Measurement of drug-induced changes in hundreds of liver proteins: developing a molecular pathology

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ABSTRACT **One of the emerging goals of toxicology is the connection of effects observed at the whole animal and histopathologic levels with events at the molecular level. Studies on the many cellular enzymes and other proteins affected by xenobiotic treatment form a key part of efforts to bring about such a connection. Recent investigations using computer-analyzed two-dimensional electrophoresis indicate that it is possible to detect both quantitative and qualitative changes in hundreds of tissue proteins in the course of rodent toxicology experiments. Databases characterizing these proteins and their responses to treatment with model compounds provide the framework for a mechanistic interpretation of cellular effects. The approach offers a systematic method for the comparison of in vivo and in vitro test systems as regards metabolic status and response to known effectors.—Anderson, N.L. Measurement of drug-induced changes in hundreds of liver proteins: developing a molecular pathology.**

Key Words: 2-D electrophoresis • molecular pathology • database • proteins

INTRODUCTION

Beginning in 1975, with the introduction of practical techniques for the resolution of hundreds of individual proteins (1), it has become a major goal in numerous areas of biological research to catalog the proteins of specific organisms or tissues (2, 3). The primary motivation for such a general approach has been the realization that the mechanistic basis for most biological events remains unknown in detail; if we are to penetrate the complex activities of the cell, we will need tools that can search for significant changes in many, as yet uncharacterized, macromolecules. The advent of two-dimensional electrophoresis makes this global analytical approach technically feasible, and with it, the development of databases redescribing the field of microscopic pathology at the molecular level.

Toxicology and pharmacology present in some ways the prototype fields for the application of this approach; in the general case, treatment of an animal (or cell system) with some chemical entity causes an effect on the normal function of a part or parts of a complex system. Much of present biological knowledge has been obtained through the analysis of just such artificial (poison-induced) or natural (mutation-induced) aberrant behavior. A key limitation in analyzing these phenomena has been the problem of collecting enough information about events at the molecular level to adequately describe what happens. If this problem can be solved, toxicity might be better understood and potentially avoided.

In order to begin systematically to link molecular changes with drug effects using a new analytical technology, several criteria must be met: it must be shown *1*) that a useful range of known effects produces detectable changes at the molecular level, *2*) that there is sufficient specificity associated with molecular effects to differentiate various classes of mechanisms, and *3*) that there is some basis for expecting that the molecular changes can be interpreted in a way that helps illuminate not only the details, but also the possible significance, of the events observed. In short, it must be shown that a comprehensive molecular analysis will contribute something to toxicology in its own terms. If these requirements can be met, a combination of the large body of ongoing experimentation associated with current toxicology and the power of new molecular methods may produce a substantial increase in knowledge of biological systems and, hence, in our ability to accurately determine the safety of new drugs.

In this report, we present a brief discussion of the basis for the proposed approach to toxicology and discuss some results indicating areas of immediate application.

TWO-DIMENSIONAL ELECTROPHORESIS

Analytical technology

Two-dimensional (2-D) gel electrophoresis was the first (and is so far the only) method capable of resolving a number of proteins approaching that expected to be found in living cells (2–10,000). The method, as originally elaborated by O'Farrell (1), involves the use of isoelectric focusing under denaturing conditions, followed by separation in a perpendicular direction by electrophoresis in sodium dodecyl sulfate (SDS). Hence, the proteins are separated by isoelectric point (a function of their content of acidic and basic amino acids) in one dimension (usually presented as the horizontal dimension), and by subunit molecular mass (polypeptide chain length) in the other (usually vertical) dimension. The result is a 2-D map in which discrete spots represent individual proteins (**Figure 1**), with large proteins at the top and acidic proteins at the left. The method requires use of denaturing conditions, so that enzymatic activities are not generally preserved. Nevertheless, most resolved proteins still react with appropriate antibodies following separation and blotting, and the quantities of protein separated on the gel are often sufficient to allow subsequent biochemical

