In this review we describe the use of targeted antibodies developed to facilitate studies on the expression of P450 proteins. The challenge of producing specific antibodies that distinguish between often highly related P450 proteins has led to the development of methods of antibody production to meet this need. Targeting antibodies toward the C terminus of P450 proteins has been found to be a particularly successful approach that is both rapid and efficient at producing specifically binding antibodies. Recent advances in genomic sequencing and proteomics now allow ready identification of expressed proteins. The levels and distributions of these proteins may be determined using antibody-based methods. However, for each protein to be studied, a unique antibody will be required. Consequently, some means of producing large numbers of well defined antibodies is needed. In this context, the potential of extending the approach used to produce specific antibodies against P450 proteins to the wider field of functional genomics is discussed.

Requirement of Antibodies for Proteomic Studies

Recent progress in the sequencing of the entire human genome is a landmark achievement (International Human Genome Sequencing Consortium: Lander et al., 2001). On its completion, this initiative will provide a complete catalog of human DNA sequence information. These data are currently being used very productively to develop a variety of technologies, such as micro gene arrays, designed to assess gene expression at the mRNA level. However, there is still a great need to investigate gene expression at the protein level, partly because of the often poor correlation between mRNA and protein expression, but also because ultimately, it is protein function that dictates biological processes.

Progress into the investigation of the role played by individual proteins in biochemical processes and disease would be greatly facilitated if suitable methods could be developed to permit the identification, location, quantification, and functional analysis of individual proteins. Proteomics is providing rapid advances in some of these areas, but there are still serious limitations that could be overcome by producing suitable antibodies. However, it is widely recognized that this is an area in which major developments are required before the effective advancement of proteomic studies will be possible. Conventional approaches to antibody production (either polyclonal or monoclonal) are time consuming and inconsistent with respect to affinity and, particularly, specificity of the product. Antibodies produced against intact proteins, regardless of their source (e.g., purified proteins, recombinant expressed proteins, spots from gels) often cross-react with similar proteins or contaminating proteins present in the original source preparation, considerably reducing their value for use in proteomic studies. Therefore, some means for rapid and simple production of good quality, highly specific antibodies is needed.

Anti-P450 Antibodies

In many respects, for those of us engaged in studies of the expression of P450 proteins, the problems that need to be overcome to produce useful antibodies are all too familiar. To begin with, purification of P450 proteins from tissues is not a trivial undertaking. Yields are low and purity is often only achieved after some considerable effort. The situation is made particularly difficult for human P450 proteins since the quantities of tissues available are limited and the expression levels may be low. This problem has been addressed by developing heterologous expression systems using a variety of host cells including yeast, mammalian cells, and bacteria (Friedberg et al., 1999). To optimize expression levels, it has been found necessary to modify the native P450 protein, most frequently in the hydrophobic N-terminal region that is responsible for anchoring the
protein to the endoplasmic reticulum. Their further modification by the addition of a poly-histidine tagging sequence has also been used to facilitate purification using metal-ion chelate chromatography (Modi et al., 1996). Although such systems have been successfully applied to the production and purification of a number of human P450 proteins, such work cannot be considered in any way routine nor as an approach that could be applied to produce hundreds or thousands of proteins to be used as immunogens.

Regardless of the way in which the P450 protein is purified, there is still the problem of antibody production. For P450 proteins, simple immunization of an animal to produce a polyclonal antisera is rarely sufficient. Antisera produced in this way usually cross-react with other P450 proteins present in the tissues of interest. This is due to the high degree of sequence similarity shared by many P450 proteins. One way to overcome this problem is to deplete the antisera of cross-reacting antibodies by affinity chromatography (Parkinson and Gemzik, 1991). But even though successful, this method requires both a knowledge of the proteins to which the antisera cross-react and purified or partially purified preparations of such proteins to adsorb out the undesired antibodies. More frequently, workers have turned to the use of monoclonal antibodies (Gelboin, 1993). The use of such antibodies ensures that just a single epitope is recognized. However, this does not necessarily mean that the antibody will bind to a single protein. Again, the conserved nature of P450 proteins means that an identical epitope may be present in other P450 proteins, particularly those that are most closely related in their primary structures (e.g., Wrighton et al., 1992; Edwards et al., 1993). This problem is overcome by screening out those monoclonal antibodies that bind to other P450 proteins, but again, this requires having suitable preparations to undertake such selection and, of course, this takes time and effort. Common to both polyclonal and monoclonal antibody production is the need to determine that the antibodies not only bind to the desired protein, but are also specific to that protein.

