Innovation in Animal Health Research and Development in Japan

Introduction

At the time of writing this editorial for *IAHJ* in March/April of 2020, the work coincided with the peak of the COVID-19 pandemic in many countries, and at the time, the role of pharmaceutical companies in protecting human and animal health was never so apparent. Overnight, PCR, antibody tests and antibody therapy became well-known terminologies even among the general public, and vaccines appeared to gain a regenerated importance due to the high expectations of an anxious public in terms of the protection of public health and the economy. Furthermore, diseases commonly observed with advanced age and the complications associated with infections in elderly people with underlying disease came under the spotlight. The possibility of the emergence of global human pandemics as well as the increased lifespan of us humans and our animal companions, dictates that we continuously innovate and develop new pharmaceuticals to preserve our combined health. As I elaborate below, biotechnology is one area that offers us a wide variety of tools in our search for new pharmaceuticals for the prevention and treatment of diseases in animals. I will introduce several examples from Japan to illustrate the practical applications of such research. Bearing in mind that most information in this field is proprietary information at least until commercialisation, this editorial will only use site examples which are already in the public domain.

Basic Research

First of all, it is important to give credit to basic research mostly carried out at universities and public research institutes, since that research reveals mechanisms of disease and aids us in the selection of targets for our products, be they biotech products or otherwise. Deciphering molecular pathways and mechanisms provides potential targets for our products and also gives insight into what side-effects might be expected as a result of treatment with new products. One such example is the cloning of the gene for canine interleukin-31 by Prof. Takuya Mizuno and his group at Yamaguchi University in 2009[1]. This work was important because it showed that canine interleukin-31 expresses limited homology with its human counterpart and also shows that findings in veterinary science may not always be extrapolated to human medicine and vice versa. Interleukin-31 is involved in the signal transduction that results in the pruritus observed in dogs with atopic dermatitis. A neutralising antibody product against canine interleukin-31 is now approved in many countries for the treatment of pruritus associated with atopic dermatitis in dogs. Prof. Satoru Konnai and his group at Hokkaido University also produced monoclonal antibodies against the bovine Programmed Death-1 (PD-1), an important immune checkpoint inhibitor membrane protein expressed in certain cells of the immune system, and whose function is to potentially dampen detrimental immune responses. Blocking PD-1 using the neutralising antibodies produced by Prof. Konnai increased T cell function and inhibited bovine leukaemia virus expression *in vivo*[2]. Antibodies for human PD-1 are already on the market in many countries for the treatment of various cancers and it is expected that similar antibodies for therapeutic application in companion animals will follow. The characterisation of livestock pathogens and parasites is also an area where academia and public research institutes contribute greatly to our increased understanding of infectious disease and eventually, to the generation of prophylactic vaccines. One example is the isolation and characterisation of bovine viral diarrhoea virus from Japanese cattle by Dr. Keita Matsuno and his colleagues at Hokkaido University[3]. Many of the examples of biotech innovations I give below originated either from universities and public research institutes, or collaborations between industry and academia, highlighting the importance of academia and public research institutes in animal health innovation in Japan.

Escherichia coli

Perhaps one of the most widely used methods in biotechnology is the expression of gene coding for proteins that are biologically active, purifying the proteins by chromatographic methods and applying them to pharmaceutical use. Methods vary depending on the successful matching of the target gene with the expression system which affects the activity and yield of the target protein. The *E. coli* expression system is probably the most well understood expression system and was used to produce the first Food and Drug Administration (FDA)-approved recombinant protein drug in 1982. The name of the approved drug was Humulin and the active ingredient was a recombinant human insulin[4]. Although *E. coli* is a very useful tool for the production of recombinant proteins, there are limitations as to the size and type of proteins that can be expressed in *E. coli*. However, it is still a method of choice in some fields because it is easy to use and rather inexpensive when compared to other expression methods.

E. coli is a good expression vector for bacteriophage-derived bacteria-lysing proteins called endolysins. Endolysins are able to lyse bacterial cell walls when bacteriophages emerge from the target bacteria, and have been labelled as promising substitutes for antibiotics due to the emergence of antimicrobial resistance in animal health. A group led by Prof. Koji Nishifuji from the Tokyo University of Agriculture and Technology has recently shown the potent inhibitory effects of one such endolysin on *Staphylococcus aureus* infection in a mouse model of impetigo[5]. Since most endolysins are highly selective for the bacterial host, damage to beneficial skin flora is minimised, potentially providing a high degree of safety. The potential application of endolysins in canine skin *Staphylococcus pseudointermedius* infections and in bovine mastitis due to *S. aureus* infection is also of considerable interest.

The *E. coli* system is also very useful in the production of DNA vaccines in the form of plasmid DNA expressing genes identified from the pathogen of interest. As compared to live, attenuated or inactivated vaccines, this technology allows for expression of only selected genes in the vaccine, thereby increasing the safety of the product. A DNA vaccine for West Nile Virus infection in horses was approved by the United States Department of Agriculture (USDA) in 2005 and recently, Immunomic Therapeutics Inc. (US) entered into a partnership with Zenoaq (Japan) for the development of DNA vaccines in the area of companion animal allergy[6]. Culture of *E. coli* transformed with vaccine pDNA can yield bacterial pastes of more than 1g/L from which the pDNA can be purified.

