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The immune system has evolved to respond to certain microbial products with a sequence of physiological events, termed the acute phase response, that appears to be adaptive. The molecular triggers for the acute phase response appear to be cell wall products such as endotoxin, in the case of bacteria and double stranded RNA, in the case of viruses. Both endotoxin and double stranded RNA induce a class of hormone like substances, cytokines, that drive the acute phase response. It is not known it bacterial and viral products induce overlapping sets of cytokines or if the virus response is completely dependant on interferon. Until now there has been no convenient way of assaying either serum for multiple cytokine activity, or detection of cytokine RNAs.

To circumvent this problem I have developed a polymerase chain reaction method (RNAPCR) for the detection and semi-quantitation of specific mRNA species. This method will detect mRNA in a range of 10^{-4} to 10^{-5} copies per cell allowing for a systematic analysis for multiple cytokines simultaneously. Data on the double stranded RNA induced production of cytokine mRNAs by the mouse macrophage line RAW is shown. Because the RNAPCR is strand specific, the methodology can be used to monitor the production of double stranded RNA in infected tissues. The power of this techniques is just beginning to be exploited.

(2)

Date: February 1, 1991

1st Annual Report on Contract #N00014-90-J-1311

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CONTRACTOR: Uniformed Services University of the Health Sciences and The Henry M. Jackson Foundation for the Advancement of Military Medicine

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INTRODUCTION

The molecular mechanisms by which virus infections trigger both overt symptoms such as fever as well as host response at the molecular level is unknown. This contract is responsible for investigating the molecular mechanisms occurring during the host-virus interaction that constitute the host response. One major part of the host defense is the induction of the synthesis of a large number of biological response modifiers; the cytokine cascade. The molecular mechanism we are focusing on as the trigger for the cytokine cascade is the hypothesis that double-stranded RNA (dsRNA) produced during the virus replication acts as an inducer (Carter and DeClerq, 1974) for a wide range of cytokines that alone, in combination with each other or in combination with dsRNA play a significant role in the pathogenesis of the viral disease.

We are studying in a mouse pulmonary infection model, using Influenza A strain PR/8/34, production of virus-derived dsRNA and induction of specific cytokine mRNAs. To examine the production of virus-specific dsRNA, a unique PCR method has been devised. This method is applicable to the detection any mRNA sequence, and we have chosen for study the cytokines and interferons listed in Table I. The sensitivity of this method has been established and baseline measurements of cytokine mRNAs in uninfected animals and cell lines have been determined.

MATERIALS AND METHODS

Influenza infection. Swiss Webster male mice (viral antibody free, 24-35 days old) were lightly anesthetized then infected intranasally with 32 mouse lethal doses of influenza A/PR/8/34 (H1N1). The virus was prepared to maintain mouse lethality by alternately propagating it in mice and 9-11 day embryonated pathogen free SPAFAS eggs. Sham controls received an equal volume of diluted allantoic fluid from uninfected SPAFAS eggs. Lungs for total RNA preparation from infected and sham animals were removed

at 24 and 48 hrs postinfection and rapidly frozen at -70°C.

Preparation of total tissue and cellular RNA. Total RNA from lung or tissue culture cells was prepared by the method of Chomczynski and Saachi (1987) with the following modifications. Following extraction with guanidine isothiocyanate: phenol:sodium acetate and chloroform, the nucleic acid was either isopropanol or ethanol precipitated. For removal of residual DNA certain RNA preparations were banded on cesium trifluoroacetate gradients and fractions of density greater than 1.65 g/ml were pooled and precipitated using ethanol. RNA from tissue culture cells were cleaned of contaminating genomic DNA by precipitation with 3 M lithium chloride (final concentration). This precipitate was washed extensively with 70% ethanol prior to further use. To insure that RNA preparations were free of DNA the RNA preparations were treated with RNase free DNase (Boeringer Mannhiem). Then the RNA was phenol:chloroform extracted, chloroform extracted and ethanol precipitated using sodium acetate.

