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Minireview

Development of Human Antibody Medicine by Use of Human Antibody-Producing Transchromosomic Animals.

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INTRODUCTION

In 1890, Emil von Behring and Shibasaburo Kitasato demonstrated that immunization of rabbit with heat-inactivated diphtheria toxin gave it resistance against diphtheria infection and that the sera from such immunized rabbits gave the same resistance to non-immunized rabbits. They originated serotherapy by its application for treatment of human [1]. Since appearance of antibiotics in 1935, the serotherapy has not been much used for human microbial infectious diseases. But at present time, the serotherapy is used for specific viral infectious diseases and accidental bites of vipers. In 1975, Kohler and Milstein [2] developed hybridoma technology, which made it possible to obtain large amounts of highly specific, high-affinity mouse monoclonal antibodies by the fusion of mouse splenocytes from immunized mouse with myeloma cells. From then antibody research became stellar in immunology and was very much used for developing classification makers to identify various differentiated cells, cancer cells and specialized cells. Although monoclonal antibody research had been done for more than 20 years, its use as therapeutic drugs was very limited because of its immunogenicity to humans. Since recombinant DNA technology was applied to the development of mouse/human chimeric, humanized and fully human antibody to reduce its immunogenicity to humans in the 1980s, antibody research has become popular again. Nowadays, more than 17 antibody products have been launched in the market and over 100 antibody products are under clinical development [3]. For serotherapy

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with humanized polyclonal antibody, human antibody-producing rabbit and cows are now under development. In the near future, the currently used antibody products derived from antigen-immunized horses and rabbits,and gamma-globulin products from a pool of human donors' blood will be replaced with human antibody purified from the human antibody producing animals. Human polyclonal antibody is a mixture of various antigen-specific antibodies to effectively get rid of various antigens, which is thought to be useful for protection/treatment of emerging virus-derived infectious diseases such as SARS (Severe Acute Respiratory Syndrome) and avian flu, and hospital infection such as MRSA (Methicillin Resistant *Staphylococcus aureus*) and multi-drug resistant *Pseudomonas aeruginosa*.

In this review I describe development of human antibody-producing mice and cows and discuss the potential of human antibody-producing animal derived human polyclonal antibody products for treatment/protection of bacterial and virus infectious diseases.

HUMAN ANTIBODY-PRODUCING TRANSCHROMOSOMIC MICE

We planed to develop transgenic mice carrying a whole set of human immunoglobulin (Ig) genes to produce fully human antibody in 1992. It was difficult to introduce a whole set of human Ig genes into mice by conventional transgenic procedure because the DNA size of the Ig genes was very large (which was 1~2 mega base long). We have developed a novel procedure in which a human chromosome (hCh) fragment was introduced into mouse embryonic stem (ES) cells, from that chimeric mice were produced [4]. The procedure to produce a transchromosomic (TC) mouse is shown in Fig. 1.

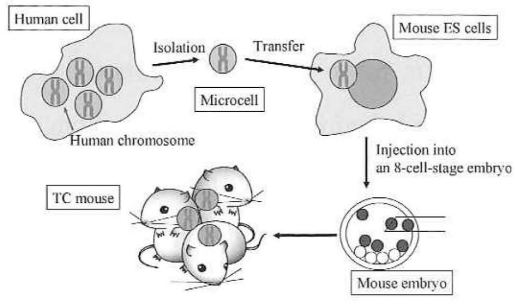

Fig.1. Generation of transchromosomic (TC) mice.

Human fibroblast cells containing a human chromosome tagged with a drug selection marker were treated with colcemid for 48 hrs to obtain microcells containing each chromosome. Such microcells were isolated by direct centrifugation of cultured flasks filled with medium containing cytochalasin, then fused with ES cells in the presence of PEG. The ES cells containing a human chromosome with a drug selection marker could be cloned under drug selection. In the chromosome-

transfer process, the transferred chromosome frequently fragmented and was maintained stably in ES cells. Those ES cells were injected into 8 cell stage embryos to generate chimeric mice, which were cross-bled with wild type mice to obtain a transchromosomic mouse containing a germline-transmitted human chromosome or its fragment.

Microcells containing hCh fragments were prepared and fused with mouse ES cells with PEG (polyethylene glycol). Then the ES cells were injected into an 8 cell stage embryo to produce a chimeric mouse. We call such a chromosome-transferred transgenic mouse 'a TC mouse'. Human antibody-producing mice were generated by cross-bleeding among the four mouse lines, which were knockout (KO) mice with disruption of Ig-heavy or Ig-light kappa chain genes and TC mice with a hCh fragment encoding human Ig-heavy or light kappa chain gene [5]. We found that a hCh#2 fragment encoding human Ig-light kappa chain gene was relatively unstable in mouse cells and could improve the human antibody-producing mouse with the following two strategies.

