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Molecular immunology—gene regulation and signal transduction

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Abstract

Research on 'molecular immunology—gene regulation and signal transduction' in veterinary species is relatively new. The reason for its novelty is that until recently there have been very few tools with which we can work. Over the last 10 years the veterinary immunology community has succeeded in generating panels of defined monoclonal antibodies (mAb) and cloned genes that has enabled such work to be started. More recently, quantitative, high-resolution analytical tools for veterinary species have begun to be developed; some of these are specific for veterinary species and others have been adapted from human or rodent systems. Of the species-specific tools that have recently been developed perhaps the most widely used are the immunoassays for cytokines, RNAase protection assays (RPAs) and in the near future oligonucleotide and EST-based microarrays. This presentation will describe some of these assays and discuss their relative advantages and disadvantages.

Keywords: Molecular immunology; Gene regulation; Signal transduction

1. Introduction

In the last two decades, fundamental research in 'molecular immunology—gene regulation and signal transduction' has been focussed on human or rodent systems because of the availability of resources, reagents and assays. Work in veterinary species has lagged far behind and until recently there have been very few tools with which we can work. The rationale for working in veterinary species in this area is that we need to understand the pathogenesis of many important veterinary pathogens or that certain species enable us to ask fundamental questions that are not possible with more conventional systems. The lack of resources has meant that veterinary immunologists have needed to generate their own reagents before any experiments can be started. Since 1985 there has been a huge effort to produce panels of monoclonal antibodies to the leukocytes of the major target species, which have been characterized in relation to their homologues in the model species. A major part of that validation has involved satisfying the criteria of the IUIS/VIC sponsored Ruminant Antigen Workshops that occurred concurrently with previous IVIS (Hopkins et al., 1993; Naessens et al., 1997). In addition, and over the same time period a relatively large number of relevant genes have been isolated, cloned and sequenced (Haig et al., 1994).

2. Analysis of cell activation

The study of lymphocyte activation is still a major area of immunology and in the past has focussed on

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human and murine systems. Many of these studies utilize species-specific monoclonal antibodies but the assessment of cell activation states is in general not species-specific. This is too large a topic to consider is detail here; so I will consider a few assays that are applicable to veterinary species.

Simple proliferation assays have been used for decades and the tritiated thymidine incorporation assay is still the most popular. The reason for its popularity is its simplicity as the incorporation of radioactive thymidine is linearly proportional to cell proliferation. However, the assay has a very steep learning curve and is not reliable for polyclonal cell populations. Gaining ground recently, for reasons of safety, reliability and practicality is an ELISA technique that measures the incorporation of BUdR. As with tritiated thymidine, BudR is incorporated into DNA during replication and the amount incorporated is measurable using an anti-BUdR ELISA.

These methods simply assess cell division; however analysis of cell activation usually requires assays of higher resolution and sensitivity. One of the most sensitive and flexible assays measures mobilizable Ca^{2+} that occurs as an initial step of activation and which triggers many of the future second messenger pathway events. Perhaps the most reliable method for measuring Ca^{2+} is the Fluo-3/carboxy-SNARF-1 method (Molecular Probes, Eugene, OR). Cells are loaded with these dyes prior to activation. Ca^{2+} release after activation is detected by light emission from the Fluo-3 on excitation in a flow cytometer (Budde et al., 1994). The SNARF-1 is affected by change in H^+ ion concentration and emits light at a different wavelengths.

A common event that occurs during the activation of many cell types is the activation of enzymes such as phospholipase A_2 and C, protein kinase C and the MAP kinases, which results in the phosphorylation of a large range of other members of the second messenger pathways. The assay systems for these enzymes are based on radionuclide-labeled substrate digestion or ^{32}P-phosphate substrate phosphorylation (e.g. Amersham Pharmacia Biotech, Amersham, UK). They are sensitive and quantitative and although established for human cells, are not species-specific. Also applicable for veterinary animals are assays based on anti-phosphoryltyrosine antibodies. These antibodies bind to phosphorylated proteins of all species that are generated during cell activation by the activity of the various activated kinases. The use of these antibodies is usually by western blots of cell lysates and therefore this system is usually only qualitative. Furthermore there are no specificity controls in this system and identifying any phosphorylated product requires much further work. This problem is not unique to any one species. However, with the use of relevant calibrated controls and suitable densitometry the assays can be made to be quantitative.

3. Measurement of cell transcripts

The measurement of mRNA transcripts has become one of the major technologies in current biomedical research. Some criticize this approach because the relative levels of mRNA do not necessarily reflect the protein product; although with the exception of a small number of cytokines it is currently not possible, in veterinary species, to measure the levels of many biologically active proteins. The reason for this defect is the lack of defining monoclonal antibodies and standardized assays, and this is slowly being remedied.

