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Bioassays – a continuously developing field

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The growth in the development of biological therapeutics has resulted in the increasing importance of bioassays to the pharmaceutical industry. The complexity of biological molecules means that physicochemical techniques alone are usually insufficient to characterize a preparation adequately to permit prediction of its potency. For this, measurement of a specific functional response induced in a biological system – a bioassay – is required. The bioassay is thus an essential tool for research and development, and is normally required by regulatory authorities in setting product specifications. At IBC's recent symposium 'Biological Assay Development and Validation (5-7 May 2003, Bethesda, MD, USA) new developments in assay techniques were discussed, along with appropriate methods of statistical analysis and current regulatory requirements.

Gerald Feldman [Center for Biologics Evaluation and Research (CBER), FDA; http://www.fda.gov/cber] and Tony Mire-Sluis [Center for Drug Evaluation and Research (CDER), FDA; http://www.fda.gov/cder] emphasized the importance of the progressive development and characterization of the bioassay during the product development process, culminating in a fully validated assay by the time of submission of the license application. Therefore, even during the early stages of bioassay development, it is important to consider the features that will ultimately need to be validated, such as the specificity, accuracy, precision and robustness.

Bioassays based on the *in vitro* responses of stable cell lines are commonly the assay systems of choice; they can provide a biologically relevant mechanism of action to test the product and are less variable, expensive and labor-intensive than *in vivo* assays. Originally, responses such as cell proliferation or secretion of a specific protein (i.e. 'late-stage' or 'end' responses) were the only measurable outcomes, but with increasing knowledge of signal transduction pathways, bioassays based on early signaling events are becoming widely used. Another development has been the creation of responsive cell lines by insertion of an appropriate exogenous receptor into a non-responsive line. Similarly, in 'reporter gene assays', conveniently measured artificial responses can be engineered by insertion of appropriate genes.

Early signaling events

Thomas Millward (Novartis Pharma AG; http://www.novartis.com) presented the development of assays for chemokines based on the phosphorylation of a signal pathway component using CHO (Chinese hamster ovary) cells transfected with the appropriate chemokine receptors. Chemokines are multipotent cytokines that enhance inflammation by inducing chemotaxis in various cell types, and inhibitors of chemokines have potential as anti-inflammatory drugs. Current assays for chemokine activity involve measuring either the migration of cells, which is low throughput, difficult to automate, highly variable and often has a low signal-to-noise ratio, or changes in intracellular calcium levels, which requires specialized equipment.

The signaling pathway of many chemokines involves the phosphorylation of the p44/42 mitogen-activated protein kinase (MAPK), and there are commercially available monoclonal antibodies (Mab) that recognize the phosphorylated MAPK. To measure chemokine and chemokine inhibitor activity, cells grown in a 96-well culture plate are treated with chemokine and various concentrations of potential inhibitors. After fixation and permeabilization, they are incubated with the anti-phospho-MAPK Mab. Following standard ELISA techniques, a secondary antibody linked to peroxidase is added, followed by tetramethylbenzidine (TMB), and phospho-MAPK concentrations are measured colorimetrically.

This assay has the advantage that it can be adapted to a number of different chemokines by inserting the appropriate receptor into the CHO cell line. Furthermore, it can be adapted to a different phosphorylated signaling pathway component by using another primary antibody such as anti-phospho-JNK or anti-phospho-JAK.

Automation

In the initial stages of development, most bioassays are performed manually. Later, automation of part, or all,

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of the process can increase sample throughput and reduce labor, cost and assay variability. Hersh Mehta (Medimmune Vaccines; http://www.medimmune.com) reported on the results of automating part of a potency assay for live, attenuated influenza virus vaccine with the aim of increasing sample throughput in order to support long-term manufacturing needs. The assay is based on the cytocidal effect of infectious virions on MDCK (Madin-Darby Canine Kidney) cells grown in 96-well plates. The potency of the vaccine is determined by measuring the dilution that is required to infect 50% of the inoculated wells, known as the median tissue culture infectious dose ($TCID_{50}$). In the semi-automated version of the assay, pipetting, sample dilution and plate washing steps are automated. The manual microscopic determination of the cell viability of each well is replaced by a spectrophotometric reading. Here, MTT dye is reduced by active viable cells to formazan, resulting in a color change from yellow to blue, and this absorbance change is used to distinguish infected from uninfected wells. Potency is calculated by automated processing of the spectrophotometric data. After optimization of parameters in the MTT methodology and in the MDCK cell culture, validation of the semi-automated method included the testing of different influenza vaccines and wild-type strains and lab to lab reproducibility.

If changes are made to a bioassay that is used for the release of an approved product, or in product development to provide data in support of a license application, it is necessary to demonstrate comparability of the results obtained by the two methods. The discussion highlighted the point that, for this reason, it might be preferable to retain some procedures used in the original method even when the new method offers alternatives with possible advantages.

Binding versus bioassays

The complexity of bioassay systems means that they are subject to many sources of variability. In cell-based assays, for example, the responses of cell cultures vary with age and culture history. Assays that measure the binding (i.e. ligand–receptor, antigen–antibody) tend to be less variable, but there is the problem that binding does not necessarily correspond to potency. Mark Schenerman (Medimmune; http://www.medimmune.com) described the development and implementation of a potency assay that is based on the binding of the product Synagis® to immobilized ligand. This required an understanding of the mechanism of action of the product and comparison of data from the binding assay with those from functional bioassays.