(1) We produced the KM mouse by cross-bleeding of the TC mouse with a hCh#14 fragment encoding an Ig-heavy chain gene with the YAC-transgenic mouse containing 50% of an Ig-light kappa variable and an entire constant region gene, which was produced by the US biotech company, Medarex Inc. [6].

(2) A hCh#2 fragment encoding human Ig-light chain gene was translocated onto a hCh#14 fragment, which was stable in mouse cells, to build a stable human artificial chromosome (HAC, see the chromosome in Fig. 2) containing both human Ig-heavy and light chain genes.

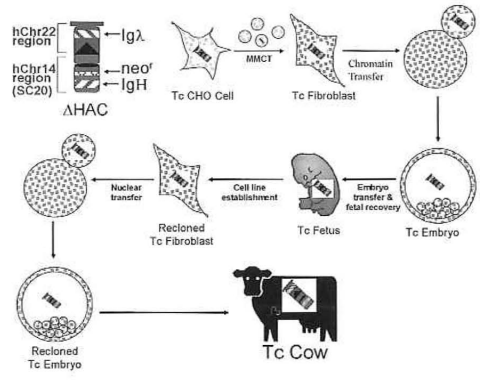

Fig.2. Generation of transchromosomic (TC) cows.

A HAC (human artificial chromosome) was prepared by translocation of a human Ch#22 fragment containing an Ig-λ light chain locus onto a human Ch#14 fragment containing an Ig-heavy chain locus. The HAC-containing microcells were prepared for MMCT into bovine fetal fibroblast cells, which nucleus were transferred into an enucleated egg by the chromatin transfer method and cultured *in vitro* to develop to the blastocyst stage. The embryo was implanted into uterus of surrogate mother. The 40 day-fetus was recovered and

the fibroblast cells were prepared from the fetus and stored. The nuclear chromatin of the fibroblast cells with the HAC was transferred into an enucleated egg to generate TC cows. This figure was modified from the original figure described in the article [7].

Various human monoclonal antibodies for therapeutic use have been obtained by the conventional hybridoma method since the KM mice and HAC mice became available [8].

HUMAN ANTIBODY-PRODUCING TRANSCHROMOSOMIC COWS

A much larger animal with a human chromosome fragment is necessary for production of human polyclonal antibody than a mouse, which is useful for production of monoclonal antibodies. It is a humanized version of the serotherapy which was originated by Bhering and Kitasato. When we started the research in 1999, ES cell lines of any other animals than mouse was not available and for embryo cloning by nuclear transfer, three species of animals (goat, sheep and bovine) were available. In cows, much IgG which is useful for antibody medicine can be obtained not only from blood but also from milk. We selected cows as a host animal for human antibody production because it produced a lot of IgG in milk, which was considered to be safe as daily food.

A HAC containing both human Ig-heavy and light chain genes was introduced into bovine fetal fibroblast cells with the microcell mediated chromosome transfer (MMCT). The HAC-containing fibroblast cells' nuclei was transferred into an enucleated egg by chromatin transfer method [9], cultured *in vitro* to the blastocyst stage and implanted into uterus of a surrogate mother (Fig.2). The HAC-containing cows produced a small amount of fully human and a large amount of cow polyclonal antibody in their blood because their endogenous Ig genes still function [10]. Then the sequential gene targeting method has been developed to produce a cow with disruption of multiple endogenous genes [11]. The procedure is described in Fig. 3.

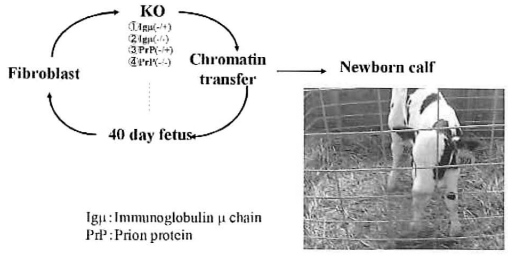

Fig.3. Multiple disruption of endogenous genes in cows.

One allele of the endogenous Ig-heavy chain μ gene in the fibroblast cell was disrupted by gene targeting and a 40-day fetus was obtained by chromatin transfer of the fibroblast cell nuclei into an enucleated egg. Then the

fibroblast cell line was prepared again from the 40-day fetus with the gene disruption, and the other allele of the Ig-heavy chain μ gene was disrupted and the 40-day fetus with disruption of the both alleles was obtained again. By such sequential gene-disruptions in the fibroblast via embryo cloning, a cow with multiple endogenous gene disruptions can be obtained.