RNAPCR methods for analysis of influenza A gene segment and cytokine mRNA levels. RNA concentrations were determined by ultraviolet (UV) spectroscopy to standardize the quantities of RNA used per reaction. The RNAPCR procedure was performed essentially as described in Jacobsen et al.(1989). The modification of using GAPDH as an internal control was performed either as described in Diamond et al.(1990) or in Gendelmen et al.(1990). Briefly the specific antisense primers were mixed with 2 micrograms of total lung or cellular RNA and heated to 70°C. After cooling on ice for 5 minutes the remaining reaction components, DTT, reaction buffer, nucleotides and water were added and mixed. Following addition of 500 units of Murine moloney leukemia virus reverse transcriptase (final reaction volume 25 ul) the reaction was incubated at 37°C for 30 minutes. The reverse transcriptase reaction was terminated and the specific cDNAs were melted off their respective mRNA templates by incubation at 95°C for 10 minutes. For polymerase chain reaction amplification of the specific cDNAs were either amplified together as in Diamond et al. (1990) or separately as in Gendelman et al. (1990). The direct co-amplification method was used if the mRNAs of interest are expressed at levels within 10-fold of GAPDH. The PCR for each cDNA is perfoimed separately if there is a greater than 10-fold difference in the mRNA levels. The PCR reactions were amplified for 20 cycles using 94°C for 90 sec, 50°c for 90 sec and 72°C for 120 sec. Following amplification the reaction products were resolved on 2.5% agarose gels, and the gels were then Southern blotted. For detection of the specific PCR products a third sequence specific oligonucleotide located between the amplification primers was used as a probe. When possible the probe sequence used spanned an exon boundary to insure specificity for mRNA amplification. The probe was labelled using ^{32}P- -ATP, and polynucleotide kinase. The labelling and hybridizations were performed as described (Saiki et al., 1987). All blots were washed at 5°C below the T_d for the specific probe. Successful PCR was defined by the presence of a autoradiographic obtained signal that

was mRNA specific. If a signal was detected in the reverse transcriptase negative controls (indicating genomic DNA contamination) then the samples were re-DNased and the experiment repeated.

Selection and testing of PCR primers. When possible PCR primers were chosen using a computer program for selection of oligonucleotides (Lowe et al., 1990). Primers for flu gene segments, cytokines and interferons were chosen within the coding region of each sequence and when possible the primers are sited on adjacent exons. For flu gene segments there is of course not a problem with DNA contamination, but the interferon α and β genes are problematic. The list of PCR primers and probes used is shown in Table 1.

RESULTS

Establishment of the RNAPCR system. The RNAPCR system was established to provide a means of detecting and describing mRNA levels in a semi-quantitative manner. RNAPCR is the method of choice when the RNA of interest is at low abundance. The study of cytokine expression and virus replication in animal tissues is an ideal system for study by PCR because at early stages of the infection such a low percentage of the cells of a tissue are involved in the disease process. This method allows us to detect and define the earliest response of the host to the virus.

As a test of the sensitivity of the PCR system, a synthetic RNA template of interferon β (IFNβ) was synthesized using SP6 RNA polymerase. After purification and UV-spectroscopy, the IFNβ mRNA was serially diluted for RNAPCR. The last dilution performed, 0.001 pg equivalent to 10^3 copies of mRNA was easily detectable. Based on these results and other experiments as little as 10-100 copies of the mRNA species of interest is detectible by this scheme.

Detection of influenza virus-specific RNA in total RNA isolated from mouse lungs. Determination of virus replication and measurement of potential dsRNA of influenza origin was performed by RNAPCR. The diagram in Figure 1 shows how the use of the specific PCR primers in the reverse transcriptase reaction are use to produce a PCR product originating from only one strand of viral RNA. Since the input genome (vRNA) is negative stranded or antisense, PCR-based detection is dependent on cDNA synthesis directed by the "sense primer". Detection of mRNA and cRNA by PCR is dependent on cDNA synthesis directed by the "antisense primer".