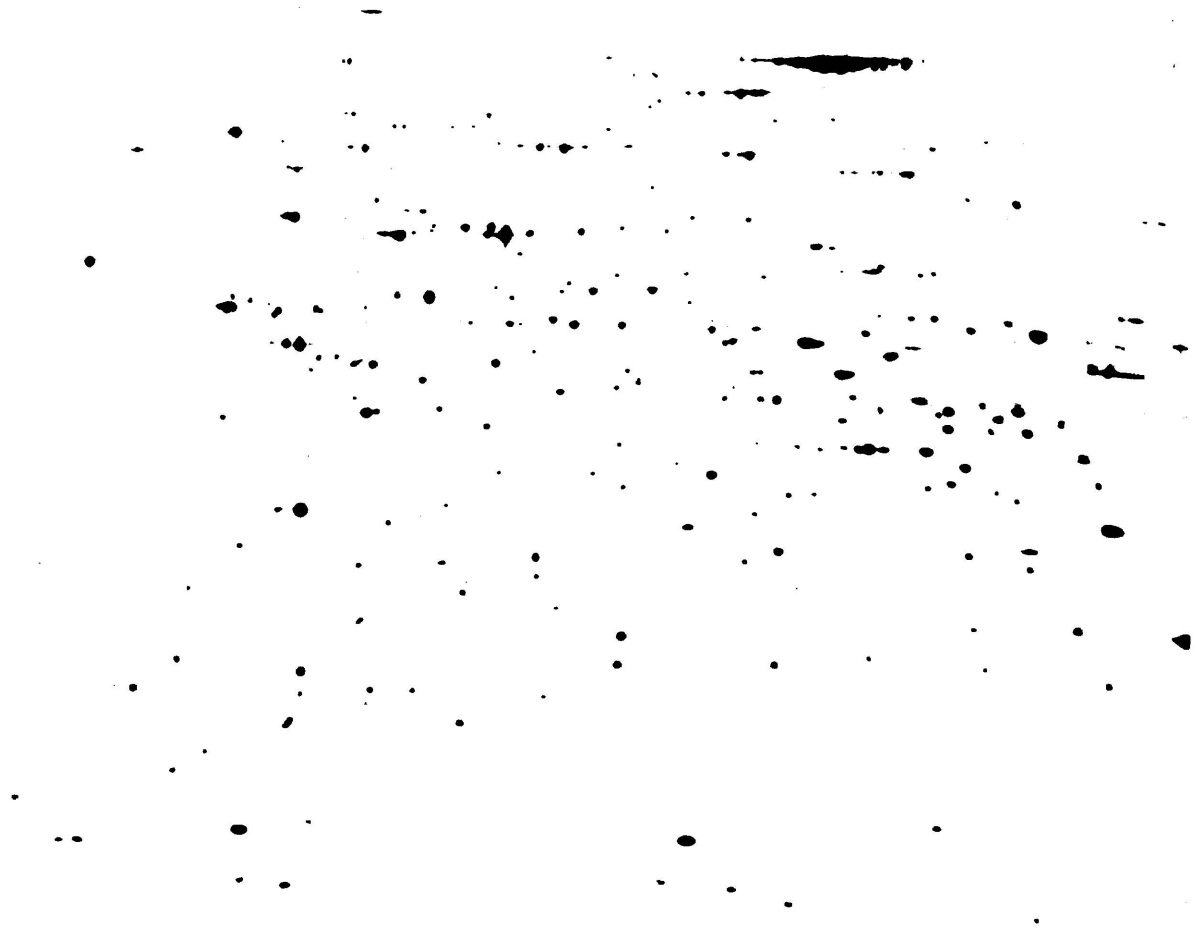

Figure 1. Liver 2-D protein pattern. A two-dimensional protein pattern of mouse liver proteins (B6C3F1). Acidic proteins are on the left, basic to the right, high molecular weight (c. 200 kDa) at the top, and low molecular weight (c. 8 kDa) at the bottom. The proteins were resolved in the 20 × 25 cm ISO-DALT® gel system and stained using colloidal Coomassie Blue. Approximately 1,200 proteins are resolved on such gels.

characterization, including amino acid sequencing. Approximately 4,000–5,000 published papers have employed the technique to examine proteins in a wide variety of biological systems.