**Anti-Peptide Antibodies**

An alternative way to produce antibodies against P450 proteins is to use synthetic peptides that represent small regions of the protein of interest as immunogens. This approach requires no protein purification to prepare the target P450 and other related forms; instead, it simply requires knowledge of the sequence of these proteins. From this information it is possible to predetermine the region of the P450 protein to which the antibody will bind. Selection of regions that vary between related forms will ensure that the antibodies will be form-specific (Edwards et al., 1991). It is also possible to target regions that may be involved in catalysis and in this way inhibit the activity of the enzyme (e.g., Adams et al., 1997). The selected peptides are synthesized and chemically coupled to a carrier protein, and then animals are immunized with the conjugate. The resultant antisera can be used directly without further processing. In this way it is possible to produce epitope-specific polyclonal antibodies. We have successfully applied this approach to the production of panels of antibodies against human and rodent P450 proteins (Edwards, 1998; Edwards et al., 1998). From our investigations into P450 proteins it is evident that the same approach holds great potential for the generation of the large number of antibodies required to match the successful application of proteomic techniques in identifying potentially important proteins.

**The C-Terminal Approach**

Although anti-peptide antibodies have been applied to studies involving a wide range of proteins, it is often perceived that this approach is overcomplicated by questions of the selection of peptide (based on the probable location within the protein and the optimum size of the peptide), the success rate in producing antibodies that bind to the target protein, and the resultant specificity of the antibodies. Hence, in different hands, success in the use of anti-peptide antibodies has been variable. However, if the aim is simply to produce an antibody against a particular protein, then it is possible to circumvent such problems.

We have devised a simple, effective and reliable approach to the targeting of P450 proteins. We have exploited their extreme C terminus (i.e., typically, the terminal four or five residues) as a target for antibody production (Edwards, 1998; Edwards et al., 1998). To do this, each peptide is coupled through its N terminus to a carrier protein to mimic the presentation of the C terminus of the target protein. This method was adopted following the finding that a large proportion of antibodies raised against a peptide conjugated to a carrier protein through its N terminus are directed toward an epitope that comprises the extreme C-terminal residues and, importantly, includes the carboxyl group of the C-terminal residue itself (Edwards et al., 1995). Antibody binding to the free C-terminal carboxyl group is critical for determining specificity, since this restricts the binding of such antibodies to the C terminus of protein antigens. Therefore, although the combination of amino acids that comprise the epitope may occur in a number of proteins, such antibodies will bind only to those proteins that contain the epitope at their C terminus, and this is rare. This assertion is based on both structural and practical observations. Structural uniqueness (or otherwise) can be ascertained for each epitope targeted by examining the C termini of proteins in appropriate databases (e.g., SWISS-PROT, EMBL, PIR, etc.). But, from a practical point of view, selectivity in the binding of such antibodies is evident from analysis of samples by Western blotting. The antibodies show remarkable specificity in binding to the target P450 in liver microsomal fraction or even crude liver homogenate samples with little evidence of binding to other liver proteins (Fig. 1), despite the large number that are present at readily detectable levels.