As shown in figure 2 both vRNA and mRNA or cRNA for gene segments 3 and 4 was present in total cellular RNA extracted from infected mouse lungs 48 hrs after infection. This RNAPCR was performed for the purpose of detection using 30 cycles of amplification and no semi-quantitative information could be drawn.

Using total RNA extracted at 24hr from sham infected and influenza infected lungs we measured the relative levels of vRNA and mRNA or cRNA using the semiquantitative RNAPCR method. As shown in figure 3 there are not dramatic differences in the levels of the plus and minus strands for gene segments 3,4 and 7. This balance of plus and minus strands improves the likelihood that there are significant amounts of dsRNA present in the influenza virus infected cell.

We also investigated the double stranded nature of the influenza RNA (Figure 2). Using mild RNase pretreatment to digest only singlestranded RNA had a negligible effect on the PCR signal (Figure 2, lanes 9-12). Harsh RNase pretreatment resulted in a total destruction of the PCR signal (Figure 2, lanes 13-16). These results indicate that the flu RNA was either double-stranded after extraction or has a secondary structure that is double-stranded-like enough to prevent digestion by the mild RNase pretreatment.

Detection of cytokine mRNAs in total RNA isolated from influenza virus infected mouse lungs. Using the RNAPCR methodology we determined the presence of specific cytokine mRNA in total lung RNA prepared from sham infected (24hr treatment) and influenza infected for 24 and 48hr. Figure 4 shows that only GAPDH was detectible in lane 1 the sham infected animals. At 24 and 48 hr post infection (lanes 2 and 3) tumor necrosis factor α (TNFα) and interleukin 6 (IL6) were quite evident. The multiple bands in the IL6 are a result of over amplification indicating the presence of a large amount of IL6 mRNA. Interleukin 1 α (IL1α) mRNA was detectible only at 24hr post infection and IL1β was not detected at either time point.

Detection of interferon mRNAs in total RNA isolated form influenza infected mouse lungs. Using the same total RNA preparations described above, we next determined the levels of IFN α,β, and mRNA. As shown in figure 5, IFNα mRNA was found at 24 and 48 hrs post infection. The interferon alpha primers and probe were specific for 1, 2, 4 and 6. No IFNβ or IFN was detected. By assaying for all IFNαs simultaneously we are treating them as a single species of cytokine. With the cloning and production of the human IFNαs researchers were able to determine activity differences between the different types (Goeddel et al., 1981 and de la Maza et al., 1985). While the murine IFNα genes have been cloned and expressed separately at least one report found no activity differences between the subspecies (Battistini et al., 1991).

Survey of cytokine production by RAW cells. To find a cell line that is extremely sensitive to dsRNA we examined the RAW cells to determine which cytokines were induced and at what concentration of dsRNA. We sought to develop a cell line based assay for dsRNA based on a cell line. Previously we attempted to use human FS-4 cells, but this failed to be reproducible. Using murine RAW cells,

as shown in figure 6, GAPDH and TNFα were constitutively expressed. There was no spontaneous synthesis of IL1α, IL6, IFNα or IL10 (IL10 data not shown) during the time course (figure 6, lanes A, B, and C). A 2 hr treatment with polyC alone did not induce any cytokine mRNAs. At 1 hr of polyI polyC (100 ug/ml) treatment no cytokine mRNAs were detected. The first response was detected 2 hrs post poly I poly C treatment and IL1α preceded IFNα and a very weak IL6 response. IL10 kinetics followed the IFNα pattern (data not shown). In an effort to determine the minimum amount of dsRNA required to trigger a response were performed a dose response curve harvesting cells for RNA at 4 hrs of polyI polyC treatment. As shown in Table 2, the lowest concentration of polyI polyC that could induce a cytokine signal was 1 ug/ml. Based upon this data this cell lines are not sensitive enough to provide a convenient assay for determination of the presence of dsRNA. We are currently working on obtaining the dsRNA dependent kinase cDNA clone so we can develop an entirely in vitro assay for dsRNA content of RNA preparations.

