Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 2, pp. 131–138, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00
Pergamon Press plc

An overview of the strengths and limitations of biological assays in quality control*

ELAINE C. ESBER

Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20892, USA

Abstract: Biological assays have been an integral part of the regulation of biological products in the United States since licensing of the first product in 1902. Bioassays have been used along with other methodologies in the assessment of identity, purity, safety, potency and stability. The technology of production, i.e. normal or recombinant, has not altered the use or value of these assays. Although many scientists and regulators would prefer to replace all *in vivo* bioassays with other assays, there is a reluctance to agree that this will be possible, at least in the near future. Scientific efforts are continuing to be focussed on the development of more precise, reliable and less cumbersome assay methods.

This paper presents an overview of the strengths and limitations of bioassays as used in quality control, followed by a discussion of specific biological products as models for these principles.

Keywords: *Quality control; biological assays; standardization; tetanus toxoid; viral vaccines; polysaccharide vaccines; allergenic extracts; factor VIII; tissue plasminogen activator.*

Bioassays in quality control

The stages involved in the process of biological standardization are well recognized [1]. Biological compounds are generally first identified by means of some type of bioassay in which one or more biological effects are recognized and measured, and a suitable unit defined. Very frequently, this activity remains important in assays that are subsequently refined. Commonly, the next step is to establish and characterize a reference standard by means of a collaborative study. In addition, various assay methods may be available and will need to be evaluated to determine the comparability of the response. Isolation and synthesis of the active component is a necessary step not only to improve the understanding of the activity, but to reach the eventual goal, if possible, of chemical measurement.

The *in vivo* bioassay, and to a limited extent the *in vitro* bioassay, are measuring the end result of a series of unknown or incompletely understood events occurring in the host

*Presented at the Symposium on "Biomolecules — Analytical Options", May 1988, Sollentuna, Sweden.

in response to a stimulus. Because they are measuring a summed response, they are also the most complex of measurement systems, and require the molecule to be structurally correct, maintaining the functionally active sites.

There are three basic components of biological assays: (1) the dose, amount or strength of a preparation under test which acts as a stimulus; (2) the biological subject (animal, human tissue, cell culture); and (3) the estimation of the nature or magnitude of the response produced, usually a change in a particular characteristic (or even death) of a host.

Statistical methods needed to analyse data resulting from biological assays have been developed to enable the assessment of the validity of the comparison of two or more preparations in a bioassay, and the estimation of the relative values or potencies, with some indication of the precision of each estimate, i.e. the dose–response relationship. Bioassays are commonly influenced by factors other than the preparations under test; these factors cannot be completely controlled.

Therefore, selection of an appropriate assay depends to a considerable extent on the information to be generated and the properties of the product to be monitored. All assay techniques have differing strengths and limitations. Different assays may quantify different portions of the response or, in fact, different forms or portions of the biomolecule. Each assay may provide different information, as well as different answers, and all may be intrinsically correct.

Bioassays have been used extensively in the regulatory control of biological products [2]. For example, bioassays are used in characterizing the cell substrate used in vaccine production or a cell line used to construct a recombinant clone. Bioassays can provide information on tumorigenic potential as well as establishing the product's freedom from adventitious agents and pyrogenic substances. Measurement of a biological effect enables standardization and the assignment of units, an assessment of stability over time, and an evaluation of identity with a concern towards the development of mutations or interfering substances.

In addition to being the most complex of measurement systems, *in vivo* bioassays may be affected by extrinsic and intrinsic factors [3]. Extrinsic factors including the age, sex, diet, health and nutritional status of the animals, as well as the conditions or methods of animal handling, can influence the results of the *in vivo* bioassay. Selection of particular animal species or strains may well result in differences in susceptibility, sensitivity, metabolism or degradation rates and volumes of distribution. Some of the differences observed may reflect the presence or absence in the animal of the relevant receptors. The *in vivo* bioassay is frequently the most cumbersome, tedious and expensive, and requires the longest time for completion of the various assay systems. In some situations, it may also be the least sensitive and that which gives the greatest inter- and intra-assay variability. The presence of endogenous viruses may, unknowingly, influence the results. And of course, the ethics of using animals for experimental purposes must be seriously considered.

