Abstract
This review paper will discuss some of the opportunities for three Rs approaches in the production and quality control of immunobiologics. Major products in terms of animal use are the polyclonal antibodies (Pab), monoclonal antibodies (Mab) and vaccines. These categories of products are also characterized by the high percentage of experimental procedures that involve substantial pain and suffering to the animals. Nowadays a wide range of approaches and test methods are available that are able to substantially refine, reduce and replace animal use. These include for instance the use of humane endpoints, replacement of challenge procedures in vaccine potency testing by serological approaches or the replacement of ascites production in Mab production by in vitro techniques. Although total replacement of animals for production and quality control of immunobiologics will not be possible within the near future, it is concluded that substantial three R’s progress can be made quite easily without the need for exorbitant human and financial resources.

Key words: antibodies, vaccines, three Rs, progress

Introduction
Concern about the use of laboratory animals in biomedical research has prompted the development of alternatives. An alternative can be defined as any method or approach that allows a) animal models to be replaced by non-animal methods; b) numbers of animals to be reduced for a particular purpose or; c) animal procedures to be refined. The Principle of the three Rs as it now known, was first introduced by Russell and Burch in their book ‘The Principles of Humane Experimental Technique’, published in 1959 (Russell & Burch, 1959). Starting in the seventies of the previous century, several initiatives have been taken to incorporate the three R’s concept into biomedical research policies. Thus, the concept is now the redline in laboratory animal laws in Europe, three R’s centres and societies have been established around the world, such as the CAAT in the US, ZEBET in Germany, FRAME in the UK and JSAAE in Japan, governmental bodies focus on the validation of Three R’s methods, such as ECVAM in Europe and ICCVAM in the US, and the three Rs are included in policy documents of large international organizations, such as OECD.

When discussing alternatives most people refer to the area of toxicology. This is remarkable, as toxicology is only a modest user of laboratory animals. For instance toxicology and safety testing required about 10% of animal use in Europe (total animal use in 2002: 10.7 million). However, far more animals were needed for the development, production and quality control of medicines and
immunobiologics. In 2002 the percentage of total animals use was 44%. Although no official data are available it might be anticipated that about 1/3 of these animals have been used for the category of immunobiologics, both for human and veterinary application. Immunobiologics are products that are derived from or produced by living organisms and which activity is related to an antigen – antibody interaction. In terms of animal welfare concern, the most relevant immunobiologicals are the polyclonal and monoclonal antibodies (Pabs and Mabs, respectively) and the vaccines. Either these products require extensive numbers of animals or production and testing is characterized by high levels of pain and distress for the animals involved. In the following paragraphs information will be provided about the use of animals for these products and an overview will be given of the opportunities for Replacement, Reduction and Refinement.

**Polyclonal antibodies (Pabs)**

Immunizing an animal with an antigen generate the production of polyclonal antibodies (Pabs). Generally, antigens are complex structures, consisting of various antigenic sites (epitopes). These epitopes will trigger the immune system to generate antibody secreting cells (B cell clones), each clone producing a (monoclonal) antibody to one specific epitope. Pabs are particularly important in the laboratory as a research tool, but also for diagnostic testing purposes. In terms of animal welfare, Pab production includes several critical steps: the adjuvant being used, the route of immunization and the bleeding of the animal (Leenaars et al. 1999).

