

Establishment of Animal Models Using Experimental Rats for Allogeneic Tissue Transplantation and Quantitative Flow Cytometric Detection of Immunochimera

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Abstract

Establishment of animal models used for allogeneic tissue transplantation, MHC phenotyping as well as quantitative detection of immunochimera were carried out in this study. Both Buffalo (BUF) and Lewis (LEW) rats were chosen as the target animals. The rats were treated with single dose cyclophosphamide (Cy) of 25 to 100 mg/kg to mimic the standard conditioning therapy. Total white blood cells (WBC) were monitored daily for up to 8 days: WBC reached the nadir by day 4 and started to recover by day 5 with an obvious rebound at day 7 of Cy treatment. Flow cytometric techniques were used to determine the haplotypes of the major histocompatibility complex (MHC) as well as quantitative detection of immunochimerism in unfractionated rat WBC. Monoclonal antibodies against the rat class-I MHC antigens RT1A^{ab} and RT1A^u were used to label the class-I MHC antigens on total rat WBC. The result showed that the BUF rats were positive for both RT1A^{ab} and RT1A^u antigens, whilst the LEW rats were negative for both. Immunochimera was mimicked in vitro by serial dilution, ranging from 1/1 to 1/10⁵ of (BUF/LEW) WBC. A sensitivity of 1/10⁴ (BUF/LEW WBC) was achieved. The results showed that there were at least 2 major MHC mismatched loci between BUF and LEW rats and flow cytometry provided a sensitive method for the detection of immunochimera in unfractionated rat WBC. We concluded from this study that both strains of rats could be used as models for allogeneic tissue transplantation across at least two major MHC-mismatches. The sensitivity of flow cytometric method was satisfactory for the detection of immunochimera.

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Introduction

Allogeneic tissue and organ transplantation has long been an important medical issue, as it provides an effective treatment alternative for a broad spectrum of medical problems. It is essential to establish the animal models used for a specific type of allogeneic tissue or organ transplantation prior to its clinical application. Rodents have been used as animal models for allogeneic cell or organ transplantation for several decades.¹ The Buffalo (BUF) and Lewis (LEW) rats were developed in the 1940s and 1960s respectively.^{2,3} The MHC haplotypes in their offspring were however unknown to our laboratory due to lack of detailed biological description from

the suppliers. It was essential to test the MHC phenotypes in the BUF and LEW rats, as we decided to use both strains of rats as the animal models for our research on allogeneic tissue transplantation. Certain monoclonal antibodies, such as RT1A^{ab}, and RT1A^u, have been developed against different alleles of RT1A, the rat class-I MHC gene, since late 1970s. Although these monoclonal antibodies have been reported to be strain-specific, discrepancies and confusion on the MHC haplotypes in both BUF and LEW rats from the reports in the literature and the information provided by certain manufacturers of the antibodies for rat MHC typing have been encountered.^{4,7} Due to the uncertainty of information on rat

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MHC haplotypes in the rat strains available to us, it was thus essential to determine the MHC haplotypes, using the monoclonal antibodies against different antigens of the rat MHC class I gene locus - RT1 A. During allogeneic transplantation, the recipients are usually prepared with either a lethal dose of total body irradiation (TBI) or certain immuno- and myelosuppressive drugs, such as cyclophosphamide, busulfan and methotrexate. Cyclophosphamide is usually used as the "gold standard" for conditioning regimen in allotransplants.⁸ Successful engraftment in allogeneic transplantation relies largely on the establishment of immunochimerism in the recipients, since immunochimera induces immunotolerance which allows both short-term and long-term survival of the engraftments, such as bone marrow, kidney, skin, heart, and liver.⁹⁻¹⁴ Reliable and sensitive techniques are required for the detection of immunochimera in the recipients to monitor the immunological chimeric status in the recipient.