Prof. Ikuo Hirono and his team at the Tokyo University of Marine Science and Technology also developed a DNA vaccine to protect cultured amberjack fish from infection with the bacterium *Nocardia seriolae* (nocardiosis)[7]. Nocardiosis causes

considerable losses in the fish aquaculture industry in Japan. The DNA vaccine produced by Prof. Hirono's team was constructed by introducing a codon-optimised version of the Antigen 85-like (Ag85L) gene of *N. seriolae* into the DNA vaccine backbone, and *E. coli* were transformed with the constructed plasmid and cultured at 1L scale. The DNA vaccine was able to protect 98% of fish in an experimental challenge study, whereas only 51% of the control PBS-injected group survived.

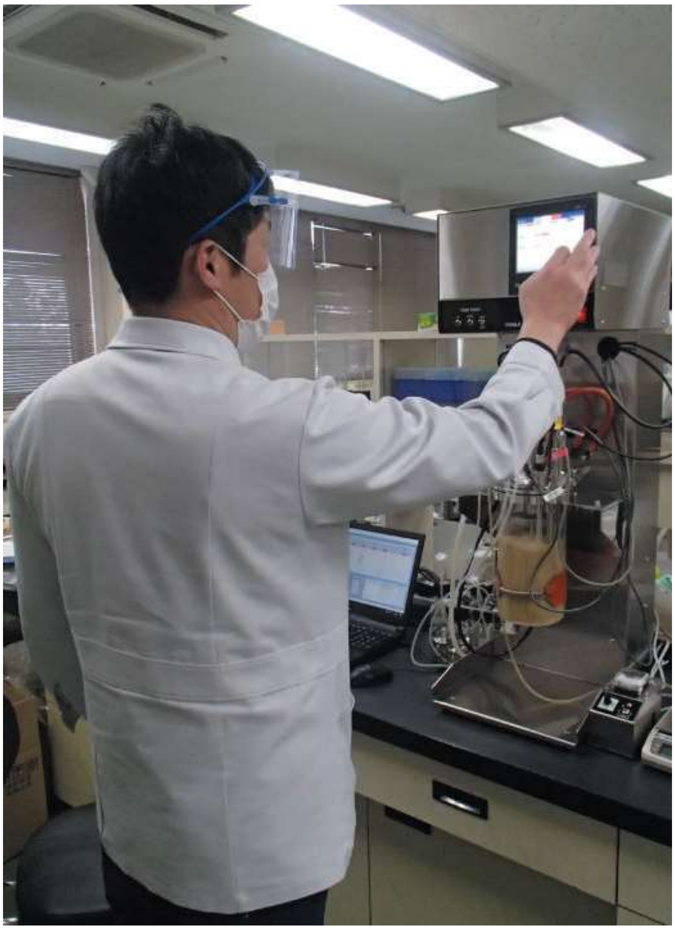
The Baculovirus Expression System

Another method successfully used in Japan to develop veterinary pharmaceuticals is the baculovirus expression system which expresses the target gene in the silkworm *Bombyx mori*, as opposed to expressing the protein in insect cell culture. The virus expressing the cloned target gene is injected into silkworms that produce the target protein in the haemolymph, which is then purified. The advantage with this system is that the scale-up of this system only involves increasing the number of silkworms at the manufacturing site and purification. The silkworms are easy to keep and manage, and totally depend on human intervention for culture since they are blind, meaning that the silkworm cannot escape into the wild. Using this method, a feline recombinant interferon omega (IFN-ω) was developed for application in cats infected with calicivirus, feline leukaemia virus (FeLV) and in dogs infected with parvovirus. The product was first approved in Japan in 1994 for feline calicivirus infection and later in Europe for FeLV, and was also found to be effective in feline infectious peritonitis (FIP)[8]. In 2005, a recombinant canine interferon gamma (IFN-γ) was similarly developed in Japan and approved for the treatment of canine atopic dermatitis[9,10]. In 2014, a desensitisation therapy for house dust mite-induced canine atopic dermatitis was approved in Japan. The active ingredient in this product is the house dust mite allergen Der f 2 produced in silkworms using the baculovirus system, which is then purified by chromatography and bound to the polysaccharide pullulan.

Recombinant bovine granulocyte-macrophage colony-stimulating factor (rboGM-CSF) was also produced by expression of the rboGM-CSGF gene using the baculovirus system and was tested in dairy cows with *S. aureus*-associated subclinical mastitis. Injection of rboGM-CSF directly into the udders induced a modest improvement in cows with early- stage subclinical mastitis[11]. GM-CSF is thought to enhance local immunity against pathogens by stimulating T cell responses and may be useful in mastitis due to antibiotic- resistant forms of *S. aureus*.