Until recently transcript measurement was performed by RT-PCR, using multiple-cycle analysis to give a degree of quantitation. However, about 5 years ago QC-PCR was developed and in recent times this has been superseded by real-time PCR. QC-PCR is a technique that involves co-amplifying a fixed concentration of sample DNA and graded amounts of competitive template bearing the same primer recognition sites as the target, but containing an internal deletion such that its product is distinguishable from the target after amplification. The target is thereby quantified in relation to the concentration of the competitor. The advantage of this method is its sensitivity but it is very expensive and time consuming to establish and it quantifies only one transcript at a time. Real-time PCR is more flexible and uses two different labeled oligonucleotides during PCR amplification, in addition to the PCR primers. The first carries a green dye at its 3′ end and the second carries red dye at its 5′ end. The sequences of the two oligonucleotides are selected such that they hybridize to the amplified DNA fragment in a head to tail arrangement so that the two fluorescence dyes are positioned closely to each other. The first dye is excited by a laser light source and emitted green light excites the red fluorochrome on the

second oligonucleotide that is lying very closely to it. The quantity of emitted red light is proportional to the amount of amplified product generated and the number of cycles required to reach a light threshold is proportional to the original quantity of template. Different fluorochromes can be associated with different oligonucleotide sets so that several genes (up to five) can be measured at once. These PCR techniques are sensitive, accurate and are ideal for the assessment of single (or few) mRNA species in multiple samples but are not suited to the analysis of many transcripts in small numbers of samples, which are more usual in the research environment.

To overcome these problems with sheep cytokines we have developed a ribonuclease protection assay (RPA) for 12 sheep cytokines (Table 1). For this assay, truncated genes of known size are cloned into an SP6/T7 containing plasmid for the production of ^{32}P-labeled riboprobes. These are hybridized with the cell message before digestion with RNAase A and T1, which digests ssRNA but not dsRNA. These labeled dsRNA fragments are then size fractionated by SDS-PAGE and quantified by phosphoimaging/densitometry. Each cytokine transcript can be identified by its particular size and quantified in relation to the housekeeping genes (Fig. 1). The advantages of this system are that it is accurate, reliable and can assess 12 transcripts at one time. The disadvantages

Table 1
Genes used in the sheep cytokine RPA

Cellular gene	Genbank accession number	Fragment size (bp)
Interleukin 1β	X54796	123
Interleukin 2	X53934	232
Interleukin 3	X18291	193
Interleukin 4	M96845	210
Interleukin 6	X68723	173
Interleukin 8	S74436	352
Interleukin 10	Z29362	272
Interleukin 12 *p40*	Af004024	332
Interferon-γ	X52640	246
TNF-α	X55152	148
TGF-β	X76916	217
GM-CSF	X53561	288
GAPDH	U94889	98
ATPase	X02813	107

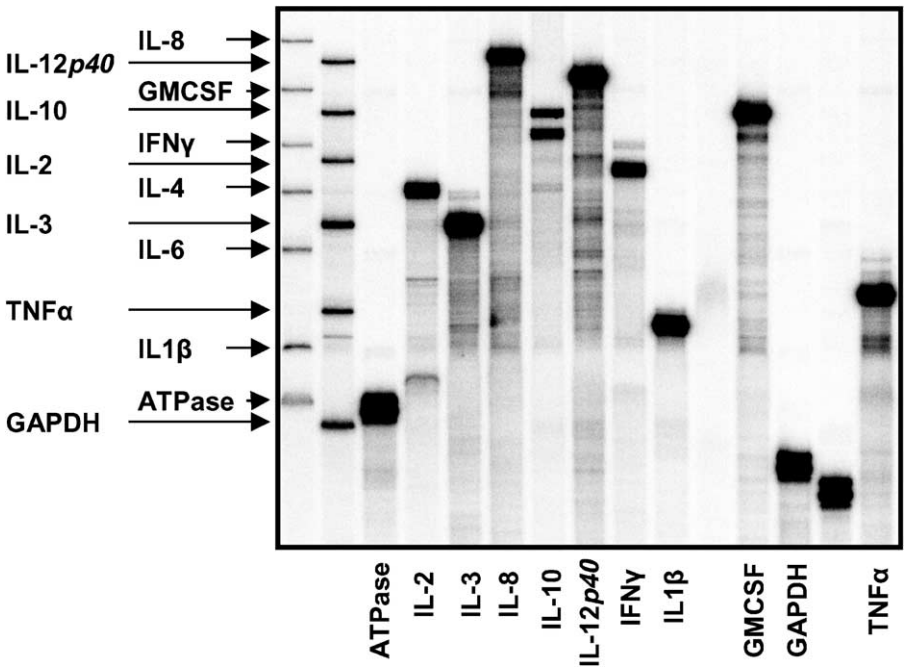

Fig. 1. Polyacrylamide gel electrophoresis of a sheep cytokine RPA. The two left-hand lanes are ^{32}P-labeled cytokine riboprobes. The other lanes represent mRNA from Con A-activated peripheral blood mononuclear cells probed with the panel of labeled riboprobes.

are that up to 5 μg of total RNA (5×10^6 cells) are required for each track and it is about 10 times less sensitive than QC-PCR.

