Spleen Lymphocyte Kinetics in Mice under Normal and Inflammatory Conditions: An Application of the Transgenic Mouse Expressing β -Galactosidase (ROSA 26)

Ken-ichi HOSOYA,^c Tadayuki TAKASHIMA,^a Ken-ichi MATSUDA,^a and Tetsuya TERASAKI^{*,a,b}

^a Department of Molecular Biopharmacy and Genetics, Graduate School of Pharmaceutical Sciences, Tohoku University;

and ^b New Industry Creation Hatchery Center, Tohoku University; Aoba, Aramaki, Aoba-ku, Sendai 980–8578, Japan; and

^c Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama 930–0194, Japan. Received June 5, 2002; accepted July 24, 2002

The purpose of this study was to develop a method for the measurement of the cell kinetics of spleen lymphocytes using the ROSA 26 transgenic mouse ubiquitously expressing β -galactosidase (β -gal). Spleen lymphocytes were isolated from ROSA 26 mice and intravenously inoculated into C57BL/6 mice under normal conditions and inflammatory conditions following lipopolysaccharide (LPS) treatment. Spleen lymphocyte accumulation in tissues was determined as a measurement of β -gal activity. Spleen lymphocytes isolated from ROSA 26 mice have β -gal activities of 1.45×10^{-4} pg per cell. A good correlation between β -gal activities and cell numbers was obtained ($r^2=0.999$) over the range 1×10^3 to 1×10^7 cells, corresponding to 70 fg to 350 pg β -gal activity. Spleen lymphocytes (4×10^7 cells) were intravenously inoculated into normal mice and subsequently each tissue was isolated and the corresponding β -gal activity measured. Spleen lymphocyte accumulation was relatively high in the spleen and lymph nodes. The accumulated spleen lymphocyte cell number was 1.39×10^7 cells/g spleen and 5.45×10^7 cells/g lymph node 1 h and 6 h after inoculation, respectively, and this remained constant up to 24 h. In the lung, lymphocyte accumulation was 3.98×10^7 cells/g tissue 10 min after inoculation then gradually fell to 7.09×10^5 cells/g tissue after 24 h. In addition, the femoral muscle following intramuscular injection of LPS showed a high accumulation of spleen lymphocytes, whereas the untreated and contralateral femoral muscle had the same level as the background. In conclusion, spleen lymphocytes isolated from ROSA 26 mice can be used to measure β -gal activity and the sensitivity is relatively high over the 70 fg to 350 pg range. This suggests that cells isolated from the ROSA 26 mouse can be applied to the study of cell kinetics.

Key words cell kinetic; cell delivery; ROSA 26 mouse; β -galactosidase

Experiments involving cell kinetics in the body and lymphocyte migration provide important information about cell delivery to specific organs and cell or tissue transplantation. One of the analytical methods for cell kinetics *in vivo* has been used for transplantation to host animals using labeled cells.^{1,2)} For this purpose, an ideal marker for cells is absolutely critical for the quantitative determination of exogenously administered cells. A number of criteria have to be satisfied: it should (1) be ubiquitously expressed in all cells (2) not affect other cell functions (3) be confined to expressing cells (4) be easily and reliably detected in the host animal after administration. Although cell labeling *in vitro* using radioactive or fluorescent compounds and gene transfection have been used,^{3,4)} the precision of detection depends on the uniformity of cell labeling and sensitivity of detection of the labeled compound. Moreover, cell labeling *in vitro* appears to change cell morphology and characteristics. The ROSA 26 mouse, a transgenic mouse expressing the bacterial *lacZ* gene,^{5,6)} may meet all these criteria allowing its use for the investigation of cell kinetics.

The purpose of the present study was to develop a method for the measurement of cell kinetics using spleen lymphocytes isolated from ROSA 26 mice. We also demonstrated the application of the system and its limitations for β -galactosidase (β -gal) after intravenous inoculation of spleen lymphocytes under the normal and inflammatory conditions.

MATERIALS AND METHODS

Animals Breeding pairs of ROSA 26, which are trans-

genic mice expressing the bacterial *lacZ* gene, were purchased from Jackson Laboratory (Bar Harbor, ME, U.S.A.). The products (6 to 10 weeks) of these pairs served as donors for spleen lymphocytes. C57BL/6 mice (6 to 10 weeks, Charles River, Yokohama, Japan), which have congenital backgrounds for ROSA 26 mice, were used for spleen lymphocytes inoculation.⁷⁾ All investigations using mice described in this report conform to the requirements established by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University.