DISCUSSION

Influenza virus is a negative stranded, single stranded RNA virus. We understand so much about the structure, antigenic drift and molecular mechanisms of growth of this virus yet; we do not have an understanding of the pathogenesis of influenza at the molecular level. We have sought to establish the existence of dsRNA of influenza origin in the tissue of infected mice. As shown in Figures 2 and 3, both plus and minus strand copies of viral gene segments are present. This was shown for three gene segments (3,4 and 7) at 24 and 48 hrs post infection by coupling the RNAPCR procedure to RNase treatment protocols. These RNase treatments served to differentiate between ssRNA and RNA with double stranded structure. These conditions were established so that polyI polyC was not destroyed by the mild digestion but was by the strong digestion. As is clearly shown in Figure 2 the strong digestion with RNase completely obliterated the RNA, even so it was undetectable by RNAPCR. The fact that there was little if any difference between the mild treatment and no treatment is most likely a result of the RNAPCR process. From our data shown here, no estimate of the percentage of ssRNA and dsRNA can be made. Proving that RNA extracted from tissue was dsRNA <u>in vivo</u> is a nearly impossible task. Once extracted from tissue free of protein the RNAs in solution will hybridize to each other or self hybridize depending on the temperature and salt concentrations used. I think these arguments are a bit silly however, since the viral RNA arose as a copy of another RNA molecule, the template strand and daughter strand had to be dsRNA at one point during synthesis. If we choose to accept this point as a fact, then there must be dsRNA of viral origin in the tissue.

A series of different cytokine mRNAs were detected in the RNA

from the lung. Each cytokine detected with the exception of IL1α was present at both the 24 and 48 hr time points. An important point to keep in mind here is that the cytokines that are not induced may well be as important by their absence as those that were synthesized. For example, IFN was not detected during the course of these experiments, yet IFN has been shown to have protective effects and is associated with recovery from influenza infection (Taylor et al., 1989).

Within the next year of this contract we intend to expand our repertoire or cytokines to include IL2, IL4,IL5, granulocyte-macrophage CSF and other cytokines associated with Th-1 and Th-2 cell types (Mosmann and Coffman, 1989). However since we are viewing influenza infection a model of overall host response, we will also develop assays for mediators that are involved in inflammation, IL8 (when the murine form is cloned) and IL9, plus special focus will be given to Il-1,TNF,IL6 and GMCSF in this light. As reported by Dinerello, IL-1 and TNF are the mediators responsible for the hypotension and shock in his rabbit acute respiratory distress model system (Review, Dinerello, 1989). In addition cytokines which are anti-inflammatory, tumor growth factor β and IL10 will be assayed to determine if these cytokine play a role in the pathogenesis.

This year we will also extend our study to a new flu model using a strain that through two different culture protocols has been developed into pathogenic and nonpathogenic forms (Nickerson and Jakab, 1989). This virus is an influenza A HK/68 (H3N2) so new primers for hemagglutinin and neuraminidase will need to be made. Since we will be able to compare pathogenesis of avirulent and virulent forms of the same virus strain we will be able to define the molecular events that distinguish these two infections. Our collaborator, Dr. George Jacob at the Johns Hopkins University School of Hygiene and Public Health has previously shown that the two forms of the virus reach the same titers in the lungs of the infected animals. By virus measurement the only significant difference between the two forms was that the avirulent species was less persistent in the lungs, seemly cleared between days 8-9 instead of day 11 in the case with the virulent form. This system will help us to further pinpoint which are the key factors in the host response involved in influenza pathogenesis.