The techniques of RT-PCR and RNAase protection are limited in the number of genes that can be examined and are non-parallel in nature. Microarray technology in contrast permits high throughput, global assessment of gene expression and by its nature generates both quantitative and qualitative data that enables parallel, comparative analyses (Staudt and Brown, 2000). At present there are no commercial sources for ruminant arrays, consequently the array we are developing in a current project is a homologous ruminant gene array consisting of approximately 300 genes (and about 50 positive and negative genes) representing the majority of sequenced ovine/bovine immuno-inflammatory genes. These include about 50 leukocyte differentiation antigens, 25 interferons/receptors and response elements, 50 cytokines and receptors, 20 growth factors and receptors, MHC molecules, immunoglobulins and T cell receptors as well as oncogenes, anti-oncogenes and anti-bacterial peptides, etc. Each gene in this array will be represented by two non-overlapping long oligonucleotides that are designed to have a similar *tm* in order to standardize the hybridization conditions (Stingley et al., 2000). Arrays can be probed simultaneously with Cy3- and Cy5-labeled cDNA representing RNA from control and test cells, respectively. The ability to produce in-house cost-effective defined chips of this nature means that precise, quantitative and statistically robust data can be obtained. After competitive hybridization the chips are washed and the arrays scanned using a confocal laser scanner. After spot signal detection the relative ratio of Cy3 and Cy5 signals on each spot is normalized and quantified using analysis software, to identify genes that are increased or decreased because of the test treatment (e.g. infection).

An advance on this oligonucleotide approach will be the exploitation of annotated expressed sequence tagged cDNA (EST) arrays consisting of up to 20,000 different genes. These resources are currently being generated for species such as cattle, sheep, swine and chickens.

4. Analysis of apoptosis

Apoptosis, or programmed cell death, plays an important role in the homeostasis and development of all tissues within an organism and is characterized by a series of stereotypic morphological changes (Wyllie, 1997). In addition it is an important protective host mechanism against virus infections and it can be triggered by other pathogens in order to modify the host environment (Howie et al., 1994). The event can be initiated by a large number of stimuli, the best known being members of the tumor necrosis receptor family, e.g. TNFR1 and fas (apo-1 or CD95). These cytokine receptors become activated after binding their ligands, which results in the generation of the death initiating signal complex associated with the intracellular 'death domain' (Webb et al., 1997).

There are three major methodologies for the assessment of apoptosis and each has advantages and disadvantages. Several companies market kits for the study of apoptosis but this article will not endorse any particular one. Fortunately these commercial kits are not, in general, species-specific and function efficiently in tissues from veterinary animals.

The most familiar feature of apoptosis is the fragmentation of DNA into about 200 bp fragments (Wyllie and Golstein, 2001). A simple assessment of DNA fragmentation by DNA electrophoresis can yield a typical 'DNA ladder'. However, this is not quantitative and is only applicable for cell lines. A more convenient technique is the TUNEL assay, which is an acronym for TdT-mediated dUTP nick end labeling. The enzyme—terminal deoxynucleotide transferase—adds hapten-tagged nucleotides to the 3′ DNA strand breaks. Enzyme or fluorochrome-tagged anti-hapten antibodies are then used to visualize apoptotic cells. The advantage of this method is that it can be used on either cell lines or in tissue sections; the disadvantages are that it is more qualitative than quantitative and occurs at a late stage in the process. TUNEL therefore underestimates the extent of apoptosis.

An earlier event is the activation, by the death initiating signal complex, of specific proteases called cysteinyl-aspartate-specific proteinases or caspases. Activated caspase 3 is the enzyme responsible for the breakdown of several cellular components related to DNA-repair. Measurement of this enzyme can therefore be used as an assessment of the apoptotic process. Colorimetric assays based on the cleavage of dye substrate yields an excellent quantitative assay for tissue culture cells, while qualitative in situ staining is

achieved by using antibodies specific for caspase-cleaved cytokeratin.

The third common assay system is based on the fact that an early event in apoptosis is the translocation of the internal plasma membrane component, phosphatidylserine to the external surface. The Ca^{2+}-dependent phospholipid binding protein, annexin V, can detect this. This is an excellent quantitative assay for tissue culture or ex vivo cells, but is unsuitable for in situ staining as fixation methods remove the phospholipid from the cells.

5. Conclusions

There is no doubt that, even today, research in veterinary species lags far behind work in humans and model rodent species. There are a number of reasons for this but most stem from the fact that the funds available for working in veterinary immunology is many times less than that available for research on model species. Consequently, the number of research workers is limited, the number and variety of reagents and other resources available are limited, the input of the commercial biotechnology industry on veterinary immunology has been minimal—we have had to produce virtually everything by ourselves—from scratch.

However, the future for molecular veterinary immunology is brighter than ever. We now have many of the defining monoclonal antibodies and cloned genes. Furthermore many have them 'in-house' and we do not have to purchase them at inflated prices from commercial sources. These, when combined with new techniques will prove a powerful combination. Finally new technologies such as functional genomics (microarray) and proteomics are becoming available in a wide variety of species and will enable 'veterinary workers' to compete with their 'human/mouse' colleagues without having to spend decades catching-up.

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