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Back to the future in immunoassay and automation

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It is tempting to think that the evolution of immunoassay technology from labour-intensive manual methods to fully automated, random-access processes represents the culmination of an era of massive development and improvement. Certainly, the achievements of the last 30 years should not be underestimated. Few involved in immunoassay development 30 years ago would have predicted that technology would advance to the point where picomolar concentrations of peptide hormones could be measured accurately in the course of a few minutes with a throughput of up to 200 specimens per hour. With present levels of automation, such activity is commonplace. This is not to say that such methods are without difficulty. We now recognise that the design of immunoassays that require completion in minutes may be considerably more demanding than that of conventional analytical methods where reactions are allowed to proceed to equilibrium (1). Perhaps the greatest difficulty with automation is that the combination of sophisticated engineering and what may be proprietary methodology places the technology beyond the scope of the majority of laboratory staff. In this situation, it may be tempting to rely on less qualified operators to keep the machines busy rather than on highly trained technologists who can understand how the analytical data are generated and so recognise problems and inconsistencies in patient results that might otherwise escape unnoticed.

While the new technologies undoubtedly present their own difficulties, I would like to consider some of the wider challenges facing the diagnostic laboratory and examine ways in which clinical biochemists can assist in meeting these challenges. To do this, I would like to consider three, widely differing, examples of clinical problems that require more than engineering solutions and, in addition, some of the approaches we can use to progress laboratory diagnostics.

Diabetes research

There is little doubt that diabetes represents one of the most significant medical problems facing developed nations today. Prevalence in Western Europe is estimated at up to 8% of the population and it is therefore hardly surprising that this generates a massive impact on health resources. In order to introduce effective management of diabetes, it is necessary that we can identify those at risk at the earliest possible stage. In the absence of routine health screening, this is not easy to contemplate. However, it is also questionable as to whether, the traditional approach of relying on fasting blood glucose measurement is effective in this regard. Recent work has shown that the onset of diabetes is associated with changes in insulin secretion in addition to the well-recognised problem of progressive insulin resistance (2). Most interesting of all is the observation that such changes may precede more traditionally recognised abnormalities of glucose tolerance. A recent epidemiological study from East Anglia (3) has shown that subjects with apparently “normal” glucose tolerance who went on to develop diabetes within 5 years actually had significantly raised blood proinsulin levels at the initial time of study, while their insulin levels were not significantly elevated. Studies such as this illustrate the fact that new methodologies are still needed in important areas of clinical diagnostics. Thus the availability of sensitive and specific assays of human proinsulin, which are suitable for high throughput screening, could well provide a new approach to the identification of diabetes at the earliest stage possible and thereby offer some hope for early treatment of this appalling disease. Such assays do not form part of the basic repertoire of any of the currently available automated systems and yet would seem appropriate for incorporation into routine laboratory testing. The fact that manufacturers of the wide range of automated immunoassay analysers available at present have not seen fit to incorporate proinsulin into their present test menus should not be seen as an excuse for ignoring the value of such test methods to the routine biochemistry laboratory and healthcare in general.

Screening of human blood for the presence of viruses

The ability to detect the presence of blood borne pathogens is vital in order to guarantee the safety of human blood used for transfusion purposes. Traditional screening tests for dangerous pathogens such as HIV and HCV have relied on the presence of circulating antibodies to these viruses. However, it has long been recognised that such antibodies are not detectable in blood for some time following initial infection. In the case of HIV, antibodies are not normally detected for 3 weeks following infection while in the case of HCV, the time lag before diagnosis may be 11 to 12 weeks. In practice, the chances of a patient receiving infected blood are very small. Never-

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theless, they are not negligible so that considerable effort has been expended in improving the diagnostic performance of screening tests.

A significant breakthrough has been achieved through the use of novel genetic probe technology which, through its dependence on complementary binding of nucleotide sequences, shares many common features with immunoassay. In recent years, a number of non-isotopic labelling technologies have introduced significant improvements in analytical sensitivity by their amplification of the signal/noise ratio monitored in immunoassay end-points. In oligonucleotide detection, there is also the opportunity to generate massive improvements in sensitivity through amplification of the target. Amplification techniques have been powerful research tools for several years but have only recently found their way into the routine diagnostic laboratory. Following a major development programme, a San Diego based company (Gen-Probe) has introduced a test system for the detection of HIV and HCV RNA in donated blood that incorporates a remarkable combination of technologies. Despite the apparent complexity of these technologies, the company plans to launch this system in a fully automated form in the near future.