Even with the recognition of these apparent "disadvantages", bioassays provide many advantages in quality control. Only tests *in vivo* can reveal the integrated biological effects of a product when the measured response is relevant to the intended action in clinical use; i.e. it may actually reflect the biological activity in man, or at least serve as a useful index of correlation. Bioassays may be very sensitive and accurate. Functional activity may provide a meaningful relationship between products, even though the structure and activity may vary. The bioassay allows for a simultaneous evaluation of

combination products and is not affected by substances that may interfere with other assays, e.g. preservatives.

Several examples can be provided to illustrate the strengths and limitations of bioassays in the regulatory control of specific products.

Tetanus toxoid

Using tetanus toxoid as a model, it is possible to present some of the complexities, difficulties and evolving issues associated with bioassay systems. The techniques established for quality control of tetanus toxoid approx. 30–40 years ago have provided a safe and effective vaccine over the years. Although there is a desire and need to adopt more current analytical techniques, as will be discussed, this change will require more information than is currently available.

Tetanus toxoid is produced from strains of *Clostridium tetani*. The organism is grown, inactivated by toxoiding, the toxoid is purified, diluted, adjuvanted and filled. Controls for identification, purity and antigen content of the toxin, as well as for contaminating blood group antigens from the growth media, employ a combination of bioassays and other analytical methods [4].

The most critical of the control tests are those demonstrating the inactivation of the highly potent tetanus toxin. Incomplete detoxification can and has occurred, with disastrous results. To date, animals have been the only suitable, sensitive model identified for controlling the adequacy of inactivation or toxoiding of the toxin. Likewise, validation of the production process and testing to assure that the toxoid is stable and that reversion to the toxin has not occurred, requires equally time tested and suitable assays.

Monoclonal antibodies can be made against many domains of the toxin [5]. Some have suggested that monoclonal antibodies might be used in quality control to specifically distinguish untoxoided tetanus toxin from toxoided tetanus toxin. Although both neutralizing and non-neutralizing monoclonal antibodies have been made against different domains of the tetanus molecule, the adequacy of monoclonal antibodies to detect only toxoided toxin has not been widely accepted. Work continues in an attempt to identify such antibodies. Dr M. C. Hardegree and her colleagues in our Division of Bacterial Products have concluded that since no *in vitro* assay, including a cell culture system, exists for tetanus toxin, animal tests will be required for some time.

In the assessment of other aspects of the protein, analytical tools are widely used. For example, HPLC has been used experimentally to evaluate the composition of different lots of tetanus toxoid [6]. High-performance liquid chromatography can be used not only to evaluate toxoid purity, but also to evaluate the final product, preservatives and other components.

With all the capabilities afforded by techniques for protein analysis and immunologic assessment, including HPLC, *in vitro* antigen–antibody reactions such as Lf determination, ELISA, PAGE and Western Blotting, however, potency determination of the tetanus antigen remains very problematical and unsettled after approx. 40 years. The potency assays for tetanus are very old assays.

In the United States, for tetanus toxoid adsorbed, a group of at least four guinea pigs are immunized with the toxoid, and a pool of serum collected 4–6 weeks after injection must contain at least 2 U ml^{-1} of tetanus antitoxin [3, 4]. Over the years, different countries have adopted different methods, different animal strains, etc. The test methods

are cumbersome, require large numbers of animals, and measure different endpoints as well.

Furthermore, a few years ago, during testing of the first and second International Reference Standards in guinea pigs or mice, the references were found to give different unitage [7]. Earlier, others had also found that variation in unitage was also observed when different mouse strains were used for testing [8]. In addition, Dr Hardegree and others have found that the ratio of potency, as determined in mice and guinea pigs, was product-dependent. These findings led her to ask the question, What is being tested in these assays? The animal? The toxoid? If the two standards varied so much in their response, how could toxoids made by a variety of methods be compared?

This leads to the question, What are we expecting potency assays to do? In the case of tetanus toxoids, there are few data to permit the correlation of the potency level in a biological assay with protection in man. However, Breman *et al.* have presented data in which a toxoid of known unitage induced varying levels of tetanus antitoxin depending on the age of the subject immunized [9].

International discussions are ongoing with the intent of refining and standardizing methodology in this area. In addition, there remains a reluctance to substitute *in vitro* methods for evaluation of the antibody response to toxoids, such as ELISA, RIA or haemagglutination, for a functional bioassay such as a toxin neutralization assay in the absence of data which validate the ability of the presence of such antibodies to provide protection in man.