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TABLE 1

PCR PRIMER LIST FOR MURINE CYTOKINES AND INTERFERONS

PRODUCT SIZE		SEQUENCE
	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH)	
195BP	SENSE	CCATGGAGAAGGCTGGGG
	ANTISENSE	CAAAGTTGTCATGGATGACC
	PROBE	CTAAGCAGTTGGTGGTGCA
	INTERFERON ALPHA	
156BP	SENSE	CTCAAAGCCTGTGTGATGC
	ANTISENSE	AAGACAGGGCTCTCCAGAC
	PROBE	CCCTGCTGGCTGTGAGGA
	INTERFERON ALPHA 1+2	
162BP	SENSE	TGTCTGATGCAGCAGGTGG
	ANTISENSE	AAGACAGGGCTCTCCAGAC
	PROBE-ALPHA1	CAGGAATTTCCCCTGACC
	PROBE-ALPHA2	TTCAGGAACCTCCTCTGA
	INTERFERON BETA	
156BP	SENSE	CCATCCAAGAGATGCTCCAG
	ANTISENSE	GTGGAGAGCAGTTGAGGACA
	PROBE	GTACGTCTCCTGGATGAACT
	INTERFERON GAMMA	
237BP	SENSE	AACGCTACACACTGCATCTTGG
	ANTISENSE	GACTTCAAAGAGTCTGAGG
	PROBE	GGAGGAACUGGCAAAAGGA
	TNF-ALPHA	
200BP	SENSE	GATCTCAAAGACAACCAACTAGTG
	ANTISENSE	CTCCAGCTGGAAGACTCCTCCCAG
	PROBE	CCCGACTACGTGCTCCTCACC
	IL-1 ALPHA	
329BP	SENSE	GGAAGATTGTCAAGAAGAGACGG
	ANTISENSE	TGAGATTTTTAGAGTAACAGG
	PROBE	CCAGATCAGCACCTTACACC
	IL-1 BETA	
196BP	SENSE	GGGATGATGATGATAACCTG
	ANTISENSE	TTGTCGTTGCTTGGTTCTCCT
	PROBE	AACCAACAAGTCATATTCTCC
	IL-6	
293BP	SENSE	ATGATGGATGCTAACAAACTGG
	ANTISENSE	GATGGATTGGATGGTCTTGG
	PROBE	AATTTCCTCTGGTCTTCTGG

TABLE 2

POLYI POLYC CONCENTRATION DEPENDANCE OF CYTOKINE MRNA DETECTION

Cytokine	PolyI PolyC Concentration			
	100 ug/ml	10 ug/ml	1 ug/ml	0.1 ug/ml
IFN α	+++	++	-	-
IL 1α	+++	++	+	-
IL 6	+	-	-	-

+++ signal visible on X-ray film after 1 hr at room temperature.
++ signal visible on X-ray film after 8hr at -70°C.
+ signal visible on X-ray film after 30hr at -70°C.

FIGURE LEGENDS

Figure 1.
Influenza virus RNA species and RNAPCR primer designations. During virus replication, three species of viral RNA are made for each gene segment. For RNAPCR, a specific primer for each gene segment must be used to initiate the cDNA synthesis step. The 5' to 3' position of this primer on each of the viral RNA species is shown. The "A" primer corresponds to the sense primer in the cytokine primer table and the "B" primer corresponds to the antisense primer. This figure is adapted from Lamb and Coppin (1983).

Figure 2.
RNAPCR detection of influenza virus RNA for gene segments 3 and 4. at 48hrs post infection.(This figure is adapted from Majde et al., 1991). Total lung RNA from sham (lanes 1-4) or virus infected samples (lanes 5-16) were either not treated (lanes 1-8) or treated with mild RNase (lanes 6-12) or treated with RNase to degrade all RNA (lanes 13-16). The conditions of mild RNase treatment were: 2 ug/ml RNase A in 0.3 M NaCl, 10 mM Tris Cl, pH 7.5, 1 mM EDTA, for 1hr at 22°C. The complete RNA digestion conditions were: 20 ug/ml RNase A in 0.1 M NaCl, 10 mM Tris Cl pH 7.5, 1 mM EDTA, for 30 min at 56°C. Prior to RNAPCR analysis of the Rnase-treated samples RNasin was added followed by addition of gene segment 3 specific primer **A** to samples 1, 5, 9 and 13; gene segment 3 specific primer **B** in samples 2, 6, 10 and 14; gene segment 4 specific primer **A** in samples 3, 7, 11 and 15; and gene segment 4 specific primer **B** in samples 4, 8, 12 and 16 (the **A** and **B** designation is explained in figure 1). The RNAPCR was then performed as detailed in the methods section and in Majde et al., 1991. A photograph of the ethidium bromide stained gel is labelled A and an autoradiogram of the Southern blot of the gel is shown in B.

