Alternatives to Animal Bioassays for Prions

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Combined PMCA and cell assays are being optimised for use as an effective, or even superior, alternative to quantitative prion bioassays in laboratory rodents

Prions and prion diseases

Bioassays that involve the use of laboratory animals are often considered to be the ‘gold standard’ for the detection and titration of certain pathogens. This holds particularly true for prions,\(^1,^2\) which are the proteinaceous infectious particles that cause fatal neurodegenerative diseases in animals (e.g. scrapie, bovine spongiform encephalopathy [BSE]) and humans (e.g. sporadic or variant Creutzfeldt-Jakob disease [sCJD, vCJD]). The BSE epidemic and resulting emergence of vCJD in the United Kingdom, and the subsequent occurrence of BSE and vCJD in other countries, have substantially increased the worldwide awareness of prions and their potential risk to public health.

Unlike bacteria, viruses or fungi, prions are pathogens that are devoid of coding nucleic acids. According to the prion hypothesis, they consist essentially of a misfolded and aggregated isoform of the host-encoded prion protein (PrP).\(^1\) Prion-forming conformers of the prion protein are referred to as PrP\(_{\text{Sc}}\) or PrP\(_{\text{TSE}}\) (‘Sc’ and ‘TSE’ are acronyms for scrapie, and transmissible spongiform encephalopathy, respectively, with the latter being an alternative name for prion diseases).\(^1,^3\) The replication of prions shows close similarities to the seeded growth of crystals, and is thought to occur by a mechanism of nucleation-dependent PrP polymerisation,\(^4,^5\) i.e. oligomers or polymers of PrP\(_{\text{TSE}}\) act as nuclei (‘seeds’) that recruit cellular prion protein (PrP\(_{\text{C}}\)) and incorporate it, in a misfolded form, into their own aggregate structure. When PrP\(_{\text{TSE}}\) aggregates eventually fall apart into smaller units, this causes a multiplication of PrP particles with proteinaceous seeding activity, and thereby a further autocatalytic replication of the pathological protein state. According to this concept, the self-replication of prions is based on their biochemical seeding activity, i.e. the ability to convert cellular protease-sensitive prion protein into misfolded, aggregated and often Proteinase K (PK)-resistant PrP (PrP\(_{\text{PrPres}}\)).

Prion bioassays and the Three Rs

For a long period of time, the infectivity of prions could be quantified only by incubation time interval assays or endpoint titrations in animals.\(^6\) Both methods rely on the transmission, often by intracerebral inoculation, of a prion infection that causes cerebral propagation of PrP\(_{\text{TSE}}\) and, eventually, neurological disease in the host animal. Mice or Syrian golden hamsters have been the most frequently used test animals in such prion bioassays. Quantitative prion bioassays in small rodents or other laboratory animals usually determine prion titres in terms of the median infective or lethal dose (ID\(_{50}\), LD\(_{50}\)) that had been present in the inoculated sample material. One prion ID\(_{50}\) or LD\(_{50}\) is the dose of prions that causes infection (in terms of PrP\(_{\text{TSE}}\) propagation or symptoms) or fatal disease, respectively, in 50% of the inoculated animals.

According to the concept of the Three Rs, as proposed by Russell and Burch,\(^7\) the use of animals in experiments should be replaced by alternative methods whenever possible (replacement), and the number of animals should be reduced to a minimum through good experimental design (reduction). Furthermore, discomfort and stress in any animals used should be minimised (refinement). To comply with these requirements in the field of prion research, particularly in terms of reduction and replacement, has long constituted a challenging task, because of the particular nature and properties of prions.