Antigens, particularly if less immunogenic, are often combined with an adjuvant to enhance or to modify the immune response and to produce antiseraum with a high antibody titre. Nowadays, more than 100 adjuvant products are available, of which only few (the aluminum salts) are approved for use in humans. The reason is that almost all adjuvant products induce adverse effects, mainly inflammatory responses and abscess formation (Leenaars et al. 1998). This is particularly true for the adjuvant most favored in the laboratory; Freund’s adjuvant (FA). FA is a water-in-oil emulsion. If the emulsion includes killed mycobacteria, the product is called Freund’s complete adjuvant (FCA), without mycobacteria Freund’s incomplete adjuvant (FIA). Several adjuvant products have claimed to produce fewer side effects. However, either these adjuvants showed to be less effective compared to FA (e.g. commercial products such as TiterMax or RIBI (Leenaars et al. 1999) or were only relevant for specific categories of antigens (e.g. ISCOMs). FA is a very potent adjuvant for a wide range of antigens (Stills, 2005). The opportunity for refinement is in the optimization of its use. First, in case of emulsions, the adjuvant-antigen volume should not exceed 0.1 ml (50% antigen and 50% FA) in small laboratory animals in case of emulsions and 0.5 ml in case of aqueous antigens. This reduces the adverse effects while still inducing an optimal immune response. Furthermore, FA adjuvant products as well as other oil adjuvant products should only be inoculated in spacious parts of the body and in parts that can easily be monitored. Thus, FA injection subcutaneously should be recommended, while intramuscular, intraperitoneal and footpath injections should be discouraged (Hendriksen & Hau, 2003).

Another critical step in Pab production is the number of booster injections. Generally, this number should be limited and blood sampling, to monitor immune response and to determine the need for booster injection, should precede booster immunization. In case of FA, FCA should only be used for primary immunization and never for booster injections. FCA sensitizes an animal for mycobacteria, which would induce major health problems in case of repeated contact.

Finally, refinement can be applied to blood sampling and exsanguination. Blood sampling should not exceed the volume that is strictly needed for antibody titration. Bleeding the animal for antibody collection must be performed under general anesthesia and is best carried out by heart puncture (Hendriksen & Hau, 2003).

Currently, no in vitro alternatives exist for Pab production. However, the number of animals can be further reduced and the method can be further refined by replacing Pab production in mammals by IgY antibody production in chicken (Gassmann et al. 1990). IgY are structurally different from mammal IgG antibodies but share most of the functional characteristics. A major advantage of using birds is that the antibodies can be harvested from the egg yolk instead of serum, thus making blood sampling obsolete (Hau & Hendriksen, 2005). Furthermore, egg yolk antibody titres are high compared to serum antibody titres, thereby limiting the number of animals needed (Svendsen et al. 1996). IgY production is favored when producing antibodies to highly conserved antigens, but production could also be extended to other antigens (Schade et al. 2001). Nevertheless, there are also
some disadvantages to IgY production such as the specific housing requirements, the limited availability of conjugated antibodies, lack of investigators experience with chicken and chicken antibodies, and IgY isolation and purification problems (Schade et al. 1996).

**Monoclonal antibodies (Mab)**

Antibodies produced by one single B cell clone are called monoclonal antibodies (Mabs). These antibodies are highly specific and do only bind to one particular epitope. This makes Mabs to indispensable tools in the research and diagnostic laboratory as well as in the clinic. In order to produce quantities of Mabs, specific B cells need to be scaled up, a process that requires a number of in vitro steps. Due to the fact that B cells cannot survive outside the body, up-scaling became only possible after Köhler and Milstein were able to immortalize these cells by fusion with myeloma cells in 1975 (Köhler and Milstein, 1975). The resulting hybridoma cells are characterized by their infinitive growth and the production of virtually unlimited quantities of Mabs.

Production of Mabs includes several steps, starting with the generation of specific B cells. Until now, this can only be done by immunizing an animal with the relevant epitope. Methods to generate epitope-specific B cells after in vitro immunization have been described (Stadler 1999) but have not been very successful to date because in vitro the vast majority of the B cells produce IgM rather than IgG antibodies (Borrebaeck et al. 1988). For immunization, BALB/c strain mice are typically used because many of the myeloma cells available for fusion have a BALB/c origin.