As is the case with most advanced analytical methodologies, considerable effort has been required to transform the 2-D approach from a small scale (e.g., 8 samples/experiment) technique to a larger scale (e.g., 100–1,000 samples/experiment) technology useful in systematic studies such as toxicology. Significant problems of instrumentation (4, 5) and standardization (6) had to be overcome to produce consistent results over large sample sets. The results presented here were obtained using the ISO-DALT® system for 2-D electrophoresis developed by the author and colleagues at the Argonne National Laboratory, and subsequently made commercially available.

Computer analysis

Because of the necessity to quantitate spots in large images, and more important, the necessity to interactively manipulate and analyze the large amounts of data that result, computers are a major component of a successful 2-D system (7, 8). While the human eye is an excellent tool for comparing two similar 2-D protein patterns, it cannot successfully search for consistent differences between two groups of a hundred patterns, nor can it provide good quantitative data on protein abundance.

Currently available 2-D analysis systems acquire high-resolution digitized images of 2-D protein patterns (typically 2048 × 2048 pixels) and reduce them to a quantitative list of protein spots observed. The results from different patterns are matched to a reference pattern, and the data are analyzed to detect differences between samples. The data presented here have been analyzed using the Kepler® 2-D software system, which also includes facilities for statistical analysis of large, multigroup experiments typical in toxicology and maintenance of data and conclusions in a relational database system.

Databases

The final element necessary to complete the 2-D approach is knowledge. Given the capability to detect changes in hundreds of specific proteins, it becomes increasingly important to know what those proteins are and what they

