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MYCOTOXIN IMMUNOASSAYS (WITH SPECIAL REFERENCE TO ELISAs)

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Abstract : Immunoassays are now in routine use for analysis of mycotoxins. This paper presents background information on antibody production and on immunoassay formats, particularly the enzyme-linked immunosorbent assay (ELISA). Applications of immunoassay technology to determination of aflatoxins, trichothecenes, ochratoxin A and others are reviewed and their performance discussed.

Introduction

In the last few years there has been an enormous increase in interest in the use of immunoassay techniques for the analysis of mycotoxins. Indeed, there are now "kits" available commercially in many countries in formats ranging from simple yes/no tests through semi- quantitative to full quantitative procedures. The reasons for the increase in interest reside in the unique advantages of antibody-based methods - their specificity, sensitivity and simplicity coupled with speed and/or high rates of sample through-puts. The advantages derive from the unique interaction of antibodies for their target molecules, an interaction of high affinity and high specificity, and one that can be manipulated so that it can be employed *in vitro* rather than in the normal *in vivo* environment.

The first immunoassay, for the polypeptide hormone insulin, was described by Berson and Yalow[1] in 1959. Clinical analysis for a wide range of analytes was revolutionised overnight. Tests that were time-consuming, of poor sensitivity and specificity, requiring large sample volumes and difficult manipulations were displaced. The current state of the art is probably best represented by the pregnancy test kits available for home use. These robust yes/no assays (commonly based on dip-stick technology) can take less than 10 minutes to perform, can detect pregnancy by the first day of the missed period and - in the best versions - contain built-in controls to detect errors in use. Though such assays are not quantitative - they do not need to be - the quantitative potential of immunoassays should not be over-looked. All immunoassays are based on sound analytical principles, a feature sometimes forgotten in the hyperbole occasionally associated with biotechnological techniques! The pioneering work of Berson and Yalow was recognised in the awarding of the Nobel Prize to Yalow.

Immunoassays are based on antibodies, the proteins synthesised by the higher animals in response to challenge by material recognised as "foreign". Such synthesis and consequent elimination of "foreign" material represent part of the defence mechanisms of these animals. Mycotoxins, because of their low molecular weight, do not stimulate the immune system and antibody production. However, covalent linkage of a mycotoxin to a suitable carrier (such as protein) that would itself be able to generate antibody production will, upon administration to a suitable animal, stimulate an immune response. Some of the antibodies so produced will be capable of binding to free mycotoxin.

It has been known since the classic studies of Landsteiner[2] that the way in which a hapten is linked to carrier has a critical effect on the specificity of the antibody response. Thus the antibodies produced have greater specificity for those parts of the hapten molecule distal to the point of conjugation. Least specificity will be observed for sites close to the point of linkage. Specificity can, therefore, be manipulated in order to generate the desired results. These results might be antibodies of broad specificity, capable of recognising groups or classes of molecules, or they might be highly specific, recognising one compound only amongst others of similar structure.[3]

Some functional groups on a potential hapten are able to be coupled directly to carrier protein. An example would be the carboxylic acid function available in ochratoxin A, which can be directly linked to available amino groups on the protein. In other cases a functional group needs to be modified before conjugation is possible. Examples would be formation of hemisuccinates or oximes with T-2 toxin or aflatoxin respectively. For a review of potential coupling methods the reader is referred to the work of Erlanger and colleagues.[4]

Polyclonal Antisera

Stimulation of the immune response leads to antibody production from lymphocytes. Each lymphocyte produces only one particular antibody molecule, thus stimulation of many lymphocytes in the body leads to production of many different antibodies - each capable of reacting with the stimulant but with different specifications and affinities. Serum obtained from stimulated animal will contain different antibodies derived from different lymphocyte cell-lines (or clones). The antiserum will be a polyclonal antiserum and its net property will be the consequence of many individual interactions.

For haptenic compounds such as mycotoxins, polyclonal antisera can have particularly high specificity and can give rise to assays of excellent sensitivity. The immune response to the hapten forms only a portion of the total response to hapten conjugated to carrier molecule. Though even this portion can mean production of a thousand or more different antibodies, such antibodies can be regarded as being the "fine-tuning" of the total response. In addition, the format of many immunoassay systems means that the contribution of antibodies of low affinity to hapten alone or to hapten plus carrier is diminished or eliminated.