Cell lines

Bioassays both *in vivo* and *in vitro* have been used extensively in the regulatory control of viral vaccines and cell lines [1]. For example, *in vivo* bioassays are used for the detection of adventitious agents, for identity tests of the vaccine virus, for monitoring the stability of viral attenuation in the case of live vaccines, or for detecting residual live virus in the case of inactivated vaccines.

Many vaccines continue to be made in primary cell cultures obtained from animals which may carry a number of endogenous infectious agents. Likewise, newer experimental vaccines, including those using viral vectors, and new cell constructs such as hybridomas and recombinant clones are made by using cell lines. Bioassay systems continue to be employed along with other methods for assessing the suitability of the cell line and the freedom from adventitious agents.

Viral vaccines — potency

In vitro bioassays have been developed for determining the potency of live viral vaccines. These measure infectivity, not antigenicity, per human dose. The dose–response relationship, defining doses inducing seroconversion, has been established in human trials, wherein the infective dose required to give $\geq 95\%$ seroconversion is quantitated.

Potency may be expressed in terms of infectious units/dose or in terms of the ratio of vaccine/reference standard $TCID_{50}$. In either case, as with all bioassays, the assay method must be validated, and the intra-test variability (precision) must be determined by use of an appropriate reference preparation.

Unlike the potency determinations of a live viral vaccine, which measure infectious titre, potency of inactivated vaccines may be able to be adequately determined by quantitating a specific antigen, i.e. one that gives rise to protective neutralizing

antibodies. For example, the potency of rabies and inactivated poliovirus vaccines is determined by measuring the response of animals immunized with the vaccine, either on the basis of their antibody response or by observing protection against a live viral challenge. Considerable progress has been achieved in developing appropriate assays to measure specific antigen content *in vitro*, e.g. by SRID and ELISA. It is critical that such *in vitro* assays quantitate the specific antigenic epitopes that are responsible for induction of protective immune responses. This specificity may be accomplished in some cases by using monoclonal antibodies or appropriately absorbed polyclonal antibodies induced with highly purified antigens.

The transition from *in vivo* to *in vitro* potency assays is well underway for both rabies and inactivated poliovirus vaccines. For polio, a recent international collaborative study has compared the standard primate test for poliovirus, as evaluated by measuring seroconversion, to (1) a potency test in rats, (2) antigen quantitation by ELISA, and (3) an antigen competition assay [10].

Of course, the successful development of a potency bioassay for an inactivated vaccine will not remove the necessity of a sensitive assay for detection of residual live virus.

The development of an appropriate bioassay for potency measurement of the experimental human immunodeficiency virus (HIV) vaccines to prevent acquired immunodeficiency (AIDs) has been hampered by the lack of suitable animal model systems. Additional research will be necessary to provide appropriate assays for this virus.

Polysaccharide vaccines

Bioassays are not always available. In these circumstances, alternate assays must be developed and validated to the extent possible. For example, the potency determination we have used for regulating the 23 valent pneumococcal polysaccharide vaccine, the meningococcal polysaccharide vaccine containing serotypes A, C, Y, and W135, the haemophilus *b* polysaccharide vaccine and the haemophilus *b* conjugate vaccine (diphtheria toxoid-conjugate) is molecular size of the polysaccharides. In these circumstances, the molecular weight was shown to correlate with antibody production in humans and in some cases with protection. This approach was accepted because the polysaccharide vaccines were reasonably pure and could be subjected to quantitation by size exclusion chromatography. Many regulatory control agencies, while desirous to achieve this goal eventually for all products, would prefer to validate the potency determination by comparison to suitable bioassays.

Allergenic extracts

Bioassays have been shown to be of value in defining "units" of allergenic activity. For years, potency determination of allergenic extracts for diagnosis of immunotherapy were based on protein content, a measurement which had little reflection on the allergen content of the material in the product. Subsequently, *in vitro* assays were developed which defined the amount of specific allergen present. Although such assays are valuable, they need to be correlated with a "potency" unit that will reflect the human response to an allergen.