The quantification of prions by using cell assays has only gradually become feasible during the past few years, and so far, only relatively few cell assays are available for this purpose.\(^8\)\(^–\)\(^12\) Furthermore, the applicability of such cell assays is often restricted, because they mostly work with just one or a few of the various prion strains that exist under laboratory and real-life conditions. Cell assays for prions typically detect PrP\(_{\text{PrPres}}\) amplification at a certain time post-inoculation, rather than cytopathic or cytotoxic effects.
Also, the development of cell-free detection methods that would be on a par with animal bioassays for prion titration was impeded for a long period of time by the unconventional chemical composition and replication mechanism of these infectious agents. The identification of seeding-active prion protein as the self-replicating principle of prions, however, has finally given rise to novel approaches for a cell-free biochemical measurement of pathological prion activity. This was possible due to the introduction of an analytical technique called protein misfolding cyclic amplification (PMCA), which mimics nucleation-dependent PrP polymerisation, in an accelerated mode, in the test tube.\textsuperscript{13} Quantitative PMCA, and related techniques, such as RT-QuIC, now permit, at least for specific prion strains, the direct titration of prion-associated seeding activity.\textsuperscript{14–17}

The detection of PrP aggregates or PrP\textsuperscript{res} (by immunohistochemistry of tissue sections, Western blotting of samples, or by other methods) was previously successful in many attempts to provide a qualitative or semi-quantitative surrogate marker for infectious PrP particles.\textsuperscript{2,18} However, in some studies (with other combinations of prions strains and host animals), such correlation was not observed,\textsuperscript{19–22} and the presence of aggregated PrP or PrP\textsuperscript{res} in itself does not provide information about the activity of the protein in terms of biochemical or biological PrP seeding and disease transmission.

Thus, for the assessment of the pathogen load in an unknown prion sample, the following indicators can be tentatively ranked according to their current significance. This produces the following hierarchy in terms of diagnostic value:

1. Lethal dose (as indicated by the transmission of fatal disease to animals).
2. Infective dose (as indicated by the transmission of PrP\textsuperscript{res} propagation or disease to animals).
3. Cell culture infective dose (as indicated by the transmission of PrP\textsuperscript{res} propagation to cell cultures).
4. Seeding dose (as indicated by the cell-free propagation of PrP\textsuperscript{res} in PMCA or other cell-free seeding assays).
5. Amount of aggregated PrP / PrP\textsuperscript{res} (as indicated by PrP detection methods).

The biochemical seeding activity and biological cell culture infectivity of prions are incongruent with the infectivity of prions in animals (or humans), so PMCA and cell assay findings cannot be used \textit{a priori} as a representative for \textit{in vivo} infectivity. This caveat limits the utility, acceptance and use of alternative methods to animal bioassays in specific areas of prion research, such as the evaluation of reprocessing procedures for medical devices for anti-prion efficacy. In a recent report on a rationale and methodology for the further reduction and replacement of prion bioas-

\textbf{Figure 1: Infectious prion rods, or scrapie-associated fibrils (SAFs), of disease-associated prion protein from the brain of a scrapie-infected hamster}

\textit{Courtesy of Muhsin Özel & Heino Diringer (Robert Koch-Institut, Berlin).}
say titrations in laboratory rodents, we have tried to address this problem.

Toward the further reduction and replacement of animal bioassays in prion research by using cell and PMCA assays

In our article, published in Laboratory Animals, we presented three pairs of PMCA and glial cell assays for different hamster-adapted prion agents (the frequently used 263K scrapie strain, 22A-H scrapie and BSE-H), as well an adaptation of quantitative PMCA to human vCJD prions.23 In this context, we described how to use our PMCA and cell assays for measuring the seeding dose (SD50) and the cell culture infective dose (CCID50), respectively, in a prion test sample. One SD50 represents the dose of prion-associated seeding activity that converts PrPc into PrPres in 50% of PMCA samples, and one CCID50 represents the dose of prions that causes infection (in terms of PrPres propagation) in 50% of inoculated cell cultures. Based on quantitative correlations empirically established in reference standards (such as hamster scrapie brain homogenates) between the seeding dose, cell culture infective dose and in vivo infectivity, SD50 and CCID50 values detected in vitro can be tentatively translated into ID50 or LD50 values.