The next step in the Mab production is the selection of the relevant hybridoma cells, a process that is performed in vitro. If the desirable hybridoma clone has been identified, the final step will be up-scaling of Mab production by expansion of that clone. Traditionally this is done in vivo by intraperitoneal (i.p.) injection of a primer (generally pristane) in mice, followed some weeks thereafter by i.p. administration of hybridoma cells. Particularly, the abdominal cavity offers optimal culture conditions for the cells, such as a constant temperature, an optimal nutrient and oxygen supply, and an optimal removal of CO2 and metabolic waste products. As hybridoma cells are tumorigenic, these cells will start to grow and to secrete Mabs. After 7 to 14 days, antibodies can be collected by abdominal puncture and tapping of the ascitic fluid. Eventually, the animals can be tapped several times, although after 2 taps the volume of ascites as well as the survival rate of the animals will start to decrease (Jackson et al. 1999).

Mab production by the ascites procedure has been criticized heavily. The method leads to significant health problems (Jackson et al., 1999, Peterson, 2000) and is believed to inflict severe suffering to the animals. Pristane acts by inducing peritonitis. Growth of the hybridoma cells results in tumor formation and infiltration of tumor cells in the abdominal organs, and, finally, ascites production results in abdominal extension, dehydration and pressure to the thoracic organs (lungs). As a consequence, several countries in Europe and individual institutes in the US have issued policy guidelines on Mab production. Generally, these guidelines limit the volume of pristane to be injected, and the maximum number of taps allowed. More recently, new innovative in vitro methods for Mab production have become available, leading to restrictions in most North European countries, such as Germany, the UK, The Netherlands and Switzerland. All these countries strongly discourage ascites production. In 1997, a ‘Dear Colleague’ letter, sent by the US National Institutes of Health to research institutes in the US stressed the fact that in vitro methods for Mab production should be preferred (NIH, 1997).

An overview of in vitro Mab production methods has been provided in several review papers (e.g. De Geus & Hendriksen, 1998; Jackson, Trudel & Lipman, 1999). Generally, the methods can be divided into single compartment cultures and double compartment cultures. In single compartment cultures cells and growth medium are in the same compartment, either with the cells as monolayer attached to the wall of the tissue culture flask (tissue culture flasks and gas-permeable bags) or in suspension as in roller bottles and in spinner flasks. These cultures allow for low densities of cells only, thus resulting in low concentrations of Mabs. Additional disadvantages are that culture conditions and consequently cell viability are far from optimal (CO2 and metabolic waste products ‘intoxicate’ the medium), and Mabs are excreted to the surrounding fluid and can only be collected by removing the medium and microbial contamination can easily occur. On the other hand, these systems are easy to handle and less expensive compared to two compartment systems. In the two-compartment systems hybridoma cells are separated from the medium by a semi-permeable membrane, thus allowing gas
exchange and exchange of small (nutrient) molecules and growth factors. A very popular system is the CELLine device with cells retained in a small cultivation chamber and a large nutrient supply compartment separated by a semipermeable dialysis membrane from the cultivation chamber. Mabs produced concentrate in the cultivation medium and can be collected relatively easily. Other two-compartment systems are the miniPERM system, which is a modified roller bottle two-compartment bioreactor, the Tecnomouse in which different hybridoma cell lines can be cultured in parallel in separate cell culture cassettes and the hollow fiber bioreactor systems. Two-compartment systems require skilled personnel and a higher investment in manpower and equipment. For some people this is a reason to argue that in vitro production is less cost-effective than ascites production. However, comparisons of in vivo to in vitro production systems, including all the costs related to Mab production have shown that in vitro production can compete to ascites production (Table 1). Furthermore, two-compartment systems produce antibody concentrations that are similar to ascites production, some systems can produced for significant time periods and the monoclonal antibodies are not contaminated with murine immunoglobulins or murine pathogens (Dewar et al., 2005).

### Vaccines

Many reading this article probably would not have done so when they had not been vaccinated. Nowadays, physicians are unfamiliar with the clinical signs of several childhood diseases that, without vaccine prevention, have high morbidity and mortality rates, such as diphtheria, tetanus and pertussis. While these diseases are relatively unknown in industrialized countries, they still are in developing countries.