Transgenic Silkworm

In 2003, Prof. Katsutoshi Yoshizato and his team at Hiroshima University and other co- workers published a paper describing the production of human type III procollagen in the cocoons of transgenic silkworms[12]. A construct that encodes a fusion protein of the gene of interest and silk fibroin is introduced into the silkworm genome which results in silkworm that produce the protein of interest in their silk. The product can then be harvested from cocoons. The transgenic silkworm can also express fluorescent protein markers such as the enhanced green fluorescent protein which allows relatively simple selection of the transgenic silkworm expressing the target protein. Unlike *E. coli*, the transgenic silkworms can allow expression of very high molecular weight proteins, and also allow for post-translational modification of the proteins such as glycosylation. The expressed protein can be extracted from the resulting cocoons using standard purification processes. More recently, Dr. Katsura Kojima and his team at the National Institute of Agrobiological Sciences produced a transgenic silkworm



that was able to produce 'high- toughness' spider silk in their cocoons[13]. The production of veterinary pharmaceuticals using this approach is also being explored. Another interesting feature of this technology is that antibodies can also be produced in the cocoons of the transgenic animals[14], possibly presenting a relatively inexpensive method for the large-scale production of antibodies. Conventional antibody production using the large-scale mammalian cell culture described below can be very expensive, and result in prices that may be prohibitive to the end-user. Dr. Yoshio Kiku and colleagues also produced recombinant bovine GM-CSF in the cocoons of transgenic silkworms and used the cytokine to show that it decreased somatic cell counts (SCC) in the milk of cows with subclinical mastitis when infused directly into the udders[15].

Transgenic Chickens

Another promising area of biotechnology is using genetic engineering techniques that generate hens which produce high levels of bioactive proteins in the egg white, which are then purified by chromatographic methods. In a recent article by Dr Isao Oishi and colleagues, the generation of ovalbumin gene-targeted hens producing human interferon beta is described. The authors used the CRISPR/Cas9 gene editing technology to introduce the interferon beta gene into the ovalbumin locus and then selected progeny to generate a flock with stable and high yields of the cytokine[16]. The authors state that as compared to earlier technologies which involved injecting retroviral vectors into chick embryos, the newer method is less laborious and more predictable in terms of yields. The technology can also be applied to generate proteins useful in animal health.

Mammalian Cell Expression Systems

Some of the most profitable human pharmaceutical products on the market, such as those developed for inflammatory disease and cancer are antibody products. The expression of genes coding for antibodies against targets of interest in mammalian cells is now a well- accepted method for the large-scale production of therapeutic antibodies. In addition to the cloning of the canine interleukin-31 gene described earlier, a number of Japanese researchers have cloned genes of interest, antibodies for which may have therapeutic or diagnostic applications. Among these is a monoclonal antibody for canine thymus and activation regulated chemokine (TARC)[17]. Using this monoclonal antibody, the levels of serum TARC were found to be associated with the clinical scores of canine atopic dermatitis (in press). The same TARC antibody may well be useful in the diagnosis of canine atopic dermatitis. Antibody products are mostly injected intravenously for systemic use, and the doses that are sometimes required for pharmacological effect can be relatively high. It is therefore important that a highly efficient expression system is available for the stable production of high antibody yields. Certain commercially available cell lines and their strains, such as the Chinese Hamster Ovary (CHO) cell line, are well characterised and used for the production of both human and veterinary pharmaceuticals. Using this mammalian cell culture system, antibody yields of 3g/L have also been reported after screening and selection of cell clones expressing high levels of the target protein, and optimisation of cell culture conditions.

Plant Expression Systems

In 2013, the Japanese company Hokusan K.K. successfully obtained Japanese marketing authorisation for its canine interferon alpha-4 product Interberry α for use in dogs with gingivitis. The product is a lyophilised powder from strawberries containing canine interferon α which is expressed in genetically modified (GM) strawberry plants. The GM strawberry plants are grown in a biosecure enclosure which does not allow escape of pollen from the GM plants into the environment, and manufacturing is performed under Good Manufacturing Practice (GMP) requirements. The product is applied by wet finger directly to the affected area to reduce inflammation and is said to have an excellent safety profile.

Innovation and Regulatory Approval

In most jurisdictions, in order to obtain marketing authorisation for a novel veterinary medicinal drug, emphasis is rightfully placed on safety and efficacy, whether the drug be intended for animals that are a source of our food or for animals that are our treasured companions. Innovative new medicinal drugs, especially those that employ genetically engineered intermediates during production, involve processes that are sometimes novel to regulatory agencies, resulting in long periods of evaluation in the process of registration. Pascal Drake and colleagues have written an informative review on the regulatory hurdles one may encounter when registering a new drug, taking as an example registration of recombinant biologics as compared to nutraceuticals derived from plants[18]. One hopes that as our knowledge and application of biotechnology evolves, the regulatory landscape also evolves to allow us to provide the best possible products at affordable prices that can be met by the consumer.

Conclusion

This editorial is by no means an exhaustive review of all the research on biotech methods and their applications ongoing at Japanese laboratories, but rather a short overview of biotechnology and its potential, taking Japan as an example.

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