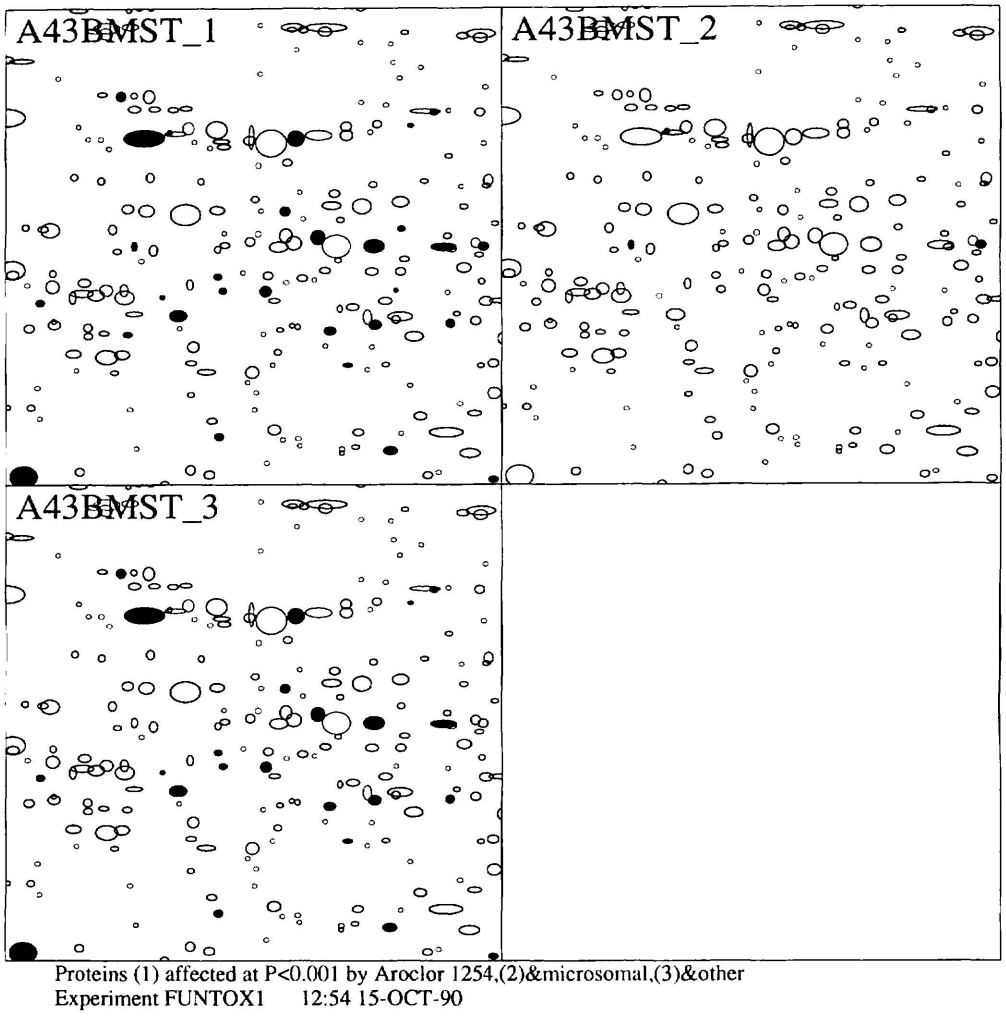

Figure 2. Montage showing effects of Aroclor 1254 in liver and analysis in terms of subcellular location. A graphical display showing statistical and database analysis of the effect of Aroclor 1254 on protein expression in mouse liver. The three panels show all spots in the same subsection of the standard mouse liver 2-D pattern, with spots possessing the appropriate characteristic highlighted (shown in black): panel 1 (upper left) shows in black those proteins whose abundance is altered at a level $P < 0.001$ in animals given Aroclor 1254 (250 mg/kg; 58 proteins in the whole 2-D pattern); panel 2 (upper right) shows Aroclor-affected proteins (from panel 1) that are also determined to be located in microsomes (6 proteins in the whole 2-D pattern); panel 3 (lower left) shows Aroclor-affected proteins (from panel 1) that are determined to be located in either mitochondria or the soluble phase (43 proteins in the whole 2-D pattern). It is evident that most Aroclor-affected proteins are not microsomal. Subcellular location data were obtained from a separate experiment via a database characterizing liver proteins. This and subsequent figures are produced by the Kepler® 2-D software system.

do. Two sorts of information are most useful: protein characterization (including identification where possible, subcellular location, sequence, etc.) and data on regulation by model compounds. Using this information, effects can be analyzed in terms of similarity to effects of other compounds and the consequences for cellular metabolism can be inferred. Results presented in this paper make use of the Rodent Molecular Anatomy and Molecular Effects Databases under development at LSB.

PROTEIN ALTERATIONS ASSOCIATED WITH TOXICITY

Aroclor 1254

Experiments with the classical microsomal enzyme inducer, Aroclor 1254 (a mixture of chlorinated hydrocarbons), provide a clear example of one of the most basic lessons to be learned from the comprehensive protein analysis approach. The consensus of more than 2,000 publications using Aroclor is that its major biological effect is an induction of cytochromes P-450. Such an induction is observed on 2-D gels of microsomes of Aroclor-treated rats (9). However, 2-D analysis of whole liver tissue demonstrated that Aroclor treatment produces statistically significant increases or decreases in the levels of hundreds of liver proteins (10). Perhaps most important, it was observed that >90% of these effects involved proteins that were assigned (in the liver database) to the mitochondria or soluble phase, not the microsomal fraction (**Figure 2** and ref. 11). Hence, potentially important metabolic alterations are produced that may, from the cell's point of view, overshadow the induction of the drug-metabolizing enzymes of the ER.