A skin test procedure for quantifying "allergy units" has been developed [11]. Multiple dilutions of a product are applied by a skin test procedure designed to calibrate the allergenic extract in allergenic units. The human response obtained in the skin test is believed to be related to allergenic activity, i.e. the interaction of the allergen with IgE

and release of various mediators resulting in erythema. The quantitative skin test, parallel line bioassay, has been shown to have a good correlation with relative potency as measured *in vitro* for a number of allergens including short ragweed, rye, Cat 1 extract and others [12]. Our scientists consider that once a reference has been calibrated by skin testing in allergy units, it is then appropriate to use in *in vitro* assays of that reference for testing and for quality control release of other allergenic extracts containing that allergen.

Hormones — potency

The potency of hormones has been controlled by *in vivo* bioassays until recently. These bioassays have been refined and subsequently replaced for some products by other assays such as HPLC, once validated. Remarkable progress has been made in this area, as presented elsewhere in this symposium.

Large glycoproteins

Assays such as physicochemical quantitation or immunoassays, although valuable in the in-process control and characterization of large glycoproteins, cannot be expected to replace functional assays for some time. Functional assays allow a comparison of the biological properties of products which are structurally similar but may differ in biological activity. This is relevant when comparisons are made between products or in the quality control of a product. For example, in stability studies, interferon gamma will lose its antiviral activity while retaining antigenic epitopes recognized in immunoassays.

Furthermore, the structure of the glycoprotein will vary with the expression vector and this may well affect activity. For example, while interleukin-2 is biologically active in both glycosylated and non-glycosylated forms, erythropoietin is not active unless glycosylated. Different cell substrates used in production will add carbohydrate molecules of varying composition in different ways because of enzyme differences. In between these extremes, there are, most likely, many subtle variations in structure, resulting in products that may well vary in properties such as tissue distribution, half-life and survival, etc.

Glycoproteins are especially difficult to analyse structurally. The primary structure of the protein portion of the molecule can be determined with a reasonable degree of accuracy. However, the assembly as well as the composition of the carbohydrate molecules is complex.

Carbohydrate chains of glycoproteins have many potential effects on the biological activity of the molecule. These include: (1) antigen recognition with cellular and subcellular uptake, (2) intracellular translocation, (3) receptor function, (4) secretion, (5) protein stability, (6) cell adhesion, (7) contact-dependent inhibition of growth, and (8) antigenicity.

At this time, it is not possible to assume that characterization by physicochemical or immunologic assays will assure the functional activity of a product.

Factor VIII

Factor VIII is a large glycoprotein of 2351 amino acids that has recently been manufactured using recombinant DNA technology [13]. Coagulation experts have been considering for some time the methods by which it might be quality controlled. For years, Factor VIII concentrates, used for treating patients with haemophilia A, have been made from human plasma. The control and standardization of Factor VIII

concentrates derived from plasma have been heavily dependent upon biological assays, and the general principles of biological standardization [14].

The bioassays available for Factor VIII are *in vitro* assays and include the one-stage partial thromboplastin time (PTT), and the two-stage thromboplastin generation test (TGT). These methods have enabled, with some accuracy, the identification and standardization of a unit that allows a one-to-one correlation with the clinical therapeutic effect, as well as allowing comparison of activity between manufacturers' products. Factor VIII concentrates, until recently, have been less pure with specific activities ranging from 0.3 to 5 IU mg^{-1} protein. With the use of affinity chromatography, recent preparations have specific activities as high as 3500 IU mg^{-1}.

Although these *in vitro* bioassays have considerably enhanced the development of therapeutic fractions, coagulation experts caution that there must always be concern about the specificity of coagulation tests and the assumption that the therapeutic benefit of Factor VIII is correlated with its *in vitro* potency.

The road has not been uncomplicated. Although there has been concern that references and assays may be measuring different properties, it must be noted that the development of *in vitro* bioassays, reflecting clotting activity and the complex series of events involved in clot formation, has been an invaluable substitution for the original whole blood clotting times. These original assays were performed by puncturing haemophilic patients and observing the time required for clot formation.

The new technology has not solved all of the problems. Even recombinant DNA derived Factor VIII is a heterogeneous mixture of structurally related molecules which can be processed to yield a biologically active product [15]. Whether the *in vitro* bioassays and the currently available international reference standard will be acceptable for assessing recombinant Factor VIII activity is unknown. Many believe that they will provide a reasonable means to quality control these products.

Whether physicochemical assays will eventually be able to replace functional assays of Factor VIII is unknown. It seems more likely that these techniques will allow further characterization and quality control of this glycoprotein.