Many different animal species have been used to generate polyclonal antisera - from guinea pigs to donkeys and horses. No general consensus exists to the relative merits of any one of these, though claims are often made for one or other. Large animals can give rise to large serum volumes than smaller animals, though this benefit is counter-balanced by ease of access. In the authors experience, rabbits can give rise to quite sufficient amounts of serum (sufficient even for commercial kit production) whilst still being easy to manage. Ease of management also means that more than one animal can be immunised against any one immunogen, overcoming the considerable variation in inter-animal response observed.

Good quality polyclonal antisera against haptens can be obtained within six months following the first immunisation - it can also take considerably longer! Many texts are available to give details of procedures.[5,6]

Monoclonal Antibodies

Monoclonal antibodies, as the name implies, are the product of a single antibody-producing cell-line or clone. The ability to produce such antibodies *in vitro*[7] has become so important to many areas of research that Kohler and Milstein were awarded the Nobel prize for their pioneering work. Monoclonal antibodies are the products of antibody-producing spleen cells (which would not normally survive for long *in vitro*) given immortality by fusion with tumour cells. The resultant hybridoma will continue to divide and increase in number whilst producing antibody. Monoclonal antibodies each contain identical molecules able to react in the same way with target sites and they are available in the large quantities required for widespread dissemination.

The disadvantage of monoclonal antibodies for hapten work is that it can be extremely difficult to realise antibodies with properties as good as those produced by polyclonal methods. Thus the monoclonals may not be as specific or have as high affinity. The reason for this discrepancy is that the properties of a polyclonal antiserum may often be determined by the influence of a handful of high specificity, high affinity antibodies. Searching for these cells producing quality antibodies in order to make hybridomas is not a process favoured statistically, hence the problem. Since the production of monoclonal antibodies is also comparatively expensive, there should be good reasons for choosing this route rather than the polyclonal one as far as antibodies to haptens is concerned.

As before, many suitable books are available should more detail of procedures be required.[8,9]

Types of Immunoassay

Many different formats of immunoassay (complete with many different acronyms!) have been described. To further complicate matters, terminology is often not as exact as it might be. The basic form of immunoasay is an equilibrium reaction of unknown sample analyte, a fixed

amount of labelled analyte and a limited number of antibody binding sites, giving rise to antibody-bound and antibody-free phases:

$$An + An^{*} + 2Ab \rightleftharpoons AbAn + AbAn^{*}$$

The fixed amount of labelled analyte competes with the unknown analyte for the limited number of binding sites available. Little unknown analyte means more label being present in the bound phase: lots of unknown means less label in the bound phase. Provided the two phases can be separated and the label in one or other quantified, the amount of unknown in the sample can be determined by reference to the behaviour in the assay of known standards.

The most common form of label used in mycotoxin immunoassay work is an enzyme. Radiolabels have been used, as in the early clinical work, but because of their lack of availability, the difficulty of working with and disposing of radioactivity, and the superior properties of alternatives, they have not found widespread use. Of the non-isotopic methods, the use of enzyme labels with colorimetric points has proved particularly popular.

The most common procedure for separation of bound and free phases is to immobilise one of the assay components to a solid phase. Particles such as Sepharose have been used, but microtitration plates have become almost universal for quantitative work. These plates, usually of polystyrene, usually contain 96 wells each of capacity around 0.3ml. The plates can be used with fully automated equipment or away from the specialised laboratory "in the field". Associated equipment at both levels is comparatively inexpensive. Reagents are normally stable.

The combination of enzyme label and solid phase immunoassay results in a procedure known as an enzyme-linked immunosorbent assay, ELISA. Two common forms of ELISA are the direct, competitive ELISA and the indirect ELISA. In the direct ELISA, antibody is immobilised and exposed to enzyme-labelled analyte and unknown (or standard) analyte. After a suitable period of incubation, non-antibody bound material is washed away and the amount of enzyme-label that is antibody bound can be determined. In an alternative form of direct ELISA, a standard amount of analyte is immobilised and the antibody is enzyme-labelled.

The indirect ELISA also requires immobilisation of a fixed amount of analyte, but incubation takes place with antibody and unknown analyte (or standard). The resultant degree of antibody binding to the immobilised phase is then determined by addition of an enzyme-labelled, species-specific second antibody. This antibody raised, for example, in goat, is able to recognise antibodies from a different species - rabbit, for example. Though the indirect ELISA appears more complex, it does have certain advantages. The incubation of food extract is clearly separated from that of enzyme, resulting in reduced possibilities for interference of food extract with enzyme activity. In addition, the primary antibody can be used at considerably higher dilutions than is possible when

immobilising antibody in the direct ELISA, again reducing potential for interferences to occur.