Isolation and Transplantation of Spleen Lymphocytes

The spleen was isolated from ROSA 26 mice and minced using two slide glasses. Then, fragments of minced spleen were passed through 70 μ m-nylon mesh and a suspension of cells was added to density separation medium (Lympholyte-M, Cederlane, Ontario, Canada) and centrifuged (1500g, 20 min, at room temperature) according to the manufacturer's protocol. The resulting spleen lymphocyte layer was removed and adjusted until it contained 8×10^7 cells/ml phosphate buffer saline (PBS). Isolated spleen lymphocytes (4×10^7 cells) were injected into recipient mice *via* the tail vein.

β -Galactosidase (β -Gal) Assay Tissue samples were isolated from recipient mice over the period from 10 min to 24 h after intravenous inoculation of the spleen lymphocytes. Inflammation of the femoral muscle of the recipient mice was produced by intramuscular injection of 20 μ g (67 μ g/ml PBS) lipopolysaccharide (LPS, WS typhosa 0901, Wako Pure Chemicals, Osaka, Japan). Each tissue sample, except for blood, was homogenized using Ultra Turrax (Kubota, Tokyo, Japan) equipment and then centrifuged (18500g,

* To whom correspondence should be addressed. e-mail: terasaki@mail.pharm.tohoku.ac.jp

20 min, 4 °C). The resulting supernatant was incubated at 48 °C for 60 min to inactivate endogenous β -gal-like activity. This treatment reduced the background level of β -gal activity in the tissue, but did not affect the β -gal activity derived from the bacterial *lacZ* gene.^{8,9)} After heating, samples were centrifuged (18500 *g*, 20 min, 4 °C) and the resulting supernatant was used for the β -gal assay. The β -gal activity in each sample was determined using the β -Gal Reporter Gene Assay Kit (Boehringer Mannheim, Mannheim, Germany). The chemiluminescence of each sample was measured in a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany). In the presence of β -gal, the β -galactopyranosyl group of 3-(4-methoxyspiro[1,2-dioxetane-3,2'-tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenyl- β -D-galactopyraoside (AMPGD) is cleaved to form the protonated form of 3-(4-methoxyspiro[1,2-dioxetane-3,2'-tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenol (AMPD) at pH 7.5. When the pH is shifted from 7.5 to 12, AMPD is changed to the anionic form and then 1,2-dioxetane fragmentation occurs with an increase in chemiluminescence.^{10,11)} This assay could detect as little as 2 fg β -gal (Sigma, St. Louis, MO, U.S.A.). The standard curve for relative light units versus β -gal had a very high correlation coefficient over the range 2 fg to 200 pg ($r^2=1.00$, $n=3$).

Data Analysis The spleen lymphocyte migration number (cells/g tissue) was estimated from the following equations:

$$Y = aX^b \quad (1)$$

where Y , X , a , and b are β -gal activity (pg), cell number, β -gal activity/cell (pg/cell) and coefficient, respectively. The parameters, a and b were estimated by fitting using the non-linear least squares regression analysis program, MULTI.¹²⁾

$$\text{cell number (cells/g tissue)} = \{(U - U_{bg}) / (U_{cell} \times C_q)\}^{1/b} \quad (2)$$

where U , U_{bg} , U_{cell} and C_q are the β -gal activity (β -gal/g tissue) in the recipient tissue sample, background (β -gal/g tissue), β -gal activity of one spleen lymphocyte of ROSA 26 mouse, and quenching coefficient in each tissue sample solution, respectively. The quenching coefficient was determined as the ratio of each tissue extract and buffer, i.e., 200 pg β -gal/ml tissue extract using lysis buffer (β -Gal Reporter Gene Assay Kit) vs. 200 pg β -gal/ml lysis buffer. The quenching coefficient for each tissue sample was as follows: 0.788 ± 0.004 (lymph node), 0.577 ± 0.015 (muscle), 0.490 ± 0.014 (spleen), and 0.397 ± 0.052 (lung) ($n=3$). Lymph nodes were isolated from brachium, groin, and small intestine. Muscle was isolated from femur.

Unless otherwise indicated, all data represent the mean $\pm$ S.E.M. An unpaired, two-tailed Student's *t*-test was used to determine the significance of any differences between two groups means.

RESULTS AND DISCUSSION

Spleen lymphocytes isolated from ROSA 26 mice exhibit β -gal activity (Fig. 1). A good correlation between β -gal activity and cell number was obtained ($r^2=0.999$) over the range 1×10^3 to 1×10^7 cells, corresponding to 70 fg to 350 pg β -gal activity, suggesting that the *lacZ* gene in the cells remains constant. The resulting value for β -gal activity per spleen lymphocyte was estimated to be 1.45×10^{-4} pg/

Fig. 1. Relationship between β -Galactosidase Activity and Spleen Lymphocyte Number of ROSA 26 Mice

Each point represents the mean $\pm$ S.E.M. ($n=3$). The error bar is smaller than the size of the symbol.