Most vaccines in health care programs are produced according to traditional procedures: in-activation or attenuation of the virulent micro-organism or detoxification of the toxin thereof. The procedure requires that each batch of vaccine produced is tested for safety and potency. Some of these tests are based on animal models. In terms of numbers being used, most animals are needed for the (regulatory required) quality control of inactivated vaccines and toxoids. Particularly the test to demonstrate that the vaccine batch produced induces protective immunity after administration is animal demanding. This is only so for inactivated or detoxified vaccines. Efficacy of live vaccines is determined by counting or titration of the number of live particles.

The potency tests for the inactivated vaccines are based on principles, quite often already established in the early days of vaccine development. One of the most used approaches, the so-called ‘parallel-line’ potency test, is to immunize groups of animals with serial dilutions (three or four) of the vaccine under study and a reference vaccine with known potency, respectively. Generally, these ranges of dilutions include groups receiving a low, an intermediate, and a high vaccine dose. After a number of weeks, the animals are challenged with the virulent micro-organism or toxin, and specific clinical signs or death are recorded for the observation period given. Vaccine doses are chosen in such way that up to 100% protection is expected in the animal group that received the highest vaccine dose and almost 0% protection in the animal group receiving the lowest vaccine dose. Based on the information obtained from the various groups, a dose-response curve can be plotted, both for the vaccine under study and for the reference preparation, and the dose that protects 50% (ED50 = Effective dose 50) of the animals is calculated. Finally, the potency of the vaccine under study can be expressed in that of the reference preparation, this on the condition of parallelism and linearity of the dose response curves. It will be clear from the above that

### Table 1. Mab production: in vivo versus in vitro (Peterson N, 1999)

<table>
<thead>
<tr>
<th></th>
<th>Flasks &amp; Gas-permeable</th>
<th>Gas-permeable</th>
<th>Mini-fermentor</th>
<th>Hollow-Fiber</th>
<th>Ascites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate</td>
<td>300-900mL/flask</td>
<td>600 mL/bag</td>
<td>7 mg/day</td>
<td>3-6 mg/day</td>
<td>1-3 mL/tap</td>
</tr>
<tr>
<td>Purity</td>
<td>75-90%</td>
<td>75-90%</td>
<td>60-65%</td>
<td>35-75%</td>
<td>25-65%</td>
</tr>
<tr>
<td>Concentration</td>
<td>0.01-0.3mg/ml</td>
<td>0.01-0.2mg/ml</td>
<td>2-3 mg/ml</td>
<td>0.5-4 mg/ml</td>
<td>1-5 mg/ml</td>
</tr>
<tr>
<td>Cost</td>
<td>$0.50-2/mg</td>
<td>$0.50-2/mg</td>
<td>$1-3/mg</td>
<td>$1-10/mg</td>
<td>$1-2 mg/ml</td>
</tr>
</tbody>
</table>

Values differ with different hybridomas

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the parallel-line potency test requires large numbers of animals (up to 140/test) as well as induces substantial levels of suffering.

Attention is nowadays given to the development of methods that could replace, reduce and refine the use of animals. The reasons for this trend are divers (Hendriksen, 2005):

- Moral and ethical concerns about animal use.
- The questionable relevance of some animal models, or poor reproducibility within and between laboratories.
- The fact that animal tests are time consuming and interfere with the limited shelf life of vaccines.
- New developments and strategies in vaccine production such as standardization of production processes and the introduction of Good Manufacturing Practice (GMP), Quality Assurance (QA) and in-process control that make extensive quality control of the final product less relevant and even superfluous.

A brief overview will be presented of what has been achieved so far:

**Refinement**

A particular approach to refinement is the use of humane endpoints, which means that an animal will be humanely destroyed as soon as sufficient indication is obtained that the challenged animal is insufficiently protected and will not survive the observation period. Thus for rabies vaccine potency testing death as an endpoint could be replaced by specific clinical signs (Cussler et al. 1999). For whole cell pertussis potency testing it could be shown that specific clinical signs as well as a decrease in body temperature < 34.5 °C is predictive for death and could be used as criteria for euthanasia (Hendriksen et al. 1999). The use of humane endpoints is now generally accepted in major pharmacopoeias such as the European Pharmacopoeia.