Direct and indirect forms of ELISA can be used in quantitative and semi-quantitative modes. In quantitative format, the optical densities of the coloured end points are determined and recorded. In the semi-quantitative form several possibilities are available. Colour can be matched to pre-determined colour charts by eye to give an idea of the range in which sample concentration appears. Alternatively, simple yes/no tests are possible in which colour exceeding a certain density is recorded as being positive and in excess of a given concentration. An important example of this type of assay is the card or spot test. Two sample ports are provided in a credit card-sized, plastic coated card. A drop of sample extract is applied to the appropriate spot and is drawn through the upper surface by capillary action into an absorbent layer beneath. The upper surface contains immobilised antibody which binds to putative analyte in the sample. In the next stage, a drop of an enzyme-labelled analyte solution is applied. This too is drawn through the antibody layer, but binding of analyte (thus enzyme) will only occur if the level of analyte in the sample was low enough to leave vacant sites. Addition of substrate in the third stage produces colour change according to the amount of enzyme present, itself determined by the amount of analyte present in the original sample. The other spot on the card is usually for a control sample to check that procedures have been correctly performed.

In the examples described antibodies have been an integral part of the analysis process. They can also be used as a method of abstraction of analyte prior to analysis by alternative procedures. In these cases, an immuno-affinity column of immobilised antibodies is used as a substitute for the normal clean-up procedures employed prior to thin-layer or high performance liquid chromatographic procedures.[10] As these columns are not strictly analytical in function they do not form part of the scope of the present article.

Immunoassays for Aflatoxins

Several different strategies have been reported for coupling of aflatoxin B_1 to protein. The most common - and, in the experience of the author - the method most likely to produce antibodies of the desired properties, involves oxime formation prior to conjugation.[11-17] In this way, antibodies specific for aflatoxin B_1, or aflatoxin B_1 and B_2, or for aflatoxins B_1, B_2, G_1 and G_2 can be obtained.

An alternative approach is to attach the aflatoxin B_1 to protein through the furan end of the hapten. Several possibilities have been tried proceeding through the 2,3-dihydro-2-hydroxy derivative, known as aflatoxin $B_{2\alpha}$, which can be cross-linked directly to protein with tetrazobenzidine[18] which can be linked to protein after ester formation with glutaric anhydride.[19] Opening the furan ring of aflatoxin $B_{2\alpha}$ prior to conjugation has been an attractive proposition because of the potential for generating antibodies of very broad specificity for the aflatoxins.[20-22] Another possibility is the conjugation to protein of

the 2,3-dichloride derivative synthesised from aflatoxin B_1.[23] Antisera produced from conjugates through the furan ring will be unable to distinguish aflatoxins B_1 and B_2, and often M_1 as well.

Antibody preparations against aflatoxin G_1,[24] aflatoxin Q_1,[25] and aflatoxin B_2[26] have also been prepared.

There has been considerable interest in the application of immunoassays to the determination of aflatoxin M_1 (the hydroxylated mammalian metabolite of B_1) in milk. Regulatory authorities in some countries have set action levels for aflatoxin M_1 as low as 10 ppt (10ng per 1). The production of antisera specific for aflatoxin M_1 (as opposed to assays of broad aflatoxin specificity) has required formation of the M_1 oxime prior to conjugation.[27-31] It must be said that immunoanalysis of aflatoxin M_1 in milk has not shown the degree of success that has been observed in other areas of aflatoxin analysis. Sample extraction seems to be necessary if the required sensitivity is to be obtained, presumably due to the presence in milk of interfering lipids.

Immunoassays for Trichothecenes

Immunoassays for trichothecenes have received considerable attention recently, partly because of the difficulties experienced in analysing these compounds by conventional chemical methods. T-2 toxin has generated most interest. Nearly all reports have described the production of T-2 protein conjugates synthesised after T-2 hemisuccinate formation. Such conjugation leads to subsequent production of very specific antisera. Comparatively insensitive radioimmunoassays[32-34] have given way to ELISAs some 3 orders of magnitude more sensitive.[35-38]

Monoclonal antibodies against T-2 toxin[39] have performed poorly when used for analytical purposes compared to polyclonal equivalents - so much so that novel immunoassay technology was developed in order to boost performance.[40,41] Further work will no doubt improve the situation.

Diacetoxyscirpenol antibodies have been generated after derivatisation through the hydroxyl function.[42-45] Again, the antibodies were of high specificity. The most sensitive of the assays,[44] the indirect ELISA with polyclonal antibodies, was applied to determination of the toxin in wheat.

