are present in tissues and cells.

A variety of antibody conjugates and detection systems have been used in immunohistochemistry and like the ELISA both direct and indirect methods of antibody labelling can be used. Peroxidase and alkaline phosphatase antibody conjugates are commonly used. When combined with the appropriate substrates, these antibody conjugates produce a localized colored product that can be seen under microscopic examination. A more direct method of visualizing cytokines can be obtained by using FITC

or rhodamine conjugates that fluoresce when exposed to UV light (Carson, 1990).

Although immunohistochemical detection of cytokines is a commonly used technique in pathology research, it suffers from major limitations with respect to the study of cytokine production. The first and most important is that immunohistochemical demonstration of a cytokine requires that it be present in a cell-associated form. Since most all cytokines have been shown to be secreted proteins, immunohistochemical techniques may grossly underestimate the amount of cytokine molecules that have been produced because this technique only detects cell-associated cytokines (Hofler et al., 1988). Furthermore, this method can not distinguish between cells that have produced cytokines and cells that have internalized cytokines. This may lead to false-positive results concerning the cellular localization of cytokine-producing cells (Sander et al., 1991). The concurrent analysis of cytokine gene transcription or cytokine secretion using other techniques can provide supportive information when analyzing cytokines by immunohistochemical techniques (Chensue et al., 1991; Kasahara et al., 1991).

Immunohistochemistry does offer two unique capabilities that are not found with other cytokine assays. The most obvious advantage is that cytokine production can be localized to individual cells and specific anatomical compartments, and

the proximity of cytokine-producing cells to pathogenic agents can be determined (Nickoloff et al., 1991; Arnoldi et al., 1990). Another attractive advantage is that multiple labelling and detection systems can be used on the same sample to allow several antigens to be simultaneously detected. This capability has many applications including detecting more than one cytokine or phenotypically characterizing different cytokine-producing cells (Chensue et al., 1991; Nickoloff et al., 1991).

In Situ Hybridization

In situ hybridization is a technique that uses the principles of hybridization methodology to locate specific nucleic acid sequences at the single cell level (Hofler et al., 1988; Coghlan et al., 1985). It was first described as a method of locating DNA sequences on isolated chromosomes (Gall and Pardue, 1971), and it has since been modified to detect mRNA in distinct cell populations (Angerer and Angerer, 1981). In situ hybridization is highly analogous to immunohistochemistry in that both techniques look at different aspects of the same process; the in situ analysis of gene expression. Immunohistochemistry uses specific antibodies to detect the protein product of a cytokine gene whereas in situ hybridization uses nucleic acid probes to detect the cytokine mRNA. The in situ detection of mRNA is a unique and powerful

method of transcript analysis that combines high sensitivity and specificity with precise anatomic localization.

The most obvious advantage in using in situ hybridization for studying cytokine gene expression is the ability to localize a hybridization signal in histologically complex tissues. In addition to providing information about the cytokine-producing cell, this technique also offers some technical advantages. Extracting RNA from tissues is not necessary, which eliminates the need for large amounts of tissues and the technical skill in handling labile RNA preparations. Moreover, the cytokine mRNA molecules of interest are not diluted by the mRNA from numerous background cells that are not expressing the cytokine gene. Finally, the tissue fixation procedures that are used for in situ hybridization eliminate much of the health risk that is associated with clinical specimens (DeLellis and Wolfe, 1987).

Combining in situ hybridization with immunohistochemistry provides a unique method that allows the detection of cytokine and cytokine mRNA molecules in the same tissue (Holfer et al., 1988; Shivers et al., 1986). These techniques can be used to verify that a cytokine is being synthesized by a cell and that the cytokine has not simply been acquired from another source (Mielke et al., 1990; Howell et al., 1991). The phenotypic characterization of cells expressing cytokine mRNA can also be determined (Peuchmaur et al., 1990). Another application of

these combined techniques is to locate cytokine gene transcripts in relation to the presence of pathogenic agents (Arnoldi et al., 1990). The combination of in situ hybridization and immunohistochemistry permits unique insights into the mechanisms of cytokine gene regulation that are not afforded by any currently available method of cytokine analysis.

The major disadvantages associated with in situ hybridization is that it is technically demanding and it requires knowledge and skill in both histology and molecular biology. Despite these many difficulties, in situ hybridization offers many advantages over other methods of cytokine analysis and it has been widely used in the single-cell detection of cytokine gene transcription.

SUMMARY

There are many techniques available for studying cytokine production and each method has its own capabilities and limitations. Bioassays lack specificity but provide information about cytokine biological activity. Immunoassays are highly specific, but they detect cytokines that may or may not be active. Analyzing cytokine mRNA levels gives specific information about cytokine gene activation, but does not reveal any information about cytokine secretion. Finally, in situ methods of cytokine analysis localize cytokine production to a single-cell, but these assays are difficult to develop. It is apparent that no currently used method of analyzing cytokine production provides comprehensive data, and cytokine production may need to be evaluated by multiple approaches in order to obtain useful information.

The virtual explosion of research in cytokine biology in the past decade has been facilitated by the development of methods to analyze cytokine production. However, it is apparent that the continued development of new and improved methods and reagents is needed. This is especially true in the area of veterinary research where the study of cytokines in domestic animals has been limited by the lack of species-specific reagents.

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PAPER II. CLONING BOVINE CYTOKINE cDNA FRAGMENTS AND MEASURING BOVINE CYTOKINE mRNA USING THE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

ABSTRACT

Bovine cytokine-specific primers and the reverse transcription-polymerase chain reaction (RT-PCR) were used to clone cDNA fragments that were specific for bovine IL-1α, IL-1ß, IL-2, and IFN-γ. Specificity of the cDNA fragments was verified by sequence analysis based on known bovine IL-1α, IL-1ß, IL-2, and IFN-γ gene sequences. In addition, RT-PCR was used to monitor cytokine mRNA expression in concanavalin A and lipopolysaccharide-stimulated bovine peripheral blood mononuclear cells (PBMC) and the results were compared to those obtained by measuring PBMC cytokine secretion using biologic assays. IL-1 activity in lipopolysaccharide-stimulated PBMC cultures was similar at 12 and 24 h, although the activity decreased by approximately 40% at 48 h. IL-2 and IFN-γ activity in supernatants of concanavalin A-stimulated PBMC cultures was low at 12 h and reached maximum levels at 48 h. RT-PCR transcript analysis detected an increase in IL-1α, IL-1ß, IL-2, and IFN-γ mRNA expression that was usually correlated with the detection of these soluble cytokines by the bioassays. These results indicate RT-PCR is a sensitive and effective method of obtaining cDNA probes and that this technique can be used to monitor bovine cytokine mRNA expression.