In our experience with antibodies targeted against the C terminus of P450 proteins, the only proteins with C termini that are similar enough to allow antibody binding are other P450 proteins that are members of the same family or subfamily. This is the case for the human forms of the CYP2C subfamily, which all have identical C termini. Thus, the antibody targeted against the C terminus binds to all forms (Table 1). Obviously, this antibody is limited in its specificity. However, since CYP2C18 is expressed at only a very low level in human liver, and CYP2C8, CYP2C9, and CYP2C19 can be separated by SDS-polyacrylamide gel electrophoresis (Goldstein et al., 1994), then it is still possible to determine levels of each P450. In fact, since the epitope is identical in all of the CYP2C forms, it is possible to compare levels between these forms and quantify them using a single standard. The C termini of CYP3A4 and CYP3A7 are also identical, and so the antibody targeted against this region recognizes both forms (Table 1); but since CYP3A7 expression is largely limited to fetal tissue, the antibody targeted against this region recognizes both forms (Table 1); but since CYP3A7 expression is largely limited to fetal tissue, the C terminus of CYP2A6 varies from that of CYP2B6 in just one residue, with the four C-terminal residues being identical. This appears to be sufficient to allow the antibody to bind to both forms (Table 1). However, the variation in the C termini of the other seven forms of human P450 studied is such that all other antibodies bound to just a single P450 and to no other proteins in human liver microsomal fraction (Table 1).

We have also produced a CYP3A7-specific antibody by targeting an internal sequence of CYP3A7. This antibody has been used to examine the expression of CYP3A7 in the liver of 59 normal human donors by immunoblotting. In 58 of these, no evidence of expression was found. In one sample an immunoreactive protein was present, but we have yet to confirm whether this is CYP3A7 (unpublished data). Thus, although CYP3A7 mRNA is expressed at detectable levels in the majority of adult livers (Schuetz et al. 1994; Burk et al. 2002), this does not appear to be the case for CYP3A7 protein.
FIG. 1. Specificity of binding of C-terminal directed anti-peptide antibodies.

Samples of human liver were separated by SDS-polyacylamide gel electrophoresis using 9% gels containing the following: lane S, reference standard (5 μg); comprising a pool of human liver microsomal fraction from six previously characterized donors; lanes 1, 3, and 5, liver homogenate from three different donors (50 μg); and lanes 2, 4, and 6, liver microsomal fraction from the same respective donors (5 μg). The immunoblots were developed with either anti-Val-Ile-Pro-Arg-Ser antiserum to detect CYP2E1 or anti-Thr-Val-Ser-Gly-Ala antiserum to detect CYP3A4 as described previously (Edwards et al., 1998). Note that in each of the blots, in both the microsomal fraction and the homogenate samples, no bands other than the target P450 are detected.

TABLE 1

Human P450 proteins that have been targeted with antibodies against their C termini and the specificity of such antibodies

The reactivity of these antibodies has been described previously (Edwards et al., 1998).

<table>
<thead>
<tr>
<th>Human P450</th>
<th>C Terminus</th>
<th>Antibody Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>-Glu-Leu-Ala</td>
<td>specific to CYP1A1</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>-Pro-Phe-Ala</td>
<td>specific to CYP1A2</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>-Glu-Ala-Ser</td>
<td>specific to CYP1B1</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>-Phe-Ser-Ala</td>
<td>specific to CYP2B1</td>
</tr>
<tr>
<td>CYP2B2</td>
<td>-Phe-Ser-Ala</td>
<td>specific to CYP2B2</td>
</tr>
<tr>
<td>CYP2D1</td>
<td>-Arg-Glu-Asp</td>
<td>specific to CYP2D1</td>
</tr>
<tr>
<td>CYP2D2</td>
<td>- Ala-Val-Ala</td>
<td>specific to CYP2D2</td>
</tr>
<tr>
<td>CYP2D3</td>
<td>-Val-Ile-Pro</td>
<td>P450 not detected</td>
</tr>
<tr>
<td>CYP2D4</td>
<td>-Val-Pro-Arg</td>
<td>specific to CYP2D4</td>
</tr>
<tr>
<td>CYP2D5</td>
<td>-Arg-Glu-Asp</td>
<td>specific to CYP2D5</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>-Val-Ile-Pro</td>
<td>specific to CYP2E1</td>
</tr>
<tr>
<td>CYP3A1</td>
<td>-Ile-Ile-Thr-Glu</td>
<td>specific to CYP3A1</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>-Val-Ile-Asp-Glu</td>
<td>specific to CYP3A2</td>
</tr>
</tbody>
</table>

 binding of the antibodies with only the respective target forms being detected (Table 2). In all, 10 antibodies were produced against the C termini of rat P450 proteins that bound to single forms of P450 and to no other proteins in rat liver microsomal fraction (Table 2).