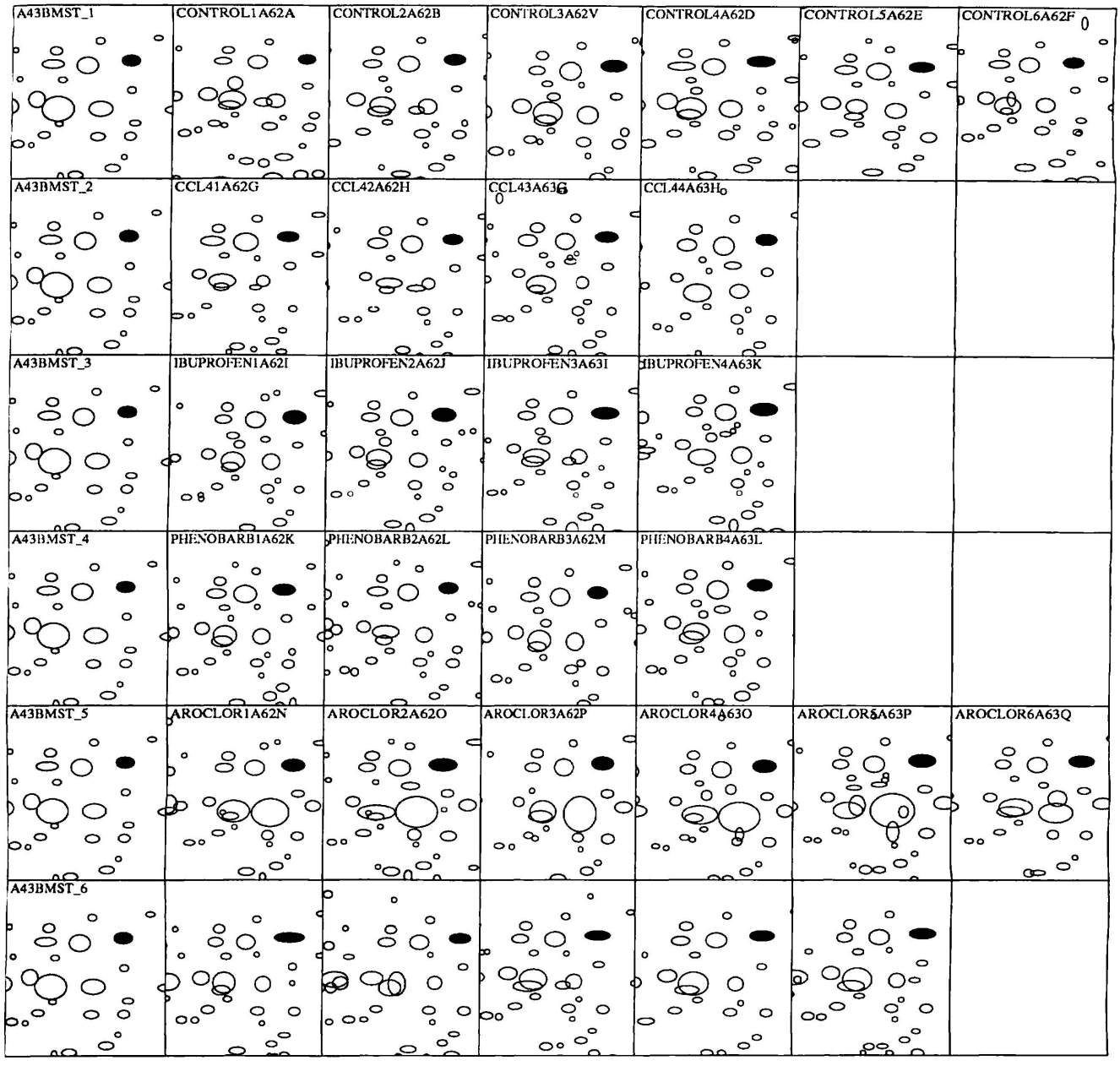

Figure 3. Montage of individual mouse patterns showing effects of various treatments on cytoplasmic epoxide hydrolase. A graphical display showing the same small section of a series of different 2-D gels, each representing the results of analyzing liver tissue from a different mouse. Each row constitutes an experimental group: row 1 is the control group, row 2 was treated with carbon tetrachloride, row 3 with ibuprofen, row 4 with phenobarbital, row 5 with Aroclor 1254, and row 6 with cycloheximide (11). The spot identified in the mouse liver database as cytoplasmic epoxide hydrolase is highlighted (spot 49, shown in black). The protein is well resolved and is quantitated with an average within-group coefficient of variation (standard deviation divided by the mean) of 13%, competitive with many conventional enzymatic and immunoassays. Figure 4 shows induction of this protein by ibuprofen and Aroclor.

Comparison of various classical toxic agents

High-dose treatment of rodents with different classical toxicants produces different kinds of protein pattern effects (11). **Figure 3** shows a small region of the 2-D pattern surrounding the enzyme epoxide hydrolase (cytoplasmic form) taken from a series of patterns corresponding to mouse liver samples from animals treated with vehicle, carbon tetrachloride, ibuprofen, phenobarbital, Aroclor 1254, or cycloheximide. **Figure 4** shows the abundances of eight interesting proteins in the animals of all these experimental groups. The data show that these proteins are regulated differently in response to different agents, and that each agent has a signature of effects at the protein level. For example, accumulation of proalbumin (#44) is increased by carbon tetrachloride (which disrupts cellular membranes, including Golgi) and decreased by cycloheximide (an inhibitor of protein synthesis). Epoxide hydrolase (#49) is induced by both high-dose ibuprofen and Aroclor but not by the other agents (most of the effects of ibuprofen in this experiment have turned out to be identical to those of the peroxisome proliferator, clofibrate).