Tissue plasminogen activator (tPA)

Unlike Factor VIII, which induces clot formation by participating in an enzyme cascade, tPA induces clot lysis by the conversion of plasminogen to plasmin in the presence of fibrin.

Tissue plasminogen activator is a serine protease with a molecular weight of 65,000. The amino acid sequence has been determined; the protein is 527 amino acids in length [16]. Multiple forms exist which have biological activity and are generated through specific cleavage mechanisms during production. The two-chain tPA consists of the A chain (heavy chain), which contains the fibrin-binding site, and the B chain (light chain), which contains the protease region.

As with many of the larger complex glycoprotein molecules, the complexity of the native structure as well as the multiple domains required for full activity (fibrin-binding site, protease active site) argue for close monitoring with biochemical and functional assays to ensure product consistency, potency and safety.

Conclusions

In summary, whether discussing a vaccine or a therapeutic agent intended for

administration in physiologic or pharmacologic doses, the selection of an assay for quality control must be carefully considered. Various assay methods have differing strengths and limitations. In selecting an assay for quality control, we must ask what we are expecting the assay to do.

The principles of biological assays and biological standardization are well accepted. All potential changes, i.e. substitutions of binding assays for functional assays, or improvements in assays must be carefully analysed before being introduced into routine use.

Physicochemical assays may enable a reduction in the extent of *in vivo* bioassays performed, but it appears unlikely that they will serve to replace *in vivo* bioassays in the foreseeable future for the majority of biological products.

References

[1] D. P. Thomas, *Devl. Biol. Stand.* **44**, 179–183 (1979).
[2] U.S. Code of Federal Regulations, Title 21, Part 600. U.S. Government Printing Office, Washington (1988).
[3] E. A. Fitzgerald, M. C. Hardegree and C. R. Manclark, in *The Mouse in Biomedical Research,* Vol. IV, *Experimental Biology and Oncology* (H. L. Foster, J. D. Small and J. G. Fox, Eds), pp. 1–10. Academic Press, New York (1982).
[4] U.S. Department of Health, Education and Welfare, *Minimum Requirements: Tetanus Toxoid*, 4th revision, Appendix A. National Institutes of Health, Bethesda (1952).
[5] J. G. Kenimer, W. H. Habig and M. C. Hardegree, *Infect. Immun.* **42**, 942–948 (1983).
[6] M. C. Hardegree, personal communication.
[7] J. Lung and G. Nyerges, *J. Biol. Stand.* **12**, 121–130 (1984).
[8] M. C. Hardegree, M. Pittman and C. J. Maloney, *Appl. Microbiol.* **24**, 120–126 (1972).
[9] J. G. Breman, G. G. Wright, L. Levine, W. C. Latham and K. P. Compaore, *Bull. WHO* **59**, 745–752 (1981).
[10] Report of the WHO Consultative Group on Poliomyelitis Vaccines, BLG/Polio/87.1. Geneva (1987).
[11] P. C. Turkeltaub, in *Allergy, Principles and Practice* (E. Middleton Jr, C. E. Reed, E. F. Ellis *et al.*, Eds), pp. 388–401. Mosby, St. Louis (1988).
[12] P. C. Turkeltaub, in *Proceedings of the Fifth International Paul Ehrlich Seminar on the Regulatory Control and Standardization of Allergenic Extracts*, Arb. Paul Ehrlich Inst. (1987) (in press).
[13] W. I. Wood, D. J. Capon, C. S. Simonsen, D. L. Eaton, J. Gitschier, B. Keyt, P. H. Seeburg, D. H. Smith, P. Hollingshead, K. L. Wion, E. Delwart, E. G. D. Tuddenham, G. A. Vehar and R. M. Lawn, *Nature* **312**, 330–336 (1984).
[14] D. L. Aronson and D. P. Thomas, *Scand. J. Haematol. Suppl.* **41(33)**, 71–78 (1984).
[15] D. L. Eaton, P. E. Hass, L. Riddle, J. Mather, M. Wiebe, T. Gregory and G. A. Vahar, *J. Biol. Chem.* **262**, 3285–3290 (1987).
[16] D. Pennica, W. E. Holmes, W. J. Kohr, R. N. Harkins, G. A. Vehar, C. A. Ward, W. F. Bennett, E. Yelverton, P. H. Seeburg, H. L. Heyneker and D. V. Goeddel, *Nature* **301**, 214–221 (1983).

[*Received for review 1 July 1988*]