Therefore, in the majority of cases the antibodies bound to only the targeted protein, and where binding to other proteins was found, this was to a highly related protein.

The binding of such antibodies is strongly affected by small alterations of the C-terminal region. Often the antibodies do not bind to related protein antigens that differ by just a single amino acid in the C terminus. However, the precise structural determinants of the specificity of such antibodies, including the size and composition of the epitope, have yet to be fully determined, and we intend to explore this further in future work. Unlike most antibodies, this is possible here since the epitopes are composed of contiguous amino acids, and this should permit the epitopes to be defined using a systematic approach.

Such is the structural relationship between P450 proteins that the C termini of some proteins are conserved between species. Consequently, a single antibody may bind equally to the same form of P450 from a variety of species. This is the case for CYP2E1, where the C terminus (Val-Ile-Pro-Ser) is the same in human and rat (Tables 1 and 2), and also mouse, hamster, rabbit, and cynomolgus monkey. Interestingly, the C terminus of marmoset monkey CYP2E1 is extended by two residues (Val-Ile-Pro-Ser-Val) and as a result is not detected at all by immunoblotting using the anti-Val-Ile-Pro-Ser-Arg antibody (Schulz et al., 1998). This illustrates one important aspect of the properties of such antibodies, i.e., that antibody binding is not reliant on just the occurrence of a combination of amino acids, but also on their location at the C terminus.

Reliability and Utility of the Approach

Overall, we have immunized a total of 38 rabbits with different peptides that represent the C termini of various P450 enzymes, as well as some other proteins (see below). In every case to date, antibodies were produced that bound to the target protein (e.g., Edwards, 1998; Edwards et al., 1998, and references therein); thus, the approach is highly reliable. The antibodies bound equally well to native and denatured proteins, showing that access of the antibodies to the C terminus is not compromised by structural constraints. This was expected since the extreme C terminus of a protein is usually highly flexible and exposed as shown by X-ray crystallographic studies of many proteins including P450 proteins.

In the rat, among those examined, only CYP2B1 and CYP2B2 have identical C termini, and the antibody against the C-terminal peptide bound to both forms equally (Table 2). There is a close similarity between the C termini of CYP2D1 and CYP2D5 with only one difference occurring in the terminal amino acid. Antibodies raised against five-residue peptides, representing the C termini of each form, showed strong reactivity with the target form and weak reactivity to the other form. To direct the antibodies more toward the terminal residues, the peptides used for immunization were reduced in size to 3-amino acids. This was successful in improving the selectivity of the
of producing specific antibodies and is a very useful adjunct to the C-terminal approach. Such antibodies can also be targeted toward regions important for enzyme activity and cause inhibition (e.g., Schulz-Utermoehl et al., 2000). C-terminally directed antibodies do not inhibit the activity of P450 enzymes.

For potential applications in proteomics, particularly in expression profiling, there is the need to produce extensive libraries of hundreds or possibly thousands of antibodies. Since the production of such quantities of polyclonal antibodies appears to be a rather daunting prospect, perhaps recombinant antibody technology can be applied. The use of recombinant libraries of single-chain variable region antibodies (Winter et al., 1994) has advanced the speed of antibody production by overcoming the need to immunize animals. Since the recombinant antibody approach is adaptable to automation and, hence, the production of large numbers of antibodies (de Wildt et al., 2000), it might be possible to produce large numbers of highly specific antibodies against defined C-terminal epitopes in this way.