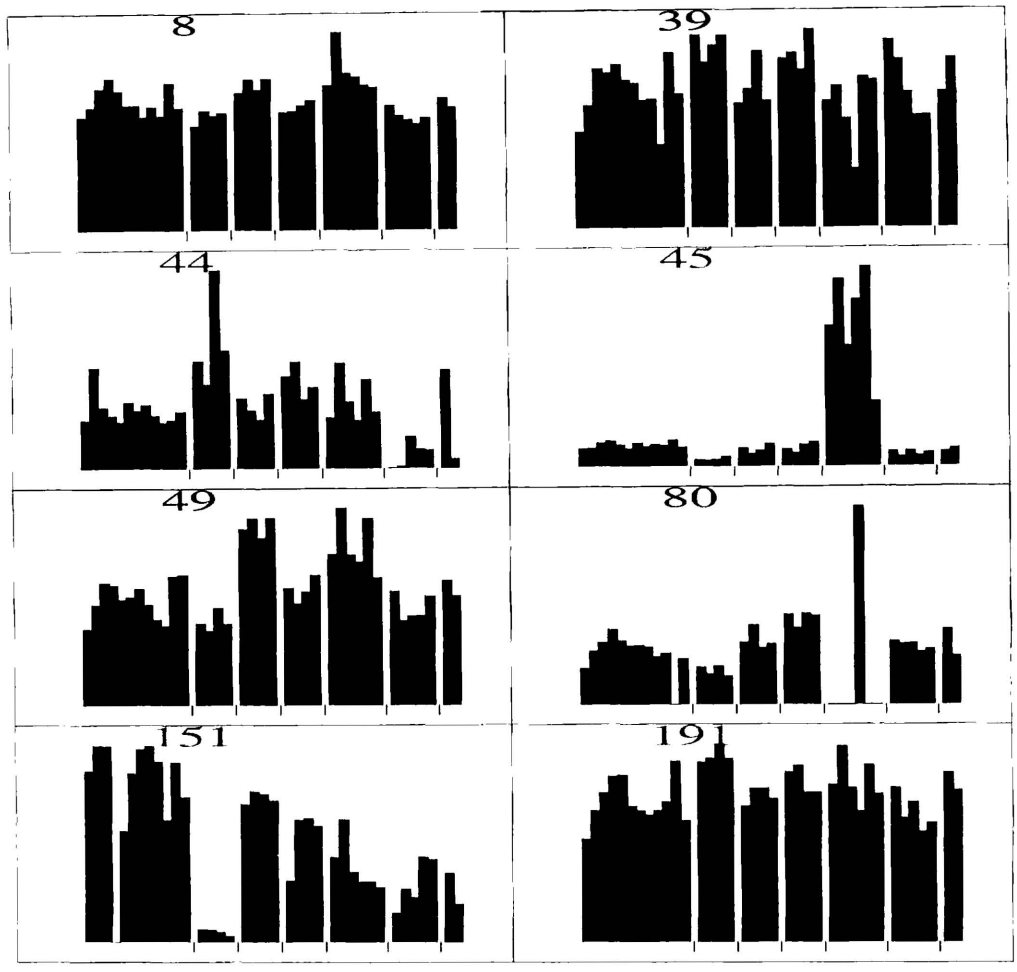

Figure 4. Bar graphs showing abundance of eight interesting proteins in mice of different treatment groups. Eight proteins were selected: a major mitochondrial matrix polypeptide (#8), β actin (#39), proalbumin (#44, the precursor form synthesized by the liver), an unidentified protein induced strongly by Aroclor 1254 (#45), cytoplasmic epoxide hydrolase (#49), cytochrome b_5 (#80), ornithine aminotransferase (#151), and tropomyosin (#191). For each protein, the bars show the abundance in each animal's 2-D pattern, with gaps separating the experimental groups (group 1 is the control group, group 2 was treated with carbon tetrachloride, group 3 with ibuprofen, group 4 with phenobarbital, group 5 with Aroclor 1254, and group 6 with cycloheximide).

Other examples

Numerous experiments in a range of in vitro systems have examined the effects of treatments including steroid hormones (12), heat shock (13), 5-azacytidine (14), interferon (15), phorbol ester tumor promoters (16), and antimitochondrial antibiotics (17). In vivo studies have examined various psychoactive agents (18), inducers of drug metabolism (9), peroxisome proliferators (19), decalin (20), and alcohol (21). Taken together, these and other similar results appear to support the hypothesis that many, if not most, pathologic phenomena are accompanied by (and may be due to) changes in the protein composition of target tissues.

IDENTIFICATION OF PROTEINS AS TARGETS FOR DRUG DEVELOPMENT

It is worth considering the potential impact of comprehensive protein information on drug discovery. In general, if a given manipulation of the cell results in the induction of certain proteins, then there is a reasonable chance that these proteins are involved in compensating for the effects of that manipulation. This is true because of the tendency of the cellular control systems to maintain metabolic homeostasis. Hence, if a compound inhibits an important enzyme, the resulting buildup of the enzyme's substrates and depletion of its products may induce the cell to produce added quantities of the enzyme. Often a series of enzymes organized as a pathway will be affected together. Such an effect can be exploited using the 2-D approach as a means to identify, through the simple fact of their induction, a series of proteins that may be useful candidates as targets for further drug development.

An interesting example of the power of this approach can be found in the metabolic pathway synthesizing cholesterol in the liver. Control of this pathway is an important goal of drug development because of the limited success of altered diet and bile-acid sequestrants in reducing blood cholesterol in many high-cholesterol patients. HMG-CoA reductase, thought to be the rate-limiting enzyme in the pathway, can be inhibited by the drug, mevinolin (lovastatin), resulting in a decrease in liver

cholesterol biosynthesis. Treatment with this drug also causes an induction of the reductase and the preceding enzyme in the pathway (HMG-CoA synthase).