Figure 3.
RNAPCR detection of influenza virus RNA for gene segments 3, 4, 7 and the GAPDH control mRNA in total RNA from sham and 24hr infected samples. For RNAPCR either primer **A** or **B** was used to initiate the reverse transcriptase reaction using total RNA from sham infected (S) and infected (I) lungs. Not that the control GAPDH is shown as in the primer **B** columns. Following amplification as described in the methods section the reactions were electrophoresed and Southern blotted. An autoradiograms of the reaction products are shown.

Figure 4.
Detection of cytokine mRNAs in total RNA from sham infected, 24 hr and 48hr influenza infected samples. RNAPCR for detection of GAPDH, IL1α, TNFα, IL1β and IL6 was performed as described (Methods). The oligonucleotide primers used for each cytokine are shown in Table 1. Southern Blots of the reactions are shown.

Figure 5.
Detection of Interferon mRNAs in the samples of total RNA. Using the same RNA preparations used in Figure 4, GAPDH, IFNα, β, and were amplified using the oligonucleotide primers listed in Table 1. Southern blots of the reactions are shown.

Figure 6.
Kinetics of cytokine and IFN production by RAW cells.
Cultures of RAW cells (5 X 10^6) were treated with buffer (lanes A, B, and C), 100 ug/ml Poly C (lane D), and 100 ug/ml polyI polyC (lanes E thru I) For 1 hr then washed with PBS and refed with fresh media. Culture fluids were harvested for cytokine titrations and RNA preparations at 1hr (lanes A and E), 2hrs (lanes B, D and F), 4hrs (lane G), 8hrs (lane H) and 24hrs (lanes c and I). RNA was prepared and RNAPCR was performed as described in Methods. Autoradiograms of Southern blots are shown.

Figure 1

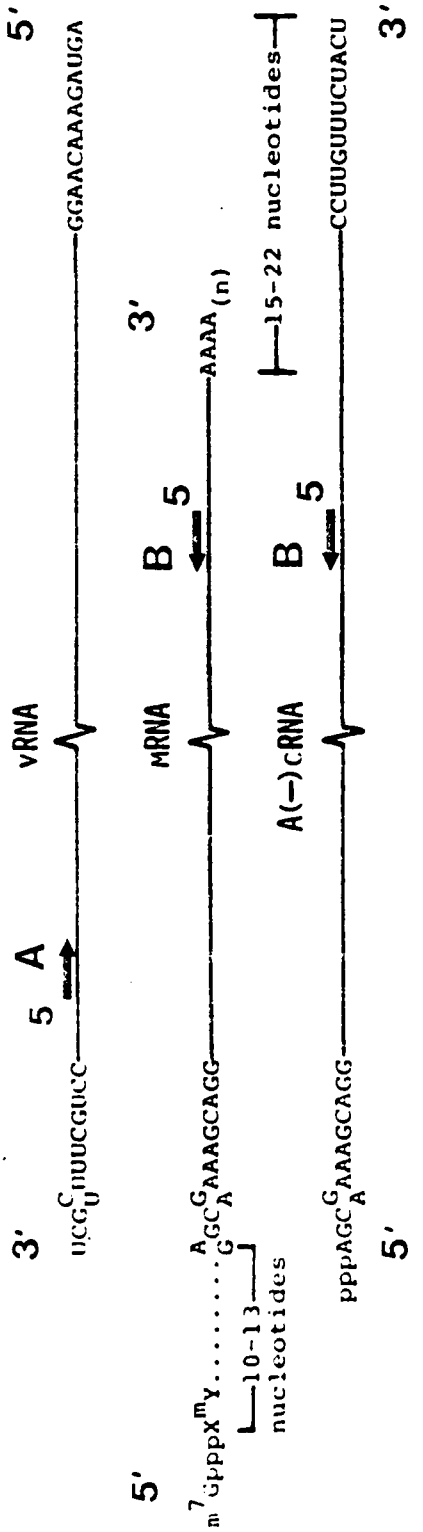
5'
3'
vRNA
mRNA
A(−)cRNA
A
B
B
5
5
3'
5'
5'
3'
15-22 nucleotides
10-13 nucleotides