INTRODUCTION

Biologic assays that use cytokine-dependent cell lines provide a very sensitive and quantitative means of measuring cytokine production (Gillis et al., 1978; Rubenstein et al., 1981). However, these assays lack specificity and they only detect secreted cytokines and not cytokines that are membrane-bound or cell-associated. In addition, samples that are assayed for a specific cytokine often contain many different cytokines that may have a co-stimulatory or an inhibitory affect on the indicator cell lines that are used in biologic assays (Helle et al., 1988; Grabstein et al., 1986; Ranges, et al., 1988).

Cloning cytokine genes has helped circumvent these problems associated with biologic assays by providing species-specific nucleic acid probes for detecting cytokine gene transcripts. Assays using nucleic acid probes are very specific and they can be used in a semi-quantitative manner to detect the presence or absence of cytokine mRNA.

The cloning of cytokine genes in cattle has lagged behind the cloning of these genes in laboratory animals and humans. Sufficient homology occasionally exists between cytokine genes of different species to allow the use of heterologous probes to detect bovine cytokine mRNA (Jensen and Schultz, 1991; Stevens et al., 1992). However, special hybridization and washing conditions must be used to compensate for the

imperfect homology, which limits the specificity and sensitivity of the probes (Sambrook et al., 1989). The development of bovine-specific cytokine nucleic acid probes would eliminate this problem and provide specific and sensitive reagents for studying the bovine immune system. The cloning of IL-1α, IL-1ß, IL-2, and IFN-γ genes in cattle (Maliszewski et al., 1988; Cerretti et al., 1986a; Cerretti et al., 1986b) has provided the necessary information for developing bovine-specific cytokine nucleic acid probes that can be used in assays to monitor bovine cytokine gene transcription.

The polymerase chain reaction (PCR) is a powerful technique for detecting the presence of specific DNA sequences and amplifying specific DNA fragments for subsequent analytical manipulations (Saiki et al., 1988). Reverse transcription-PCR (RT-PCR) involves reverse transcribing mRNA into cDNA and using this molecule as a template in the PCR (Kawasaki et al., 1988). RT-PCR is a sensitive method of detecting, subcloning, and characterizing specific gene transcripts.

This paper describes using the RT-PCR and bovine cytokine-specific primers to amplify and clone cDNA fragments that are specific for bovine IL-1α, IL-1ß, IL-2, and IFN-γ. RT-PCR was also used to detect bovine cytokine transcripts in mitogen-stimulated peripheral blood mononuclear cells (PBMC)

and the results were compared to those obtained by measuring PBMC cytokine secretion using biologic assays.

MATERIALS AND METHODS

Cattle and Isolation of PBMC

Four 3-year old non-pregnant Holstein cows were used in this study. Blood was obtained by jugular venipuncture and PBMC were isolated by density gradient contrifugation (400 x g, 30 min) using Histopaque 1083 (Sigma, St. Louis, MO). The PBMC were washed twice in phosphate buffered saline by centrifugation and placed in RPMI 1640 medium (Gibco BRL, Grand Island, NY) containing 25 mM hepes, 2 mM L-glutamine, 5% fetal bovine serum (Hyclone, Logan, UT), 5 x 10^{-5} M ß-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin. This supplemented medium is subsequently called RPMI.

RNA Extraction from Mitogen Stimulated Bovine PBMC

PBMC obtained from individual cows were incubated for 17 h (37°C in 5% CO_2) in 100 ml of RPMI media (1 x 10^6 cells/ml) alone and in RPMI containing either 1 µg/ml concanavalin A (Con A; Sigma, St. Louis, MO) or 10 µg/ml Escherichia coli 055:B5 lipopolysaccharide (LPS;Sigma). After 17 h of incubation, total RNA was isolated from each culture by extraction with guanidine isothiocyanate as described (Chomczynski and Sacchi, 1987).

Amplification of Bovine Cytokine cDNA Fragments

An aliquot of each PBMC RNA sample containing 5 μg of total RNA was added to a separate tube, precipitated with ethanol, and resuspended in 10 μl diethyl pyrocarbonate (DEPC)-treated sterile distilled water. 500ng oligo$(dt)_{15}$ primers (Promega, Madison, WI) were added and the mixture was heated at 70°C for 10 min followed by a quick-chill on ice. The samples were reverse-transcribed in a 20 μl reaction mixture containing 1X reverse transcription buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl), 50 mM dithiothrietol, dATP, dCTP, dTTP, and dGTP each at 125 nM, and 200 units Moloney murine leukemia virus RNase H^- reverse transcriptase (Superscript, Gibco BRL, Gaithersberg, MD). The mixture was incubated at 37°C for 1 h followed by a 5 min incubation at 95°C.

Four μl of each of the resulting reverse transcription reactions was used as a template in a 100 μl PCR that consisted of 1X PCR reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCL_2$, .OO1% gelatin), dATP, dCTP, dGTP and dTTP each at 200 μM, 2.5 U AmpliTaq DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT), and 200 ng each of the upstream and downstream PCR primers for the appropriate bovine cytokine (Table I). Bovine cytokine-specific primers were 20 base oligonucleotides and were chosen so that they span the coding region of the cytokine transcripts based on the published

Table I

Nucleotide Sequence of upstream and downstream PCR primers for bovine IL-1α, IL-1ß, IL-2, and IFN-γ