Materials and Methods

Experimental Rats and Treatment with Cyclophosphamide (Cy)

Both male and female adult Buffalo and Lewis rats were purchased from the Animal Holding Units, National University of Singapore. The rats were divided into 4 groups and a single dose of Cy (Glaxo-Wellcome) of 25 mg/kg, 50 mg/kg, 75 mg/kg and 100 mg/kg was given intravenously to each group of rats. Total white blood cells (WBC), haemoglobin (Hb), and platelets were monitored and recorded daily using an automated haemocytometer (K-4500 Haematology Analyser, Sysmex, Japan) for up to 8 days. Two out of 6 (33.3%) LEW rats died of skin infection. All procedures used in the rats were strictly carried out according to the International Guiding Principles for Animal Research.¹⁵

Determination of Rats MHC-Loci Using Unfractionated WBC

Whole blood of 100 to 200 µl was obtained from the tail veins by routine methods from both BUF and LEW rats. The red blood cells (RBC) were lysed using the FACS lysing solution (Becton-Dickinson, USA). The WBC were suspended at a concentration of 10^7 /ml in $1 \times$ PBS complemented with 1% bovine serum albumin. The antibodies against the rat Class-I MHC antigens included biotinylated rat-anti-rat RT1A^{ab} (IgG2b, Pharmingen, San Diego, USA) and pure mouse-anti-RT1A^u (IgG2b, Bio-Source, CA, USA) were used for primary antibody labelling, whilst avidin-fluorescein isothiocyanate⁻ (avidin-FITC, Becton-Dickinson, USA) and FITC-conjugated sheep-anti-mouse (SAM) (Silenus, Australia) was used for secondary antibody labelling. 5 µl of each primary antibody was added into 10 µl of cell suspension in an eppendorf tube and incubated at room temperature in dark. The cells were then washed twice

with PBS and then labelled with secondary labelling reagents for a further 20 minutes at room temperature in the dark. The cells were then washed once with PBS and fixed with 2% formaldehyde before further analysis. Mouse and rat IgG2b (Pharmingen, USA) was used as immunoglobulin isotype controls for both RT1A^{ab} and RT1A^u. A flow cytometer (FACSCalibur) and computer software (CellQuest, Becton-Dickinson, San Jose, USA) were used for data acquisition and analysis.

Quantitative Detection of "Immunochimera"

Upon successful detection of the major MHC loci in the rats, immunochimera was mimicked *in vitro* by serial dilution. The WBC from BUF rats were mixed into WBC from LEW rats ranging from 1/1 to 1/10⁵ (BUF/LEW) cells. The labelling procedures for both primary and secondary antibodies and flow cytometric detection were identical to that described in "Determination of Rats MHC-Loci Using Unfractionated WBC".

Results

Response to Treatment with Cyclophosphamide

All the blood cell components in both BUF and LEW rats showed a dose-dependent pattern. The total WBC of the BUF rats reached the nadir by day 4 in the rats that received 75 mg/kg and 100 mg/kg of Cy and started to recover by day 5. There was an obvious rebound of total WBC by day 7 of Cy injection (Fig. 1a). Compared with the BUF rats, the LEW rats showed a slightly different pattern of response to the treatment. The nadir of total WBC was only observed on day 4 in the 100 mg/kg group, and the WBC started to recover on day 6 of Cy injection. A lesser rebound of WBC was observed in the LEW rats (Fig. 1b). Two LEW rats died of skin infections secondary to the severe myelosuppression and no death was seen in the BUF rats.

Determination of Rats MHC Phenotypes

The results showed that the BUF rats were positive for both RT1A^{ab} and RT1A^u antigens, whilst the LEW rats were negative for both. The results of flow cytometric MHC typing are shown in Figure 2.

Quantitative Detection Of Immunochimera

Both positive and negative populations of cells were detected in the "chimeric" samples with serial dilution ranging from 1/1 to 1/10⁴ (BUF/LEW) WBC, and a sensitivity of detection of 1/10⁴ (BUF/LEW) WBC was achieved (Fig. 3). Further attempt was made to increase the sensitivity of detection beyond 1/10⁴, but it was unsuccessful.

Discussion

The rats have been used as experimental models since allogeneic tissue transplantation was first pioneered almost half a century ago.¹ Researches on allogeneic

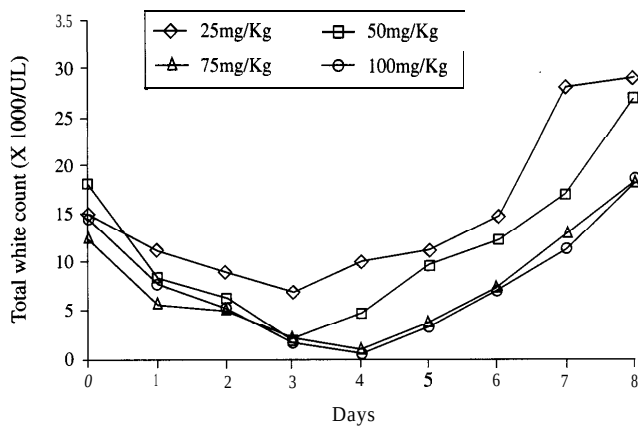


Fig. 1a. Total white blood cells (WBC) in Buffalo rats treated with cyclophosphamide.