Synagis® is a humanized monoclonal antibody used for the passive immunotherapy of RSV (respiratory syncytial virus). Synagis® acts primarily through binding to the F protein on the surface of the virion. (The Fc region of the antibody and complement were shown to play no significant role.) For the binding assay, purified F protein is coated on 96-well plates to which Synagis® is then added. Unbound Synagis® is washed away, and the remainder, bound to the F protein, is measured by standard ELISA techniques, adding anti-human-HRP conjugate followed by peroxidase substrate. The 'F protein binding ELISA' was shown to be comparable to a functional, cell-based microneutralization bioassay for multiple lots of the product. Based on these results, together with animal and human pharmacokinetic data showing batch to batch consistency of the product, the F protein binding ELISA was established as the product potency assay. It was emphasized, however, that use of a binding assay to evaluate potency might not prove widely applicable to other products with more complex mechanisms of action, and also that Synagis® would continue to be monitored using animal models and bioassays as a characterization test.

Gene therapy products

Assessing the potency of gene therapy products is particularly challenging. Their mode of action comprises several steps, each of which involves a separate biological activity. Tong-Yuan Yang (Schering-Plough Corporation; http://www.spcorp.com) described the development of three separate biological assays to test the infectivity, expression and potency of an adenovirus type-5-based gene therapy vector, SCH 412499 (Adv-p21). This vector delivers the gene for human cyclin-dependent kinase inhibitor p21, an inhibitor of cell cycle progression, and is used to prevent fibroproliferation in post-surgical glaucoma patients. All three assays are based on fluorescence-activated cell sorting (FACS).

To assess infectivity, the concentration of adenovirus in cultured cells that have been incubated with the virus is measured using fluorescein-labeled α-adenovirus antibody. To assess the expression of the p21 kinase inhibitor, fluorescein-labeled antibody against p21 is used, measuring the amount (mass) of immunoreactive p21 produced. The potency of the expressed p21 is assessed from its activity in arresting the cell cycle. This is measured as the reduction in the number of cells in S-phase, by FACS analysis of propidium iodide stained cells. All three assays, assessing the potencies of the different biological actions of the product, are used to monitor product quality and are required for batch release testing.

Throughout the meeting, further interesting case studies illustrated the

advances in the methods and technology being applied in bioassays. The importance of appropriate statistical analysis of data was demonstrated in a number of presentations. It was emphasized that, for this, it is essential to ensure that the assay design permits the appropriate data to be obtained, and early consultation with a biostatistician can be helpful.

The symposium provided a lively forum for attendees with little previous experience in bioassays who learned about some of the common pitfalls to avoid in assay design and validation, as well as for experienced bioassayists with specific problems to share or resolve. The meetings are currently held annually in the USA and in Europe.

Clinical genomics comes of age

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The promised benefits of clinical genomics stretch from the earliest phase of drug discovery, to the diagnosis and treatment of individuals, and even to already-marketed medications. Clinical genomics investigators apply the large-scale study of genes, using actual clinical materials and associated clinical information, to identify and characterize the genes that are most relevant to the origins and progression of disease. This information can then be used to speed the design and development of new and improved therapeutics, identify novel biomarkers for earlier diagnosis, and select treatments that, based on the patient's genetic makeup, are most likely to succeed and cause the fewest adverse events for that individual. According to the speakers at the *2003 Clinical Genomics Symposium Series* (Princeton, USA; 13 June), clinical genomics and related bioinformatics have matured sufficiently for molecular study of human disease to speed up and improve success rates in discovery and development of novel compounds. At the same time, organizational changes have encouraged the dissemination and application of molecular knowledge to improve pharmaceutical productivity.

Targeting cancer by subtype

The subtyping of cancer, based on differential gene expression profiles, has begun to illuminate the molecular differences between tumours. These differences help to predict individual response to chemotherapy and other medications, and also the likely course of the disease – prognosis provides little solace without the possibility of treatment alternatives. Todd Golub, of the Whitehead Institute (http://www-genome.wi.mit.edu) and the Dana-Farber Cancer Institute (http://www.dfci.harvard.edu), has directed many studies to subtype cancer at the molecular level, to identify novel targets for drug discovery and screening and to improve diagnosis. He presented results showing several cases in which expression-profiling of tumour tissue has enabled rapid identification of molecular targets for screening of novel compounds that are moving rapidly into clinical trials.

Citing more traditional means of classifying hematologic cancers, and using TEL/AML1 translocations in certain childhood leukemias as one example, Golub then demonstrated that identification of such translocations can be predictive of disease outcome. He and his colleagues have extended such molecular–clinical classifications to include global gene expression, and believe that such classifications could also be used to improve diagnosis and treatment. In one example, the FLT3 tyrosine kinase gene was identified as overexpressed in certain leukemias (i.e. mixed lineage leukemia) that are now classifiable with RNA expression profiling. Like the recent success of Gleevec© in treating chronic myelogenous leukemia, in which a translocation event (Bcr-Abl) pointed to an overactive kinase whose inhibition proved to be a successful therapeutic strategy, it was hypothesized that inhibition of FLT3 might be a viable therapy. Subsequent studies of human MLL cells grafted into mice, showed that an available small molecule inhibitor of FLT3 causes regression of the tumour. The stage is now set for testing treatment of MLL patients with a small molecule inhibitor of FLT3: Significant clinical potential with very recent origins in a clinical genomics approach.

Golub described what is now termed a 'Global Cancer Map', showing that the gene-expression profiles of a broad

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