Firstly one allele of the endogenous Ig-heavy chain gene in the fibroblast cell was disrupted and 40-day bovine fetus was obtained by chromatin transfer of the fibroblast cell nuclei into an enucleated egg. Secondly the fibroblast cell line was prepared again from the 40-day fetus, and the other allele of the Ig-heavy chain gene was disrupted and 40-day fetus was obtained again. By such sequential gene-disruptions in the fibroblast via embryo cloning, a bovine with multiple endogenous gene disruptions can be obtained. Technologies to produce a HAC-containing cow to express human polyclonal antibody and to produce a cow not to express its endogenous either heavy chain μ or prion protein, have been established. In the very near future, the human polyclonal antibody-producing cows will be commercially available. Recently we have demonstrated that prion gene-disrupted cows could be born without any obvious problem and that mutated prion protein (BSE pathogen) could be amplified *in vitro* in the brain homogenate from a wild type cow but not in that from a prion gene-disrupted cow [12].

EFFICACY OF TC-MOUSE DERIVED HUMAN POLYCLONAL ANTIBODY

Our plan is to produce tranchromosomic cows bearing the entire human Ig-heavy and light chain loci, which will produce fully human polyclonal antibody as described above. We have tested the potential of this approach using the TC mouse to produce human polyclonal antibody. We tested the efficacy of human polyclonal antibody derived from the TC animals in feasibility studies using anti-sera and purified gamma globulin from TC mice immunized with formalin-fixed *Pseudomonas aeruginosa* (PA) or vaccine preparation of Japanese encephalitis virus (JEV) [6]. The TC mouse-derived anti-sera and purified gamma globulin against PA showed *in vivo* neutralizing activity of PA (Table 1). On the other hand, conventional human-derived plasma or human gamma globulin purified from human donors failed to show neutralizing activity. The *in vitro* neutralizing titer (compensated with the concentration of IgM and IgG in the sample) of TC mouse-derived anti-sera against JEV was 80-fold higher than that of human-derived plasma (Table 2).

These results suggest that TC animal-derived human anti-sera and human gamma globulin against pathogens may show much better efficacy than gamma globulin derived from pools of human donors.

Table 1. The effect of anti-sera against *Pseudomonaus aeruginosa* on the death of mice challenged by incoculation of *Pseudomonas aeruginosa.*

PBS(300μl)	Human plasma	TC-derived serum (1/4 dil)	Human IgG (20μg)	TC-derived human IgG (20μg)
5/5	5/5	1/5	5/5	2/5

The TC mice were immunized by ip injection with about 100 mg per mouse of *Pseudomonas aeruginosa* (PA) that had been fixed with 1% formalin for more than 24 hrs and washed with phosphate-buffered saline (PBS). Challenge involved inoculation of PA (6.6 x 10^7 cfu) into a mouse (5-week old ICR) 30 min after anti-serum injection. Mortality (dead mice/treated mice) was determined 4 days after PA inoculation.

This table was reproduced from the original article [6].

Table 2. *In vitro* neutralization activity of anti-sera against Japanese encephalitis virus (JEV).

	Titer (dilution)	Titer compensated[a]
Human plasama	29	2.8
TC-derived serum	971	224.8

[a]The titer values were compensated with the concentrations of IgM and IgG contained in the serum samples.

The TC mice were immunized four times at 2-week intervals with Japanese encephalitis virus (JEV) vaccine prepared by the Chemo-Sero-Therapeutic Research Institute. Freund's adjuvant was included with the immunogen. Immobilized human or TC serum (56℃ for 30 min) diluted with buffer was mixed with the same volume of JEV (2,000 PFU/ml) solution and incubated at 37℃ for 90 min, and then titrated using a plaque assay on Vero cells. The neutralizing titer represents the dilution of the serum sample showing 50% plaque reduction compared to the control.

This table was reproduced from the original article [6].

CONCLUSION

The rapid progress of recombinant DNA and transgenic animal technologies has led to the invention of human/mouse chimeric,

humanized, and fully human antibodies. These strategies can greatly reduce the immunogenicity of monoclonal antibodies in humans. Currently, some therapeutic chimeric, humanized and fully human antibodies are already being marketed in the United States and elsewhere. We expect the human antibody-producing transchromosomic mouse, 'KM mouse ™ ' to be of major importance in the derivation and development of fully human antibodies.

We also showed that the human antibody producing TC mouse-derived anti-sera and gamma globulin had much higher titers and efficacy for the neutralization of pathogens *in vitro* and *in vivo* than human serum and gamma globulin prepared from human donors. These results lead us to expect that human antibody containing anti-sera and human gamma globulin produced in TC cows will show much higher efficacy against pathogens and will be safer for use in humans than human antibody preparations derived from pools of human donors.

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