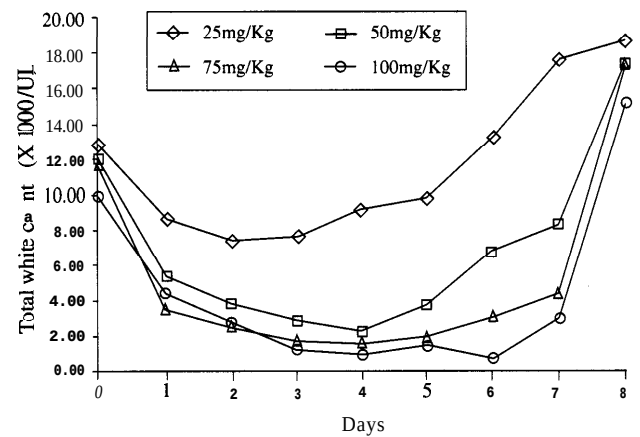


Fig. 1b. Total WBC in Lewis rats treated with cyclophosphamide.

Fig. 1. Figures show the response of both BUF and LEW rats to a single i.v. injection of cyclophosphamide at different dosages. The WBC showed a dose-dependent pattern.

tissue transplantation across the major HLA mismatches, aiming at ultimate clinical applications in certain surgical aspects, such as allogeneic tissue and limb transplantation required in hand, orthopaedic and plastic surgery, are being carried out in various centres. It is essential to establish animal models which meet all the requirements for our research. Experimental rats were chosen as models as they could surgically be manipulated with lesser difficulties than smaller animals like mice, or larger animals like pigs. Cyclophosphamide is one of the classical drugs used for immunosuppression as well as

for conditioning in allografts in both clinical practice and animal research.^{7,16-18} Our results showed that high dose Cy of 75 to 100 mg/kg, approximately equivalent to 2 to 3 g/m² in human, could be reasonably tolerated by both BUF and LEW rats, since only 2 LEW rats died of myelotoxicity in this study. Both LEW rats died due to skin infection secondary to myelosuppression. Lower dosages may not be adequate for sufficient immunosuppression required for the induction of immunotolerance; however this hypothesis can only be proven by further studies.

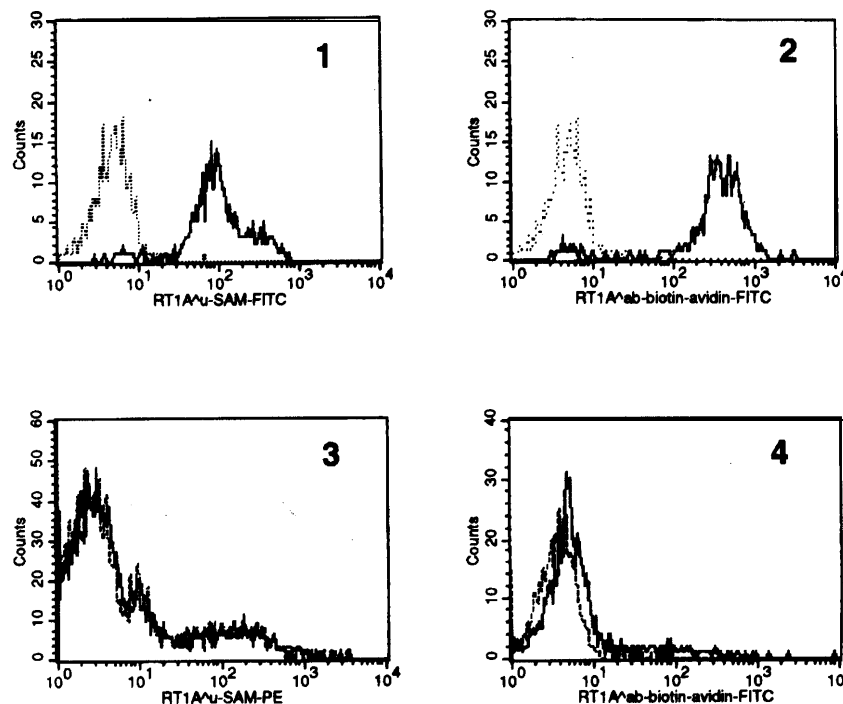


Fig. 2. Flow cytometric histogram showing the rat MHC class-I antigens RT1A^u and RT1A^b in both BUF and LEW rats. The peaks under dotted lines represent WBC stained with mouse/rat IgG2a as controls, and the solid lines represent the WBC stained with Mabs against RT1A^u and RT1A^b and secondary staining with sheep-anti-mouse-FITC for RT1A^u and avidin-FITC for RT1A^b monoclonal antibodies. The WBC from the BUF rats showed positive reaction to both RT1A^u and RT1A^b (1 & 2), but the LEW rats were negative for both MHC antigens (3 & 4).