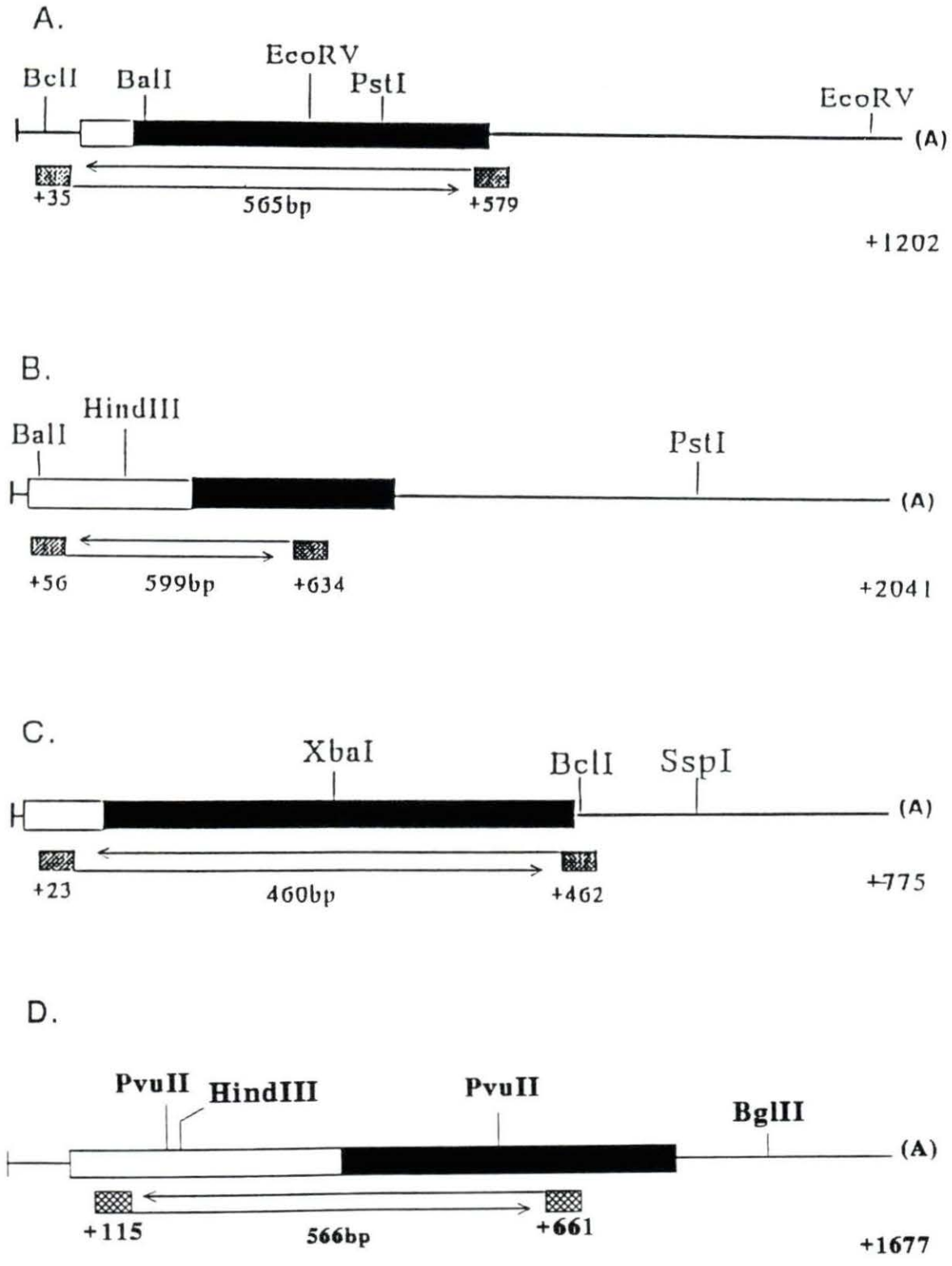
Cytokine		Oligonucleotide	Predicted Product(bp)
IL-1α	(sense)	5'ATGGCCAAAGTCCCTGACCT 3'	596
	(anti-sense)	5'CGTCTTCATTTTGAGCACTC 3'	
IL-1ß	(sense)	5'CTACAGTGACGAGAATGAGC 3'	566
	(anti-sense)	5'GGAAGTAGACCCCAAAGTCT 3'	
IL-2	(sense)	5'AGATACAACTCTTGTCTTGC 3'	457
	(anti-sense)	5'AGTCATTGTTGAGTAGATGC 3'	
IFN-γ	(sense)	5'CTGATCATAACACAGGAGCT 3'	564
	(anti-sense)	5'GACCATTACGTTGATGCTCT 3'	

sequences for bovine IL-1α, IL-1ß (Maliszewski et al., 1988), IL-2 (Cerretti et al., 1986a), and IFN-γ (Cerretti et al., 1986b) cDNAs (Figure 1). IFN-γ and IL-2 cDNA were amplified from reverse transcribed RNA that was obtained from Con A-stimulated PBMC. The IL-1α amd IL-1ß cDNA was amplified from reverse transcribed RNA that was obtained from LPS-stimulated PBMC. Cycling parameters for all primer sets were a presoak at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 37°C for 1 min, and 72°C for 2 min. After 35 cycles, the reactions were incubated for 72°C for 5 min. Aliquots of each PCR were analyzed by electrophoresis using a 1.5% agarose gel.

Cloning Bovine Cytokine cDNA Fragments

The amplified products were cloned into the PCR cloning vector PCR1000 (Invitrogen, San Diego, CA) according to the manufacturers instructions. Briefly, the unpurified PCR product was ligated into the PCR1000 cloning vector and the ligation products were transformed into competent E. coli strain INVα (Invitrogen, San Diego, CA) by standard procedures. Putative clones were initially screened by restriction analysis. Positive clones were confirmed by sequence analysis using an Applied Biosystems Model 373A automated DNA sequencer by the Iowa State University Nucleic Acid Facility (Ames, IA). The bovine cytokine cDNA fragments were then subcloned into pGEM-4Z(+) using standard procedures

Fig. 1. Partial restriction map of the bovine IFN-γ (A), IL-1α (B), IL-2 (C), and IL-1β (D) cDNA. The open box represents the coding region for the precursor protein and the shaded box represents the coding region for the mature protein. The location of the upstream and downstream PCR primers are indicated under each map with a ■ along with the numerical base position from the published sequence (Cerretti et al, 1986b; Maliszewski et al., 1988; Cerritti et al, 1986a). The size of the amplified fragment is indicated in base pairs (bp).


A.
BclI
BalI
EcoRV
PstI
EcoRV
(A)
+35
565bp
+579
+1202
B.
BalI
HindIII
PstI
(A)
+56
599bp
+634
+2041
C.
XbaI
BclI
SspI
(A)
+23
460bp
+462
+775
D.
PvuII
HindIII
PvuII
BglII
(A)
+115
566bp
+661
+1677

(Promega, Madison, WI).

Probe Generation by In Vitro Transcription

Labelled single stranded RNA probes specific for the bovine cytokine cDNA inserts were generated by producing run off transcripts from the pGEM(-4z) vector as described by the manufacturer. Vector DNA was removed by digestion with RQ 1 RNase-free DNase (Promega, Madison, WI) at 1 U/μg of template DNA. The undigested RNA transcripts were purified by Sephadex G-50 spin column chromatography (5 Prime→3 Prime, Inc., Boulder, CO).