Conclusions

The challenge of producing specific antibodies that distinguish between often highly related P450 proteins has lead us to develop methods of antibody production to meet this need. We have found particular success in targeting the C terminus of P450 proteins. This approach was found to be both rapid and efficient at producing specifically binding antibodies. Furthermore, the binding of such antibodies to related proteins is potentially predictable. It is possible that this work can be extended more widely to the field of functional genomics. The human genome contains 30,000 to 40,000 protein-expressing genes (Lander et al., 2001), and methods are now needed to determine the levels and distribution of the expressed proteins. The approach described here is capable of producing antibodies that bind to the majority of these proteins and, in theory, with a great deal of specificity.

When Dr. Gillette and colleagues organized the first meeting on “Microsomes and Drug Oxidations” in Bethesda in 1968 (Gillette et al., 1969), they could not have imagined the complexity of the P450 system. The approach described is helping us better understand the critical functions of this fascinating group of enzymes.
Donald Davies received a Bachelor’s degree in chemistry in 1962 from the University of Wales (UK). In 1965 he received a Ph.D. degree in biochemistry from St. Mary’s Hospital Medical School, University of London under the supervision of Professor Tecwyn Williams for studies of drug metabolism.

From 1965 to 1967 Professor Davies was a Visiting Fellow in the Laboratory of Chemical Pharmacology at the National Institutes of Health (United States) where he was a member of Dr. Gillette’s Section working on mechanisms of drug oxidation in the very early days of research on P450. In 1967 he returned to the UK to join the Department of Clinical Pharmacology at the Royal Postgraduate Medical School (London, UK) and was appointed Professor of Biochemical Pharmacology in 1980. In 1987 he succeeded Professor Colin Dollery as head of the department (at what is now Imperial College London). Recently, he resigned from this position to become Director of Research at ML Laboratories plc but continues to be a member of the professorial staff at Imperial College London. A major element in his research on mechanisms of drug action and toxicity has been his studies of human P450 enzymes, an interest he developed while working with Dr. Gillette.

Alan Boobis received a Bachelor’s degree in pharmacology in 1971 from the University of Glasgow (UK). In 1974 he received a Ph.D. degree in pharmacology from the University of Glasgow (UK) under the supervision of Garth Powis working in the area of drug metabolism. His doctoral work involved studies of the influence of changes in hemodynamics in the liver on drug disposition and the effects of lipid peroxidation on P450 integrity. In 2003, he received an OBE for his work on the risk assessment of pesticides.

Professor Boobis then worked as a Fogarty Visiting Fellow for two years with Dr. Dan Nebert at the National Institutes of Health (Bethesda, MD). During this time he studied the effects of modulating factors on benzo[a]pyrene-DNA adduction and was also involved in an investigation of genetic and developmental factors on P450 expression and activity. In 1976 he joined the department of Clinical Pharmacology at what was then the Royal Postgraduate Medical School (London, UK), as a Medical Research Council research training fellow under the supervision of Professor Donald Davies. He was then appointed to a tenured lectureship and subsequently promoted to Professor of Biochemical Pharmacology, his current position (at what is now Imperial College London). Recently, he also took over as director of the Department of Health Toxicology Unit at Imperial College London. His current research interests include the regulation of human P450 enzymes by genetic and environmental factors and the development of novel biomarkers of toxicity.

Robert Edwards received a Bachelor’s degree in applied biochemistry in 1977 from Brunel University (UK). In 1982 he received a Ph.D. degree in biochemistry from Guy’s Hospital Medical School, University of London. In 1984 he joined the Department of Clinical Pharmacology at the Royal Postgraduate Medical School (London, UK) to work with Alan Boobis and Donald Davies on the characterization of P450 enzymes. Here, he developed an anti-peptide approach for the production of antibodies against the major forms of hepatic P450 expressed in human and other species. In 2000 he was appointed as Research Lecturer at Imperial College London. His current research activities include the application of the anti-peptide antibody approach to proteomics as well as a continuing interest in the characterization of P450 enzymes by immunochemical methods.