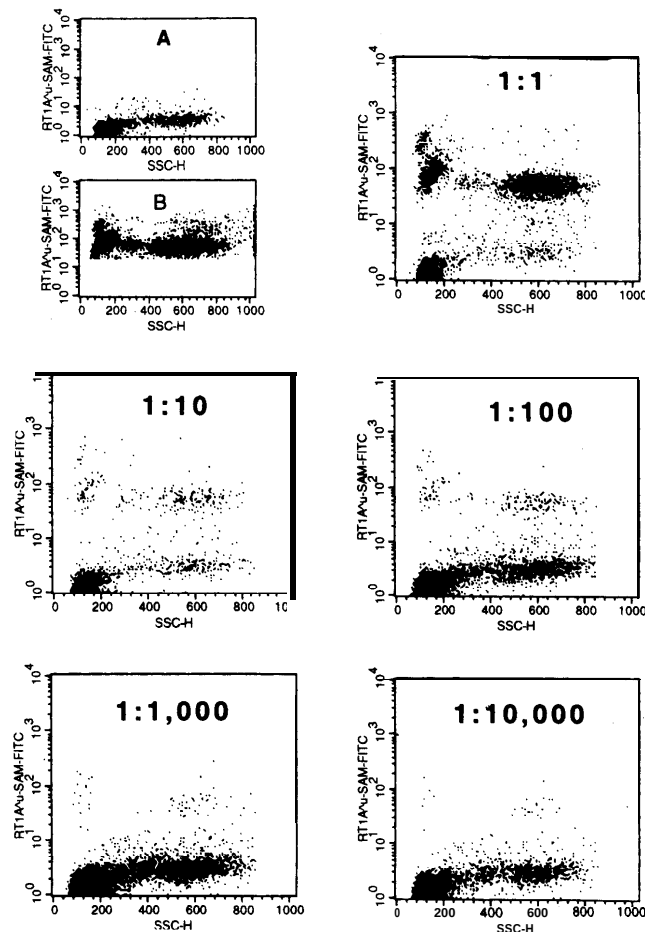


Fig. 3. Flow cytometric detection of immunochimera. Yaxis = Logfluorescence intensity. A: Negative control with WBC from LE Wrat: The LE W WBC do not show any reaction with anti-RT1A (Log fluorescence intensity is less than 10^1). B: Positive control with WBC from the BUF rats: The BUF WBC reacted with anti-RT1A with Logfluorescence intensity greater than 10^2 . The sensitivity of detection in this serial dilution is about $1/10^4$.

The MHC phenotyping was essential to this research, since no information of the MHC phenotypes on both BUF and LEW rats was available to us from the suppliers. This has led to the experiments on MHC phenotyping in the rats. The results showed that there were at least 2 major mismatches of the class-I MHC locus between the BUF and LEW rats, demonstrating that both strains of rats were suitable models for the next phase of our research. Our study has also shown that it was possible to use lysed whole blood for antibody labelling and flow cytometric techniques for rat MHC phenotyping. Certain laboratory procedures have been established during this study: MHC phenotyping using lysed whole blood has simplified the antibody labelling procedure compared with the conventional labelling methods using the mononuclear cells by density gradient separation. This also allows fast, less labour-intensive and inexpensive MHC-typing and detection of chimera in our future research.

Long-term engraftment in allogeneic cell or tissue

transplantation relies largely on the establishment of immunochimerism in the recipients.¹⁹⁻²¹ Transplantation tolerance and the long-term acceptance of allografts in the absence of immunosuppression remain as an elusive goal in humans. Different techniques, such as flow cytometry and polymerase chain reaction (PCR) using probes to different VNTR (variable number tandem repeats or microsatellites), have been used for the detection of immunochimera in the recipients of allografts with variable log sensitivity.^{9,11,12,21} The results from this study suggested that flow cytometric technique could be used as a sensitive method for the detection of immunochimerism in experimental rats.