Figure 2

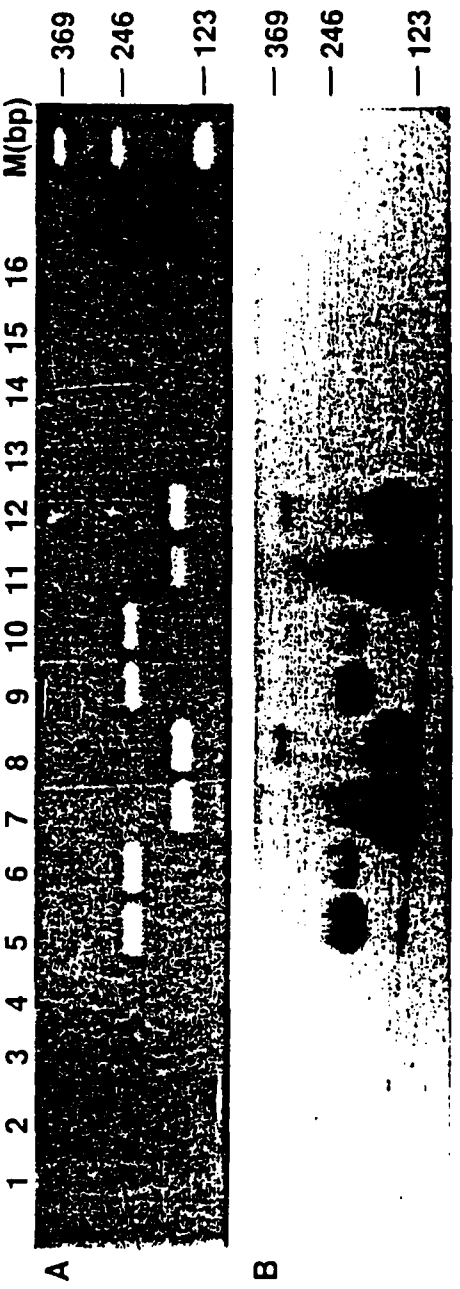

Figure 3.