In essence, analyses are carried out in three stages. In the initial stage, the blood sample is introduced into a buffer capable of lysing any virus particles present. Paramagnetic particles, to which are attached oligonucleotides that will capture the desired target sequences, are then added and, after a reaction period, are washed free of any contaminating material. In the second stage, the captured sequences are subjected to transcription mediated amplification (4) to generate sufficient target for routine identification. In the final stage, the target is reacted with a complementary probe labelled with a chemiluminescent acridinium ester. The hybridisation takes place at a temperature of around 60º, following which a selection step inactivates label that is not incorporated into hybrids. This procedure is based on the observation that acridinium bound to nucleotide duplexes is resistant to hydrolysis whereas that attached to the probe alone can be readily hydrolysed to a non-chemiluminescent derivative in the course of a few minutes (5). Following the selection step, the sample is introduced into a luminometer where the chemiluminescent reaction is initiated and the light intensity recorded. This hybridisation protection assay (HPA) takes around 50 minutes from the introduction of the labelled probe to its detection in the luminometer.

A further refinement in the system involves the use of two acridinium labels that can be quantified independently based on their individual kinetics of light emission. This dual kinetic analysis (DKA) allows two reactions within the same tube to be monitored simultaneously. The ability to monitor two reactions in this way is very significant since it makes it possible to avoid false negatives that might result from a failure of the amplification reaction. Thus every analysis incorporates amplification and detection of a control sequence in addition to that of any HIV or HCV target present in the test sample.

Results of this test method are highly encouraging. As little as 100 copies of viral RNA can be routinely detected with 100% diagnostic sensitivity. As a result, the interval between the time of infection and subsequent detection can be significantly reduced. In the case of HIV, this allows positive identification of an infection at an average of 16 days prior to the appearance of detectable antibody and 7 days earlier than the appearance of detectable p24 antigen. In the case of HCV, the new system yielded positive results at an average of 33 days before the appearance of detectable antibody. Consequently, of 6 million blood donations screened so far, as part of a validation programme, 3 have tested positive for HIV and 20 for HCV that would otherwise have remained undetected by conventional screening tests. In time, this new technology will have a profound impact on improving the safety of blood donations throughout the world.

Environmental diagnostics

The potential impact of environmental pollution on human health is frequently discussed. Apart from situations producing acute toxicological effects, this impact may be extremely difficult to assess. In the absence of any clear symptoms of derangement, or where effects only become apparent after long-term exposure, the association between a pollutant and its impact has been frequently derived through epidemiological studies or mere circumstantial evidence.

The science of genomics, whereby specific genetic responses are used as indicators of changes in cellular function in response to external forces, is now receiving considerable attention. In the pharmaceutical industry, genomics has been applied both as a tool for identifying new compounds with potential pharmacological effects as well as a means of detecting side-reactions to pharmacological agents. In my own laboratory, we have been exploring the possibility that through a study of appropriate genetic activity, we can identify environmental pollutants and also assess their impact on the biosphere. As an example we chose to study the problem of endocrine disruption by oestrogen mimics. This subject has understandably received wide media attention in recent years and now attracts considerable research activity. Since 1990, there has been a sharp increase in the number of reports of adverse effects of endocrine disrupting chemicals on fish, reptiles, birds and other wildlife (6). These effects include abnormal reproductive function, abnormal reproductive behaviour, demasculinisation and decreased hatching success. Effects on humans are more difficult to assess though it has been postulated that the fall in sperm count, observed in countries such as Denmark during the last 50 years, may relate to exposure to endocrine disrupting chemicals released into the environment (7) though the issue remains controversial (8).