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REFERENCES

1. Billingham R E, Silvers W K. Inbred animals and tissue transplantation immunity. *Transpl Bull* 1959; 6:399-406.
2. Iseri O A, Gottlieb L S, Hegsted D M, Vitale J J. Vitamin B12 and methionine metabolism in the Buffalo rat liver and Morris 5123 hepatoma. *Lab Invest* 1972; 27:226-35.
3. Lewis P D. The fate of the subependymal cell in the adult brain, with a note on the origin of microglia. *Brain* 1968; 91:721-36.
4. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975; 256:495-7.
5. Kohler G, Milstein C. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur J Immunol* 1976; 6:511-9.
6. Hard D N, Fabre J W. Problems in the use of erythrocytes for RT1A typing studies, probably due to quantitative differences in RT1A antigen expression in different strains. *Transpl Proc* 1981; 13:1329-32.
7. Hart D N, Newton M R, Reece-Smith H, Fabre J W, Morris P J. Major histocompatibility complex antigens in the rat pancreas, isolated pancreatic islets, thyroid, and adrenal. *Transplantation* 1983; 36:431-5.
8. Thomas E D. A brief review of bone marrow transplantation-1996. In: McArthur J R, editor. *Haematology 1996. Education Programme for the 26th Congress of International Society of Haematology*. Singapore: University of Washington School of Medicine, 1996:15-7.
9. Colson Y L, Wren S W, Schuchert M J, Patrene K D, Johnson P C, Boggs S S, et al. A nonlethal conditioning approach to achieve durable multilineage mixed chimerism and tolerance across major, minor, and hematopoietic histocompatibility barriers. *J Immunol* 1995; 155:4179-88.
10. Sharabi Y, Aksentjevich I, Stunt III T M, Shachs D H, Sykes M. Specific tolerance induction across a xenogeneic barrier: Production of mixed rat/mouse lymphohematopoietic chimeras using a nonlethal preparative regimen. *J Exp Med* 1990; 172:195-202.
11. Shapiro R, Rao A S, Fontes P, Jordan M, Scantlebury V I, Vivas C, et al. Combined simultaneous kidney/bone marrow transplantation. *Transplantation* 1995; 12:1421-5.
12. Lu L, Rudert W A, Qian S, Thomson A W, McCaslin D, Rao A S, et al. Growth of donor-derived dendritic cells from the bone marrow of murine liver allograft recipients in response to granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1995; 812:379-87.
13. Starzl T E, Demetris A J, Murase N, Fung J, Nakamura K, Thomson A W, et

- al. Cell chimerism permitted by immunosuppressive drugs is the basis of organ transplant acceptance. *Immunol Today* 1993; 14:326-47.
14. Fontes P, Abdul R, Demetris A J, Zeevi A, Trucco M, Carroll P, et al. Bone marrow augmentation of donor-cell chimerism in kidney, liver, heart and pancreas islet transplantation. *Lancet* 1994; 344:151-9.
 15. Howard-Jones N. A CIOMS ethical code for animal experimentation. *WHO Chronicles* 1985; 39:51-6.
 16. Mayumi H, Good R A. Long-lasting skin allograft tolerance in adult mice induced across fully allogeneic (multimajor H-2 plus multiminor histocompatibility) antigen barriers by a tolerance-inducing method using cyclophosphamide. *J Exp Med* 1989; 169:213-22.
 17. Clift R A, Buckner C D, Thomas E D, Bensinger W I, Bowden R, Bryant E, et al. Marrow transplantation for chronic myeloid leukemia: A randomized study comparing cyclophosphamide and total body irradiation with busulphan and cyclophosphamide. *Blood* 1994; 84:2036-43.
 18. Charlton B, Auchincloss Jr H, Fathman C G. Mechanisms of transplantation tolerance. *Ann Rev Immunol* 1994; 12:707-34.
 19. Nicholls B, Sykes M. Bone marrow chimerism and transplantation tolerance. *Curr Opin Immunol* 1997; 9:634-40.
 20. Markert L M, Kotstyu D D, Ward E F, McLaughlin T M, Watson T J, Buckley R H, et al. Successful formation of a chimeric human thymus allograft following transplantation of cultured postnatal human thymus. *J Immunol* 1997; 158:998-1005.
 21. Tomita Y, Khan A, Sykes M. Role of intrathymic clonal deletion and peripheral anergy in transplantation in mice conditioned with a nonmyeloablative regimen. *J Immunol* 1994; 153:1087-98.
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