Incubation of Bovine PBMC with Con A and LPS

PBMC from the four holstein cows were incubated for 12, 24, and 48 h in RPMI medium (1 x 10^6 cells/ml) alone, and in RPMI containing either Con A (1 μg/ml) or E. coli 055:B5 LPS (10 μg/ml). Cells from the cultures were harvested by centrifigation for subsequent mRNA extraction. Culture supernatants were removed and stored at -70°C until assayed for IL-1, IL-2, and IFN-γ activity using biologic assays.

Analysis of Bovine Cytokine Transcripts in Con A- and LPS-Stimulated PBMC

PBMC mRNA was extracted by guanidinium isothiocyanate extraction and oligo(dt) cellulose chromotography as described (Chomczynski, 1987, Aviv and Leder, 1972). The mRNA (0.5 μg) from each extraction was reverse transcribed as described for

total RNA, diluted (1:100), and 4 μl was used as a template in the PCR with bovine cytokine specific primers for IL-1α, IL-1ß, IL-2, and IFN-γ. Identical cycling parameters were used as described for total RNA except the annealing temperature for the IL-1α and IFN-γ reactions was elevated to 53°C. A positive control consisting of a PCR using the recombinant plasmid containing the relevant cDNA insert and a no-template negative control were included in each analysis. Aliquots of each PCR were analyzed by electrophoresis using a 1.5% agarose gel.

Southern Blot Analysis

For Southern blot analysis of amplified bovine IL-1α, IL-1ß, IL-2 and IFN-γ cDNA fragments, 5 μl of the PCR products that were amplified from reverse transcribed PBMC mRNA were electrophoresed on a 1.5% agarose gel. The gel was photographed under UV illumination and transferred to a nylon membrane (Nytran, Schleichler and Schuell, Keene, NH) by capillary action using 20X SSC (3 M NaCl, 0.3M Na_2citrate pH 7.0) as the ascending buffer. After baking (80°C for 30 min), the membrane was pre-hybridized for 30 min at 50°C in a hybridization buffer containing 50% formamide, 1.5X SSPE (0.2 M NaCl, 15 mM NaH_2PO_4, 2 mM EDTA), 1% sodium dodecyl sulfate (SDS), 0.5% non-fat dry milk, 200 μg/ml yeast tRNA, and 500μg/ml herring sperm DNA. After 30 min, 1 x 10^6 cpm/ml of the relevant cRNA probe (specific activity approximately 1 x

10^9 cpm/μg) was added directly to the pre-hybridization solution, and hybridization was performed at 60°C for 24 h. The post-hybridization washes consisted of two washes for 30 min each at room temperature. The first wash was in 2X SSPE containing 0.1% SDS, and the second was in 0.5X SSPE containing 0.1% SDS. The final wash was for 30 min at 60°C in 0.1X SSPE containing 0.1% SDS. For autoradiography, the filter was exposed to X-ray film (X-OMAT film, Kodak, Rochester, NY) at -70°C for 4 h before development.

IL-1, IL-2, and IFN-γ Assay

Secretion of IL-1 and IL-2 by bovine PBMC was quantitated by measuring proliferation of an IL-1-dependent (300B1 cells) and an IL-2-dependent (300L2) bovine T cell line as described (Stevens and Olsen, 1993). Briefly, proliferation was measured by uptake and incorporation of ^{3}H-methyl-thymidine. The proliferative responses (counts per minute) were then converted to units of IL-1 and IL-2 by probit analysis using recombinant bovine IL-1ß (American Cyanamid, Princeton, NJ) reacted with the 300B1 cells and recombinant bovine IL-2 (American Cyanamid) reacted with the 300L2 cells as a reference standard. Concentrations of IFN-γ in bovine PBMC culture supernatants were quantitated by measuring the 50% reduction of the cytopathic effects of vesicular stomatitis virus on Madin-Darby bovine kidney cells as described (Stevens

and Olsen, 1993). Briefly, the IFN-γ titer was calculated as the reciprocal of the supernatant dilution that protected 50% of the kidney cells from virus-induced lysis. Incubation of recombinant bovine IFN-γ (Ciba-Giegy, Basel, Switzerland) with virus-infected kidney cells was used as a positive control.

RESULTS

Amplification and Cloning of Bovine cDNA Fragments for IL-1α, IL-1ß, IL-2, and IFN-γ

Our strategy for cloning nucleic acid probes for bovine IL-1α, IL-1ß, IL-2, and IFN-γ involved using the PCR and paired oligonucleotide primers that were specific for bovine cytokine genes to amplify and clone the cDNA fragments. Reverse transcribed mRNA from mitogen-stimulated PBMC served as the template. The oligonucleotides used in this study (Table 1) were derived from the published gene sequences for bovine IL-1α, IL-1ß, IL-2, and IFN-γ. A partial restriction map of the bovine cytokine cDNA's with the position of the paired PCR primers is presented in Figure 1. The predicted sizes of the amplification products are given below each map.

RT-PCR performed on reversed transcribed RNA from mitogen-stimulated PBMC using bovine IL-1α, IL-1ß, IL-2, and IFN-γ primers resulted in 596, 457, 566 and 564bp amplification products, respectively (Figure 2). Some non-specific amplification occurred using the IL-1α and IFN-γ primers in both stimulated and unstimulated samples, but the predominant products in the mitogen-stimulated samples were of the predicted size. This non-specific amplification was eliminated in subsequent reactions by raising the annealing temperature from 37°C to 53°C (data not shown).