Another refinement alternative is the replacement of challenge by protective antibody titration in immunized animals. Thus, in potency testing of tetanus and diphtheria toxoid vaccines the estimation of the protective capacity of a new batch of vaccine is now based on serology instead of protection after challenge. The level of protective antibodies, induced in laboratory animals after vaccination with the batch of vaccine under study, is determined by *in vitro* toxin neutralization in cultures of Vero cells (Miyamura et al. 1974) for diphtheria toxoid and in a modified ELISA procedure (Winsness et al. 1999) for tetanus toxoid. The advantages in doing serology instead of challenge are several: a) distress in the animals is reduced from severe to minor (animals are only immunized and bled several weeks thereafter); b) serum samples can be stored and eventually be re-used if needed and c) numbers of animals can be reduced as quantitative endpoints (antibody titres) are used instead of qualitative endpoints (death or survival).

**Reduction**

An additional advantage of serology compared to challenge is that numbers of animals can be reduced as quantitative endpoints (antibody titres) are used instead of qualitative endpoints (death or survival). This allows for a reduction of about 40% of total animal use.

Another reduction approach is replacing the parallel-line assay by a single dose test. Instead of multiple doses of the vaccine under study and the reference preparation, respectively, using only one dose of both vaccines. The reason is that regulatory requirements only specify the minimum level of international units of a vaccine batch, rather than require a precise estimate of potency. Thus, in the single dose potency test it is only demonstrated, with 95% confidence, that the potency of the new vaccine batch is above the minimum level. No information is obtained about dose response linearity and parallelism of responses between the vaccine and the reference preparation. An important condition for allowing for a single-dose potency test is that this information has been made available by retrospective analysis of data of multi-dose potency test, such as has been the case for diphtheria and tetanus potency testing.

**Replacement**

As such, there are at the moment no possibilities to replace the *in vivo* potency test by *in vitro* tests for most inactivated vaccines. The immune response is a very complex process that can’t be copied in a simple *in vitro* model. However, there are other approaches under study that might lead to a total or almost total replacement of animal use by a set of *in vitro* methods and quality assurance measures. This approach is known as the ’concept of consistency’ (Metz et al. 2002). The key issue of consistency has emerged from the new generation of vaccines. These vaccines which are based on new technologies, are produced in a consistent way and the stress of quality control is on in-process monitoring rather
than on final batch testing. In-process testing is almost exclusively based on in vitro biochemical and physicochemical tests. The consistency concept has become state of the art for the new generation of vaccines. Also in the field of the conventional vaccines, continued advances in production technology have resulted in more defined and thus less variable products. This, together with the implementation of GMP and QA, makes people feel that for the conventionally produced vaccines, the extent of batch release testing should reflect the level of consistency obtained with the vaccines. Thus, a vaccine manufacturer should perform extensive testing (including animal testing) during the development phase and on the first few batches of the new product to characterize the vaccine thoroughly. However, if consistency in production is demonstrated, then testing could rely on a battery of easy-to-use in vitro assays to characterize (fingerprint) the vaccine and confirm consistency. If this approach is applied, the number of animals needed for quality control of conventional vaccines will be reduced to an absolute minimum (Hendriksen, 2005).

**Conclusions**

Immunobiologics are important products, both in medical health care programmes, in diagnostic testing and in the research laboratory. Traditionally, these products have been characterized by the use of extensive numbers of animals as well as the application of animal models with high levels of animal suffering. Also in the near future animals will be needed as complex immunological processes such as antigen processing and recognition by T cells will be difficult to transfer to relatively simple non-animal models. However, there are many opportunities to reduce and refine the use, such as in Pab production and in vaccine quality control. Furthermore, by taking advantage of the implementation of GMP and QA, and the use of new non-animal methods, new testing strategies can be developed that will shift the burden of vaccine quality control from the final product to the intermediate products. Finally, development of in vitro models will continue. This has already prompted several European countries to ban the ascites method for Mab production. Furthermore it ultimately will be successful in replacing animal models for potency testing of inactivated vaccines.

**References**


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