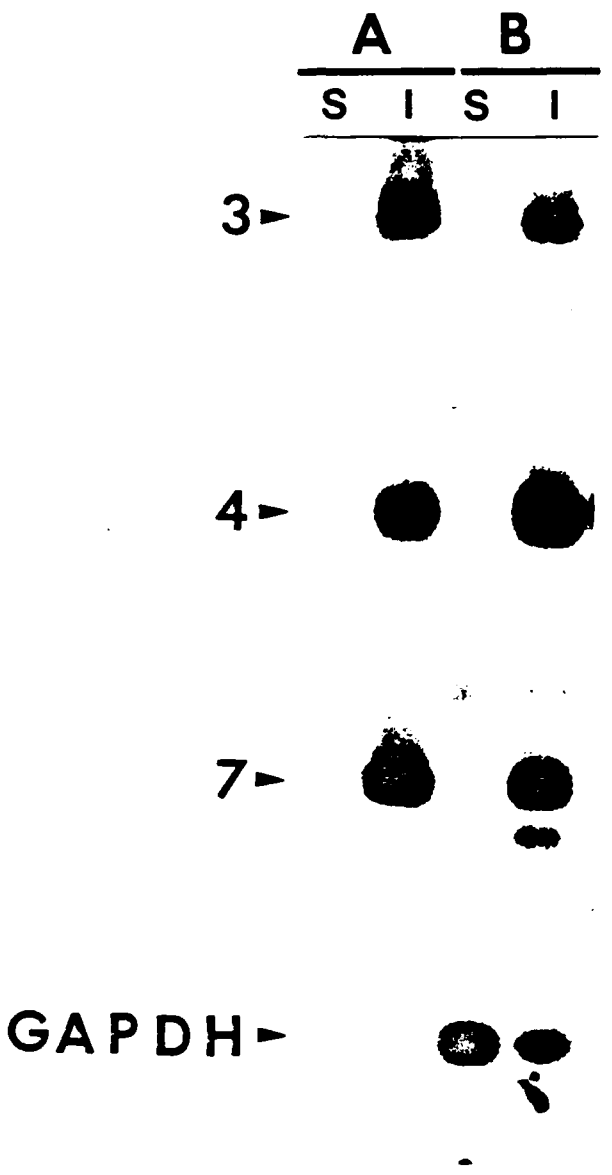

Figure 4

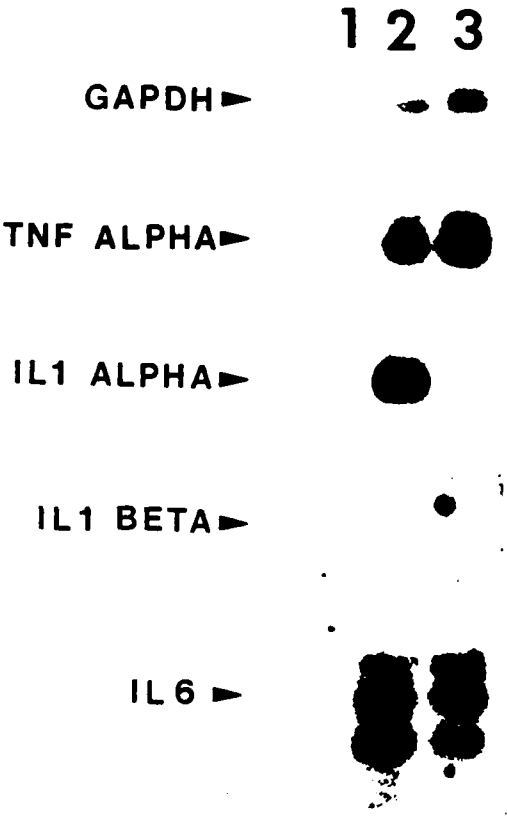

Figure 5

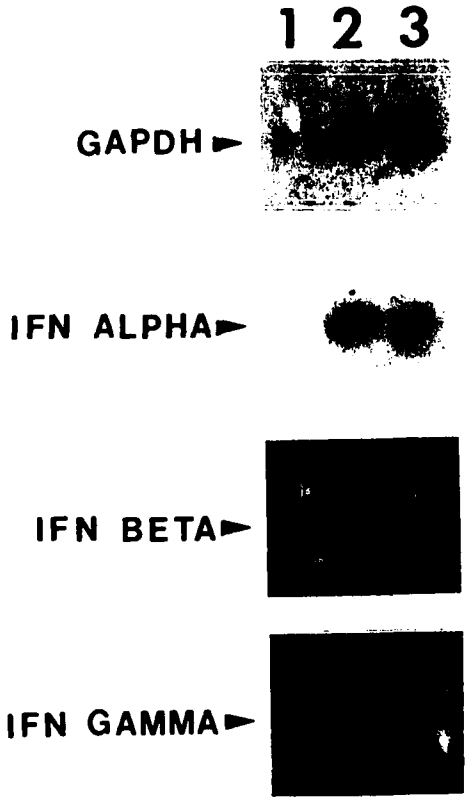

Figure 6