The putative bovine IL-1α, IL-1ß, IL-2, and IFN-γ

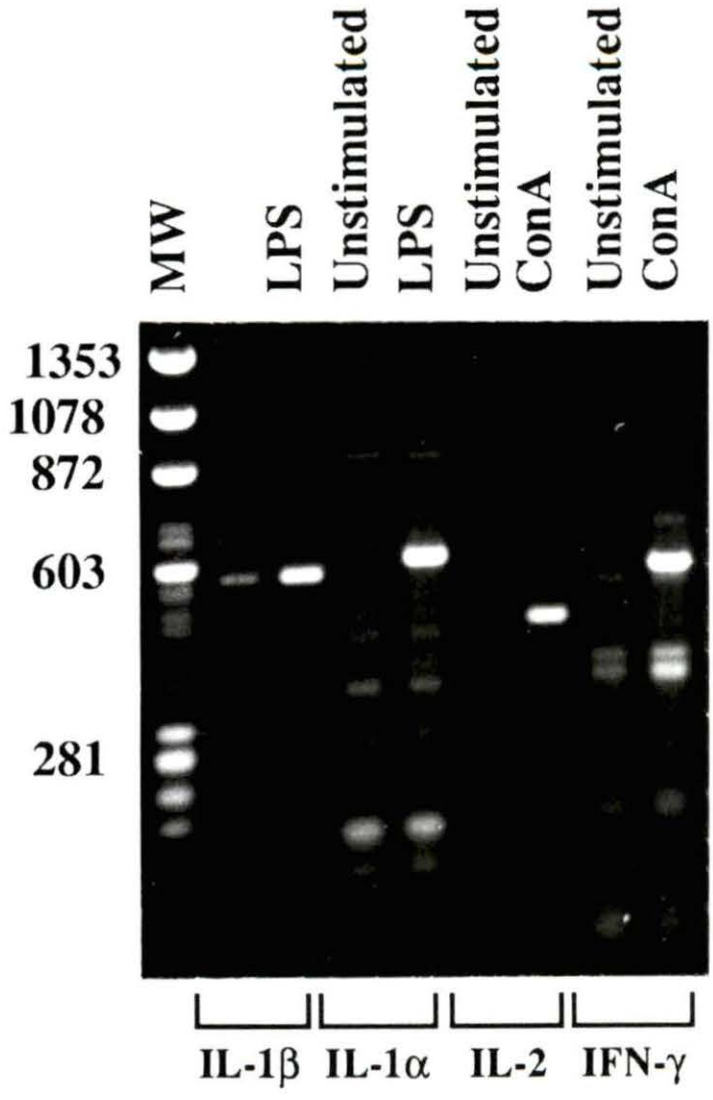


Fig. 2. Amplification of reverse transcribed total RNA from unstimulated and mitogen-stimulated bovine PBMC using bovine IL-1ß, IL-1α, IL-2, and IFN-γ specific primers.

amplification products were cloned into PCR1000 (Invitrogen, San Diego, CA) and the clones were initially screened by restriction analysis. Clones shown to contain the correct IL-1α, IL-1ß, IL-2, and IFN-γ inserts by restriction analysis, were sequenced for confirmation. Sequence analysis revealed that the PCR generated fragments were identical to the published sequences with the exception of two purine-pyrimidine transversions at base position 394 of the IL-1α insert (data not shown).

In Vitro Transcription of cRNA Probes

In vitro transcription was used to generate single-stranded RNA molecules that were specific for the bovine cytokine cDNA inserts. This is made possible by the opposing RNA polymerase initiation sites (Sp6 and T7) that flank the multiple cloning site of the pGEM-4Z vector. A typical labelling reaction yielded approximately 35 ng of RNA with a specific activity of 3 x 10^9 cpm/μg.

Comparative Analysis of Measuring IL-1 by a Biologic Assay and by IL-1α and IL-1ß Transcript Analysis Using RT-PCR

IL-1 activity in LPS-stimulated PBMC cultures was similar at 12 and 24 h, although the activity decreased by approximately 40% at 48 h (Figure 3A). Virtually no IL-1-like activity was detected in cultures of unstimulated PBMC at each time point. RT-PCR analysis indicated elevated levels of IL-1α

transcripts at 12 and 24 h after incubating PBMC with LPS. However, only trace amounts of IL-1α transcripts occurred at 48 h (Figure 4A). A low, but constant level of IL-1α transcript was detected in unstimulated PBMC, which decreased to barely detectable levels at 48 h. In contrast, IL-1ß transcripts were detected by RT-PCR at all time points in LPS-stimulated and unstimulated PBMC (Figure 4B). There was a slight decrease in the amount of IL-1ß mRNA detected in unstimulated PBMC at 12 and 48 h.

Comparative Analysis of Measuring IL-2 by a Biologic Assay and by IL-2 Transcript Analysis Using RT-PCR

IL-2 activity in supernatants of Con A-stimulated PBMC cultures was low at 12 h and reached maximum activity at 24 to 48 h (Figure 3B). Similarly, RT-PCR analysis showed that high levels of IL-2 message were present at 24 and 48 h, although it did not detect any message at 12 h (Figure 4C). No IL-2 transcripts were amplified in the unstimulated PBMC at all three incubation periods.

Comparative Analysis of Measuring IFN-γ by a Biologic Assay and by IFN-γ Transcript Analysis using RT-PCR

IFN-γ activity in PBMC culture supernatants was observed to progressively increase at 12 and 24 h and reached maximum levels at 48 h after incubation of PBMC with Con A (Figure 3C). IFN-γ mRNA was detected by the RT-PCR at 24 and 48 h, but not at 12 h after incubating PBMC with Con A (Figure 5D).

Fig. 3. Secretion of IL-1, IL-2, IFN-γ by mitogen-stimulated bovine PBMC. Cells (1 x 10^6/ml) were incubated alone or with LPS (10 μg/ml) or Con A (1 μg/ml). Cell-free supernatants were harvested at 12, 24, and 48 h after incubation. IL-1 activity (A) was assayed in supernatants from LPS-stimulated PBMC. IL-2 (B) and IFN-γ (C) activity was assayed in supernatants from Con A-stimulated PBMC. Results are presented as means ±SD. (n=4).