Our first objective was to identify suitable genes for investigation. To do this, we created a cDNA library from RNA extracted from oestrogen-exposed fathead minnows (Pimephales promelas). We were able to isolate two abundant genes, one a 4 Kb gene coding for the egg storage protein vitellogenin, the other a

1.3 Kb gene coding for the egg membrane protein ZP3. Once the sequences were established, complementary probes were designed so as to react with mRNA transcribed from these genes. Suitable labelled probes were used to develop HPAs for vitellogenin mRNA, ZP3 mRNA together with actin mRNA as an internal control. In these assays, it is possible to detect as little as 0.01 fmol of mRNA. As a consequence, tests can be carried out on a small aliquot of total RNA extracted from exposed or control fish. Results have now been obtained from several groups of fathead minnows. While juvenile and male fish had no detectable vitellogenin mRNA, mature females expressed massive levels of this transcript. More importantly, juvenile fish exposed to oestrogen for a period of 21 days expressed similar levels of mRNA to those found in mature females. Further studies in rainbow trout have demonstrated parallel effects in fish exposed for only 10-14 days.

These genetic responses provide sensitive indicators of endocrine disrupting chemicals that may be released into the environment. Perhaps more importantly, they can be used as a means of screening chemicals for their endocrine disrupting potential - before they find their way into routine use. Having established *in vivo* toxicological tests based on regulatory organisms such as the fathead minnow, it will now be possible to identify parallel responses in suitable cell lines. The availability of well-characterised responsive cell lines should then make way for a wide range of quantitative tests based on gene responses. Endocrine disruption is only one of several problem areas where such tests will yield valuable information. For example, once suitable targets are identified, it should be possible to screen chemicals for wide ranging toxicological effects in the investigation of problems such as neurotoxicity and chemical carcinogenesis. In the long term, such information will be valuable to researchers, to regulatory bodies and to industry. The ability to address environmental health issues through simple analytical procedures will provide new insights in a field, which has so far been inaccessible to conventional diagnostic procedures.

It would be a serious mistake to think that the clinical laboratory has already reached a pinnacle of development. The current level of sophistication merely reflects the fact that we have sophisticated tools at our disposal. This does not mean that the problems associated with the investigation and management of disease have in anyway decreased. The problems remain and the tools needed to investigate them are still evolving. It is certain that the range of tools available over the next few years will change. These changes will lead to new methodological developments and new instrumentation. Not surprisingly, many of the new technologies have wide-ranging applications and can lead to a breakdown of barriers between conventional disciplines. Already, HPA can be used to diagnose the presence of infectious agents, detect DNA mismatches that might be the cause of inherited diseases and monitor endocrine disruption as a result of environmental pollution. With technologies such as this available to us, it is clear that the future of clinical biochemistry will be no less demanding than it has been to date. We must not make the mistake of losing sight of the challenges and pretending that our automated systems can solve all problems without us.

Literature

1. Woodhead JS. Immunoassay design: the theory and the practice. J Clin Ligand Assay 1996; 19 (Suppl): 74-81.
2. Yoshioka N, Kuzuya T, Matsuda A et al. Serum proinsulin levels at fasting and after oral glucose load in patients with type 2 (non-insulin dependent) diabetes mellitus. Diabetologia 1988; 31: 355-356.
3. Wareham NJ, Byrne CD, Williams R, Day NE, Hales CN. Fasting proinsulin concentrations predict the development of type 2 diabetes. Diabetes Care 1999; 22: 262-270.
4. McDonough SH, Bott MA, Giachetti C. Application of transcription-mediated amplification to detection of nucleic acids from clinically relevant organisms. In Lee H, Morse S, Olsvik O eds. Nucleic Acid Amplification Technologies 1997, Eaton Publishing, Natick, MA, USA, pp 113-123.
5. Arnold LJ, Hammond PW, Wiese WA, Nelson NC. Assay formats involving acridinium ester labelled DNA probes. Clin Chem 1989; 35: 1588-1594.
6. Safe SH. Endocrine disruptors and human health-is there a problem? An update. Env Health Perspectives 2000; 108: 487-493.
7. Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 1993; 341: 1392-1395.
8. Gyllenborg J, Skakkebaek NE, Nielsen NC, Keiding N, Giwercman A. Secular and seasonal changes in semen quality among young Danish men: a statistical analysis of semen samples from 1927 donor candidates during 1977-1995. Int J Androl 1999; 22: 28-36.