Only two immunoassays for direct determination of deoxynivalenol have been described,[46,47] which is rather surprising given the interest in analysing this toxin. An immunoassay for the triacetyl derivative of deoxynivalenol has been applied to the problem with some success.[48] Again, antisera were extremely specific for their target analytes. In the case of the trichothecenes there is considerable need for a broad specificity assay because of the large number of potential contaminants. Though efforts have been made to

look at antisera against structures that might resemble a trichothecene "type" molecule,[49,50] no success has been achieved yet.

Of other trichothecenes, immunoassays have been described for application to determination of 3-acetydeoxynivalenol,[51] HT-2 toxin,[52] 3^1-hydroxy-T-2 toxin,[53] and of the macrocyclic trichothecene roridin A.[54] No doubt the number of trichothecenes for which immunoassays have been described will continue to increase.

Immunoassays for Ochratoxin A

Many different immunoassays have been described for determination of ochratoxin A, all using antibodies generated against conjugates made by linking the toxin to protein through the carboxylic acid function. In general, antibody specificity has been acceptable, though assay sensitivity has varied somewhat. Radioimmunoassays[55-57] have been out-performed by ELISAs utilising polyclonal[58,59] and monoclonal antibodies.[60] The assays have been applied to barley,[57,58,60] wheat,[59] porcine serum[57] and kidney.[61-63]

Immunoassays for other Mycotoxins

Of other mycotoxins, most effort has been directed towards setting up assays for zearalenone and sterigmatocystin. Radioimmunoassays[64] and ELISAs[65-67] have been described for zearalenone determination. Two reports of ELISAs for sterigmatocystin determination both utilised antisera against sterigmatocystin hemiacetal in order to utilise the greater water solubility of this readily made derivative.[68-69] Immunoassays for rubratoxin[70] and kojic aicd[71,72] have also been described.

Performance of Mycotoxin Immunoassays

A good mycotoxin immunoassay will use a polyclonal antisera at high dilution (greater than 1 in 10,000) or a monoclonal antibody. It will be a sensitive assay, with a limit of detection of around 10 pg, or better, and will have the required specificity. The assay will have been validated for use with the desired matrix, using a simple toxin extraction procedure and direct assay of extract or diluted extract. Recovery will be near quantitative. The assay will be precise and robust, and will show good correlation with alternative methods of analysis. Only rarely should these desirable characteristics need to be compromised!

An increasing number of publications are now appearing that show good correlations of mycotoxin immunoassay data with data obtained by alternative procedures.[17,73-76] One of these studies,[74] estimated that the microtitration plate assay for aflatoxin determination being studied was at least six times quicker to perform than the high performance liquid chromatographic determination being run in parallel - this in spite of running well below capacity at only two ELISA plates per day! The reasons for the faster rate of the ELISA

were, (i) the simple sample extraction procedure, a toxin solubilisation step, that is much quicker to perform than conventional pre-assay clean up procedures, (ii) the batch-wise operation of the ELISA allowing many samples to be analysed at once.

It would be expected that a laboratory familiar with an immunoassay would start to observe increased reliability of analytical data not just because of the simpler procedures involved, but also because of the considerably increased scope for statistical analysis of generated data. The routine use of assay precision profiles, for example, ensures that a high level of confidence can be placed in results, and that data not statistically acceptable can be rejected. Such assurance is not possible with methodology that is unable to generate sufficient results on which to make these assessments.

Future Developments

The wider dissemination of mycotoxin immunoassay reagents either in the form of commercially-available "kits" or as laboratory-based methodology will increase as more analysts come to see the benefits of the technique. There will be a role for both quantitative tests based on microtitration plates and for semi-quantitative and "yes/no" assays. Increased simplicity and user-friendliness will be important trends, though fully automated systems for analysts with large sample number through-put will also become available. In some areas, for trichothecene analysis for example, multi-analyte analysis will be the focus of continuing research. Enzyme labelled immunoassays will continue to predominate, though the demand for increased sensitivity for some analytes, such as aflatoxin M_1, will see alternatives investigated.

Immunoassays will allow analysis of mycotoxins in matrices previously regarded as difficult. An example is in the study of ingestion, absorption and metabolism of mycotoxins in humans and animals where large numbers of samples need to be generated for meaningful conclusions to be drawn, and where assay sensitivity is important. Greater understanding of the role of mycotoxins in human and animal health has economic and potential importance as well as the medical and veterinary aspects. Immunoassays for aflatoxin determination in human serum,[77-79] for example, have shown differences in levels related to country of origin. Further analytical, epidemiological and toxicological work is needed to establish the significance of such fundings.

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