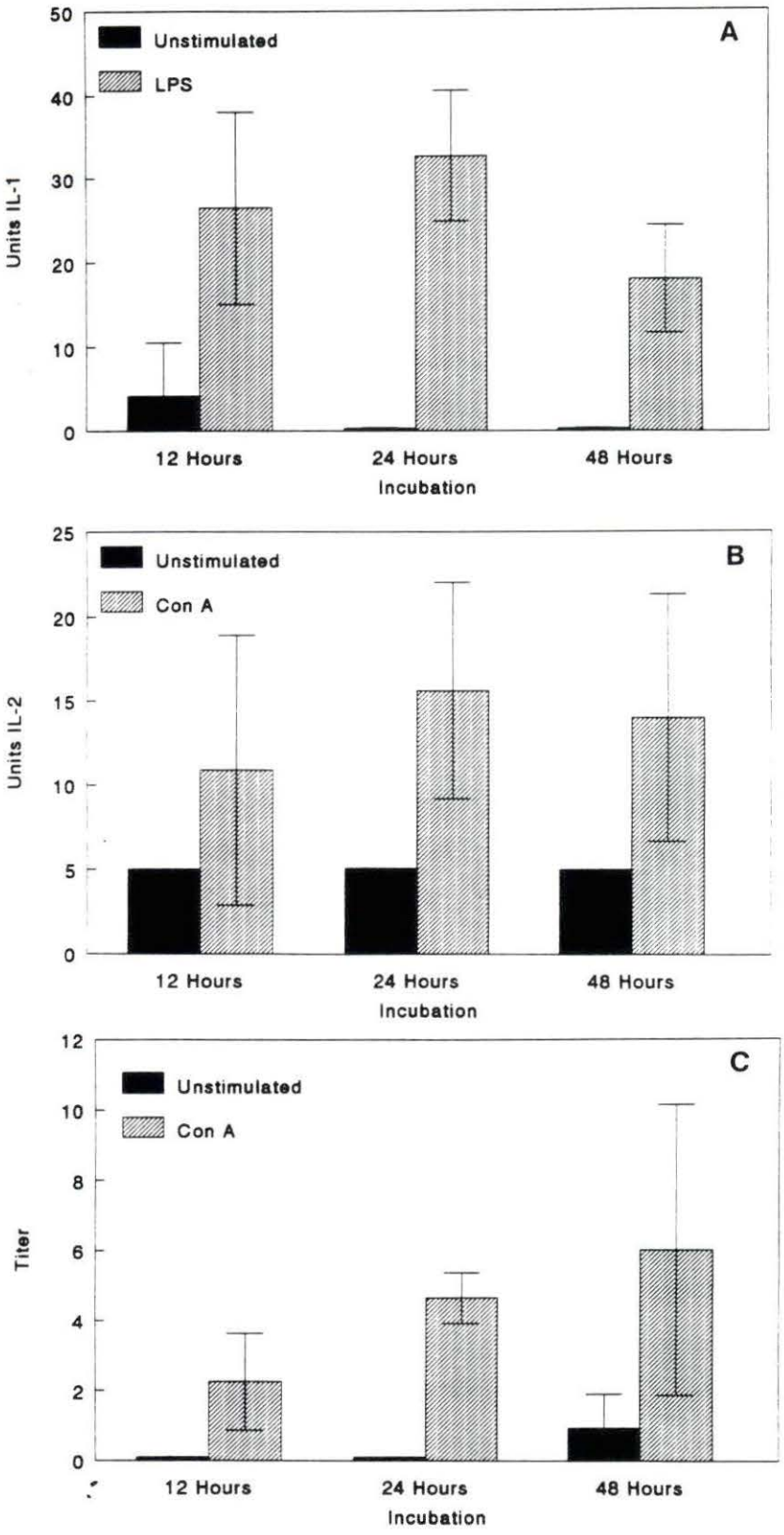
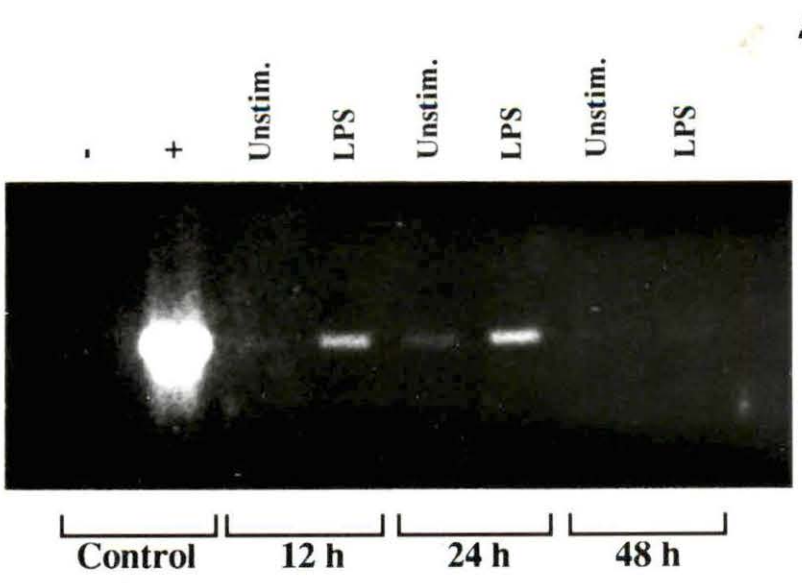

A
Unstimulated
LPS
Units IL-1
50
40
30
20
10
0
12 Hours
24 Hours
48 Hours
Incubation
B
Unstimulated
Con A
Units IL-2
25
20
15
10
5
0
12 Hours
24 Hours
48 Hours
Incubation
C
Unstimulated
Con A
Titer
12
10
8
6
4
2
0
12 Hours
24 Hours
48 Hours
Incubation

Fig. 4. Analysis of IL-1α, IL-1β, IL-2, and IFN-γ mRNA expression in mitogen-stimulated bovine PBMC by RT-PCR. Cells (1 x 10^6/ml) were incubated alone or with LPS (10 μg/ml) or Con A (1 μg/ml). At 12, 24, and 48 h after incubation, PBMC mRNA was isolated, reversed transcribed, and then amplified by PCR using bovine cytokine-specific primers. IL-1α (A) and IL-1β (B) mRNA were measured in LPS-stimulated PBMC. IL-2 (C) and IFN-γ (D) mRNA were measured in Con A-stimulated PBMC. Lanes marked (-) are a no template negative control. Lanes marked (+) are a positive control in which a plasmid containing the cloned cytokine cDNA fragment was used as the PCR template. The presented analyses are representative of the results that were obtained from analyzing mitogen-stimulated PBMC samples from 4 cattle.