In order to strengthen the significance of such indirect ID50 or LD50 assessments, we decided to perform combined PMCA and cell assays. PMCA and cell assays represent profoundly different cell-free and

Figure 2: Fluorescence labelling of non-infected primary hamster glial cells

The panels show red labelling of astrocytes with an antibody that binds to glial fibrillary acidic protein (top left), green labelling of microglia with isolectin B4 (top right), and blue labelling of cell nuclei by using DAPI (4’,6-diamidino-2-phenylindole; bottom left). The superimposition of the different labels is shown in the bottom right panel. Magnification: 200×.
cell-based test principles for the biochemical and biological titration, respectively, of prion activity in vitro. Therefore, if these assays independently deliver consistent ID50 or LD50 assessments, then this provides an important methodological safeguard that substantially backs up the overall test reliability, as compared with titrations based on either PMCA or cell assays alone. Furthermore, we proposed to empirically validate our approach of combined PMCA and cell assay measurements. For this purpose, prion titre estimates from a set of different test samples would need to be compared to the actual ID50 or LD50 levels in the respective samples. As recently shown by Pritzkow et al., this could be done, at least partly, by using bioassay data from previous in vivo studies.16

Our approach provides a concept, a methodological platform and a practical roadmap that aim to establish the combined PMCA and cell assays as an effective, or even superior, alternative to quantitative prion bioassays in laboratory rodents. If successful, this would substantially contribute toward the implementation of the Three Rs. For example, the optimal use of 13 normal hamster brains and slightly more than 2 × 10⁻⁶g of 263K scrapie hamster brain tissue in our cell and PMCA assays, is theoretically sufficient to replace the use of 60 bioassay hamsters in the in vivo titration of twelve 263K scrapie samples. However, since our in vitro techniques still require animal tissues, they currently offer only the opportunity for reduction and relative replacement in terms of the Three Rs. It remains to be seen whether further methodological advancements will provide options for the absolute replacement of animal-based prion bioassays in the future.

In any case, our assays and other seeding and cell assays can already provide substantial contributions to replacement. This particularly applies to resolving scientific questions that would previously have required prion titrations in animals, but can now be sufficiently answered by in vitro measurements of seeding activities and/or cell culture infectivities. Finally, PMCA and cell assays are also helpful with respect to reduction, since they can be used to ‘pre-characterise’ samples prior to bioassays in animals. This allows careful dose-level or sample selections, and generally improves the practical and statistical planning of animal experiments, in terms of obtaining the information of interest with the smallest possible number of animals.

Conclusions

As the PMCA and cell assays become more versatile and applicable to different prion strains, they will foster the reduction and replacement of animal bioassays in this field of research and testing. Reducing the need for animal experiments that are time-consuming, expensive, restricted in throughput and possibly problematic with respect to their ethics, will meet both the interests of prion researchers and the objectives of the Three Rs.

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The committee made 33 recommendations, and emphasised the need for “a clear restatement of the key role of the Named Veterinary Surgeon (NVS) and the Named Animal Care and Welfare Officer in animal welfare and the Three Rs, along with a clear route for escalation of concerns to the AWERB”. Again to its credit, the College “accepted all the recommendations”, admitted that there was “significant scope for improvement”, and said that it “will now move quickly to implement the recommendations”.

The need for this investigation leads to two important questions. First, why was such a situation allowed to develop at Imperial College, one of the world’s most prestigious universities? Why were the Certificate Holder, the senior academics, the project and personal licence holders, the NVS, the Home Office Inspector and others, not performing their duties up to even the minimal standards required of them?

The second question is that: if this situation could be allowed to develop at Imperial College, in what other institutions do similar problems exist and are similarly low standards considered acceptable? Professor Paul Flecknell, a member of the committee and director of the research animal facilities at Newcastle University, said that, while the report was specific to Imperial College, “every institution will pick up something we’ve said and think, ‘we should take more note of that’ ”. The Home Office, and the equivalent authorities in other countries, should insist that they do just that.

References