When this system is examined using the 2-D approach, a series of more than 10 proteins is found to be induced by mevinolin treatment (unpublished results), not counting the reductase (which is not in the pH range covered by typical 2-D gels) or the synthase (some of whose subunits are identified in the rat liver pattern). These spots are likely to represent other enzymes of the same or related pathways and, hence, identify potential additional enzyme targets for development of cholesterol-lowering agents. Such alternatives may be particularly attractive because the reductase and synthase are both strongly regulated and, hence, capable of being rapidly increased in response to treatment (the synthase subunits are among the proteins that vary most in abundance between supposedly identical animals). This behavior means that enzyme regulation in the liver may be largely capable of overcoming the presence of the drug. Alternative enzymes in this pathway that were not capable of rapid compensation might allow for increased leverage by an equally effective specific inhibitor.

RELATIONSHIP OF IN VITRO AND IN VIVO TOXICOLOGY TEST SYSTEMS

Substantial effort has been devoted to the development of numerous in vitro test systems for toxicology, including hepatocyte systems derived from a number of species. These systems may ultimately substitute for a portion of the current rodent studies if it can be demonstrated that they are in some useful sense equivalent to the appropriate tissue in vivo. How can this equivalence be demonstrated? One approach is to establish that the in vitro and in vivo systems give a similar simple response (e.g., cell death) to an extensive panel of compounds including numerous positive, as well as negative, controls. Potential disadvantages of this approach are that such a correspondence can be upset by a few marked differences in response, and that it is purely statistical rather than mechanistic.

A second approach is to try to develop an in vitro cellular system that *1*) shows a close similarity to the protein composition of the appropriate tissue in vivo, and *2*) displays protein changes with reference compounds that are equivalent to the same compounds' effects in vivo. This method of validation would be more likely to produce an in vitro system that functions in a manner analogous to that of the corresponding animal tissue. Its disadvantage is that, given the ability to compare isolated cells and the corresponding tissues, we may find that they are much more different than we would like. Such is probably the case with many hepatocyte systems (particularly human) where it is known that many of the differentiated functions, including expression of the enzymes of drug metabolism, are rapidly diminished on removal of cells from intact tissue. Nevertheless, the 2-D approach may serve as the means by which culture conditions can be systematically optimized to achieve such an in vitro-in vivo correspondence. Experiments to examine this possibility are under way.

POTENTIAL RELATIONSHIPS OF PROTEIN CHANGES TO NONGENOTOXIC CARCINOGENESIS

While it is relatively clear that genotoxic carcinogens induce formation of tumors through modification of DNA, it is much less clear how the so-called "nongenotoxic" carcinogens produce such an effect. Several lines of evidence suggest, however, that irreversible changes in gene expression may be involved; changes that might be visible in the pattern of proteins. Newbold (22) studied the effect of prenatal diethylstilbestrol exposure on the protein pattern of reproductive tract tissues in the CD-1 mouse and found a correlation between histologic changes and diminished expression of a specific 70 kDa protein. This protein was tentatively identified as a variant mitochondrial protein through comparison with LSB's mouse database; however, the database also indicated that the same mitochondrial protein shows genetic polymorphism in the CD-1 that could confound the DES-related changes. Hence, this intriguing result remains to be fully confirmed.

CONCLUSIONS

Technology has been developed over the past 15 years that makes it possible to obtain a fairly comprehensive picture of the protein composition of biological samples. Application of this approach in toxicology, begun over the last 5 years, has begun to generate data of substantial interest. Several specific uses appear particularly promising: measurement of enzyme induction, detection of protein-bound drug metabolites, and exploration of cellular metabolic pathways associated with specific therapeutic effects. As more proteins are identified in the rodent liver 2-D patterns, it will be possible to assess the induction of multiple enzymes associated with drug metabolism in a single analysis. Products of the binding of reactive drug metabolites to proteins can be detected in the 2-D pattern by virtue of the effect the metabolite moiety has on the protein's properties (and, hence, position on the 2-D map). Characterization of the proteins modified can yield important information regarding the site of reactive metabolite formation. Identification of proteins induced by compounds of a therapeutic class targeted at some aspect of liver metabolism can assist in identifying additional proteins of the target pathway and, hence, assist in development of additional drug leads.

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