A
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+
Unstim.
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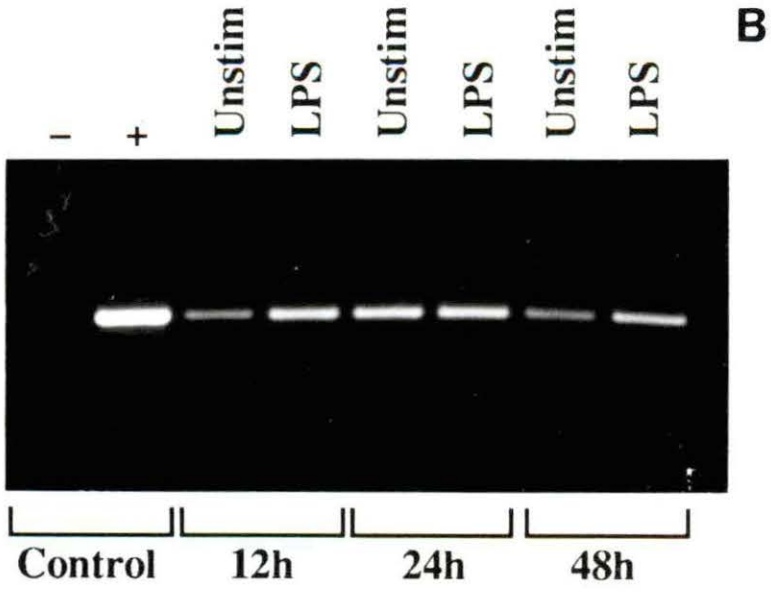
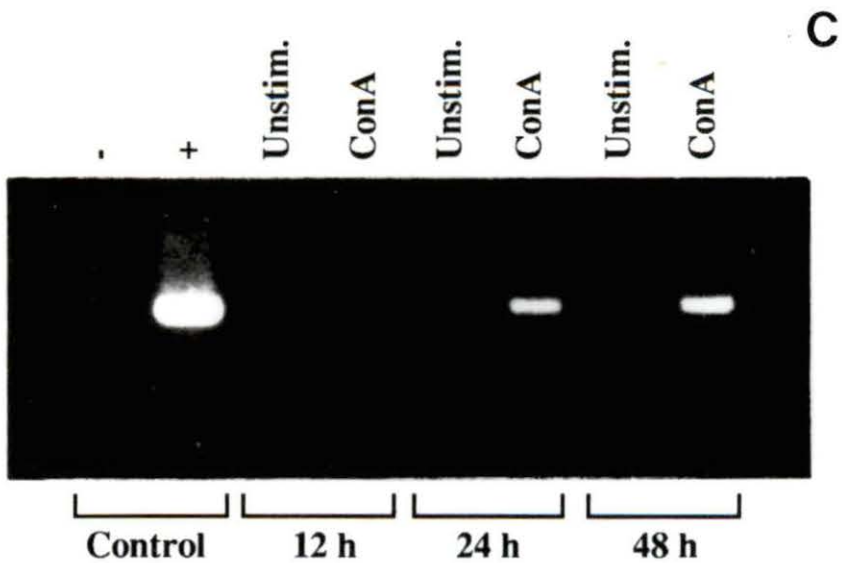
Unstim.
LPS
Unstim.
LPS
Control
12 h
24 h
48 h


B
–
+
Unstim
LPS
Unstim
LPS
Unstim
LPS
Control
12h
24h
48h



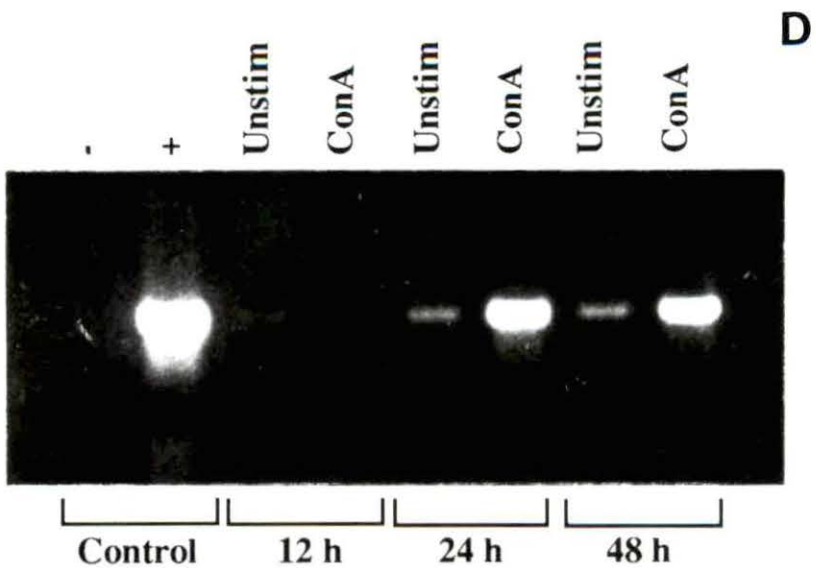


Fig. 4. Continued

IFN-γ mRNA was also detected at low levels in the unstimulated PBMC at all three incubation periods.

Southern Blot Analysis

A Southern blot using the cloned IL-1α cDNA insert as a probe showed that the amplified products in the IL-1α RT-PCR was specific for IL-1α (Figure 5). Southern blots using the cloned IL-1ß, IL-2, and IFN-γ cDNA inserts as probes also confirmed the specificity of the amplified products in the IL-1ß, IL-2, and IFN-γ RT-PCR (data not shown).

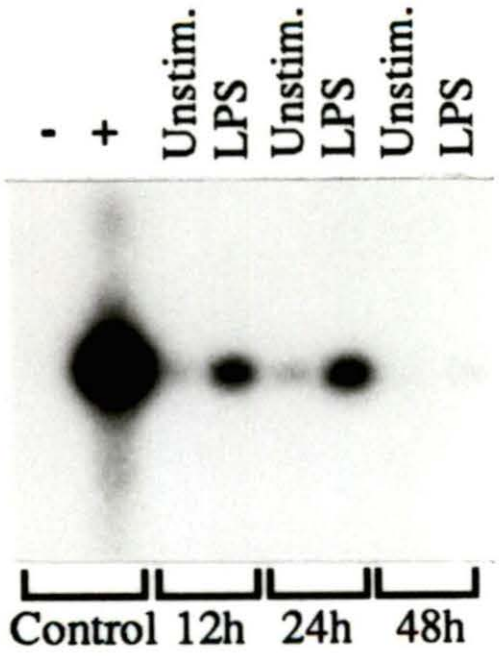


Fig. 5. Southern blot of RT-PCR analysis for bovine IL-1α. The gel from Figure 4A was transferred to a nylon membrane and hybridized to a [^{32}P] labelled probe that was specific for bovine IL-1α.

DISCUSSION

The limited availability of reagents that are specific for cytokines in cattle has hampered cytokine research in this species. In an effort to overcome this limitation, we describe using bovine cytokine specific primers and RT-PCR to develop cDNA probes that are specific for bovine IL-1α, IL-1ß, IL-2, and IFN-γ. RT-PCR was also used to evaluate bovine cytokine mRNA expression and these results were compared to measurement of cytokine secretion by using biologic assays.

To ensure that sufficient template was available for amplification in the PCR, the PBMC used in this study were stimulated with Con A or LPS to induce production of cytokine transcripts. After incubating PBMC for 17 h with mitogens, PCR products of the predicted size for IL-1α, IL-1ß, IL-2, and IFN-γ were amplified from reverse-transcribed total RNA as detected by gel electrophoresis and ethidium bromide staining. These transcripts were inducible because no product was amplified in unstimulated PBMC in the IL-2 and IFN-γ reactions and only trace amounts of the IL-1α and IL-1ß product were amplified from unstimulated PBMC. The non-specific amplification that occurred using the IFN-γ and IL-1α primer sets did not prevent the cloning of the IFN-γ and IL-1α fragments, and these ambiguous products were eliminated in subsequent reactions by elevating the annealing temperature in the PCR to 53°C.

The oligonucleotide primer pairs used in this study were chosen so that they were specific for the cDNA of interest and spanned at least one intron of the genomic sequence. This allowed the product amplified from the reverse-transcribed mRNA template to be distinguished from the product that would be amplified from contaminating genomic DNA, since the latter product would be larger in size.

The utility of RT-PCR in transcript analysis has been demonstrated in numerous studies investigating murine and human gene expression. More recently, Heussler et al. (1992) used this technique to monitor IL-2 transcript levels in Theileria parva-infected bovine cells. RT-PCR is an appealing method of transcript analysis because semi-quantitative and specific results can be quickly obtained from a minimal amount of starting material (Carding et al., 1992; Trout and Kelso, 1992). Despite these advantages, data obtained from transcript analysis must be interpreted with caution since the presence of cytokine mRNA may not correlate with the translation and secretion of a functional product (Garret et al., 1987, Chantry, et al., 1989).

In the current study, measurement of soluble cytokines by biologic assays was correlated with the detection of cytokine mRNA by RT-PCR, although discrepancies in the two assays occurred at some time points. For example, the biologic assays detected IL-2 and IFN-γ when PBMC were incubated for 12, 24,

and 48 h with Con A. However, RT-PCR revealed that IL-2 and IFN-γ transcripts were not present at 12 h, although they were present at 24 and 48 h. Transcription and secretion of human and bovine IL-2 and human IFN-γ by mitogen-stimulated lymphocytes has been shown to occur in a biphasic pattern, with the first peak of transcriptional activity occurring at approximately 1 to 1.5 h after stimulation (Grabstein et al., 1986; Weinberg, et al., 1988). It is not known whether a similar biphasic response curve occurred in the present study since the first time point of analysis was at 12 h after mitogenic stimulation. However, an early and transient peak of IL-2 and IFN-γ production could account for the detection of IL-2 and IFN-γ activity in culture supernatants at 12 h in the absence of IL-2 and IFN-γ transcripts.

Another possible explanation for the descrepencies between the biologic assays and the RT-PCR analysis for IL-2 and IFN-γ is that the biologic assays lacked specificity. The specificity of the biologic assays have been examined in a previous study that has shown neutralizing antibody to bovine IL-2 inhibits about 65 to 80% of the activity detected in the IL-2 assay and that by acid treatment IFN-γ accounts for about 80 to 90% of the activity that is detected in the IFN-γ assay (Stevens and Olsen, 1993). Therefore, the IL-2 and IFN-γ bioassays that were used in the current study primarily measure these cytokines, although neither assay is highly

specific.

The 300B1 cells that were used in the biologic IL-1 assay will respond to both IL-1α and IL-1ß (Stevens et al., 1992) and these cells can not discriminate between these two cytokines (Stevens and Olsen, 1993). The IL-1 assay detected soluble IL-1 in supernatants of PBMC at 12, 24, and 48 h after incubation with LPS. The RT-PCR analysis revealed that both IL-1α and IL-1ß transcripts were present at 12 and 24 h, and that IL-1ß but not IL-1α transcripts were present at 48 h after incubating PBMC with LPS. Therefore, IL-1 activity in LPS-stimulated PBMC culture supernatants was correlated with the appearance of either PBMC IL-1α or IL-1ß transcripts.

Both IL-1α and IL-1ß transcripts were detected in unstimulated PBMC in the absence of secreted IL-1 activity. Schindler et al. (1990) has demonstrated that adhering human PBMC to glass or plastic results in the expression of IL-1ß mRNA without detectable IL-1ß synthesis. It is currently not known whether adherence to plastic or glass stimulates bovine PBMC in a similar manner. However, this would account for the descrepencies seen between the IL-1 biologic assay and the IL-1α and IL-1ß RT-PCR transcript analysis for unstimulated PBMC in the present study since the bovine PBMC were cultured in plastic flasks.

The specificity of transcript anlysis by RT-PCR was clearly demonstrated in the current study when results of the

RT-PCR analysis for IL-1α and IL-1ß mRNA were compared with results of the IL-1 bioassay. The bioassay could not discriminate between IL-1α and IL-1ß activity, yet transcript analysis revealed that these two cytokine genes were regulated differently in LPS-stimulated bovine PBMC. Transcripts for IL-1α and IL-1ß were present at 12 and 24 h, but at 48 h only IL-1ß transcripts were present when PBMC were incubated with LPS. These differences in IL-1α and IL-1ß gene regulation could be detected by RT-PCR, but they could not be detected by the IL-1 bioassay.

In summary, bovine cytokine specific primers and RT-PCR were used to produce bovine cytokine cDNA probes and to detect cytokine transcripts in mitogen-stimulated bovine PBMC. RT-PCR detected cytokine transcripts in a specific and semi-quantitative manner and these results were usually correlated with secretion of active cytokines. Analysis of cytokine mRNA expression by RT-PCR may be useful in studying cytokine production by bovine immune cells. In addition, the RT-PCR technique conceivably could be used to measure bovine cytokine mRNA expression for any gene that has been sequenced.

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GENERAL SUMMARY

There are many techniques available for studying cytokine production and each method has its own capabilities and limitations. It is apparent that no currently used method of analyzing cytokine production provides comprehensive data, and cytokine production may need to be evaluated by multiple approaches in order to obtain useful information.

The virtual explosion of research in cytokine biology in the past decade has been facilitated by the development of methods to analyze cytokine production. However, it is apparent that the continued development of new and improved methods and reagents is needed. This is especially true in the area of veterinary research where the study of cytokines in domestic animals has been limited by the lack of species-specific reagents.

In an effort to overcome this limitation, we describe using bovine cytokine specific primers and RT-PCR to develop cDNA probes that are specific for bovine IL-1α, IL-1β, IL-2, and IFN-γ. RT-PCR was also used to evaluate bovine cytokine mRNA expression and these results were compared to measurement of cytokine secretion by using biologic assays. RT-PCR detected cytokine transcripts in a specific and semi-quantitative manner and these results were usually correlated with secretion of active cytokines. Analysis of cytokine mRNA expression by RT-PCR may be useful in studying cytokine

production by bovine immune cells. The RT-PCR technique conceivably could be used to generate nucleic acid probes and measure mRNA expression for any gene that has been sequenced.

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