Fig. 2. Time-Courses of Apparent Spleen Lymphocyte Numbers in Normal Recipient Mouse Tissue after Intravenous Inoculation

Spleen lymphocytes were freshly isolated from the spleen of ROSA 26 mice. Then, 4×10^7 spleen lymphocyte cells were intravenously inoculated into recipient C57BL/6 mice. Each point represents the mean $\pm$ S.E.M. ($n=3-4$). Key; $\blacktriangle$, lymph node; $\circ$, spleen; $\bullet$, lung.

cell. This suggests that spleen lymphocytes isolated from ROSA 26 mice can be quantitatively measured down to a level of 1×10^3 cells/sample using a β -gal chemiluminescent reporter gene assay. Spleen lymphocytes (4×10^7 cells) were intravenously inoculated into normal mice and subsequently each tissue was isolated and its β -gal activity was measured (Fig. 2). The spleen lymphocyte accumulation was relatively high in the spleen and lymph nodes. The apparent accumulated spleen lymphocyte cell number was 1.39×10^7 cells/g tissue of spleen and 5.45×10^7 cells/g tissue of lymph node 1 h and 6 h after inoculation, respectively, and remained constant up to 24 h. In the lung, the lymphocyte accumulation was 3.98×10^7 cells/g tissue 10 min after inoculation and then gradually fell to 7.09×10^5 cells/g tissue by 24 h. These results suggest that spleen lymphocytes accumulate in the immune system. In the lung, spleen lymphocytes may be trapped in the lung capillaries during the first passage. On the other hand, when LPS (20 μ g) was injected in femoral muscle, spleen lymphocytes were accumulated in the ipsilateral side of femoral muscle 24 h after cell inoculation (5.57×10^6 cells/g tissue) (Fig. 3). However, only background levels

Fig. 3. Effect of LPS on Spleen Lymphocyte Migration into the Femoral Muscle of Recipient Mice

LPS (20 μ g) was intramuscularly injected into the left femoral muscle of recipient C57BL/6 mice. Spleen lymphocytes were freshly isolated from the spleen of ROSA 26 mice. Then, 4×10^7 spleen lymphocyte cells were intravenously inoculated into recipient C57BL/6 mice 7 h after LPS administration. Each femoral muscle (ipsilateral and contralateral side) was isolated and the β -gal activity determined 24 h after spleen lymphocyte administration. Each column represents the mean $\pm$ S.E.M. ($n=3$). *, β -Gal activity was not significantly different from that of the background.

were detected in the contralateral side of the femoral muscle and the femoral muscle of untreated recipient mice. These results suggest that lymphocytes migrate and accumulate at the site of the inflammation induced by LPS. This phenomenon is agreement with the accumulation of eosinophils, lymphocytes, and monocytes used as a model of acute inflammation induced by LPS.¹³⁾ The kinetics of tissue microglial cells and macrophage engraftment using the ROSA 26 mouse have been followed over one year by Kennedy and Abkowitz.⁶⁾ Tissues from transplanted animals were histochemically stained for β -gal to identify donor cells. Isolated cells from the ROSA 26 mouse would be suitable for determining cell kinetics. Analysis of cell kinetics gives us important information about gene delivery using cells as a carrier and for cell transplantation. In addition to the reporter gene of *lacZ*, transgenic mice and rats harboring a green fluorescent protein or placental alkaline phosphatase gene have been developed.¹⁴⁾ Therefore, several detection methods are now avail-

able and each has certain advantages and limitations.

In conclusion, spleen lymphocytes isolated from ROSA 26 mice could be chased by measuring β -gal activity after intravenous inoculation of normal and LPS-treated mice. These results suggest that cells isolated from the ROSA 26 mouse are suitable for the study of cell kinetics.

Acknowledgments The authors wish to thank Dr. T. Hishinuma and Mr. M. Tomi for helpful discussions and Ms. N. Funayama for secretarial assistance. This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, and Sports, Japan. It was also supported, in part, by The Japan Society for Promotion of Science.

REFERENCES

- 1) de Groot C. J. A., Huppes W., Sminia T., Kraal G., Dijkstra C. D., *Glia*, **6**, 301—309 (1992).
- 2) Krall W. J., Challita P. M., Perlmutter L. S., Skelton D. C., Kohn D. B., *Blood*, **83**, 2939—2948 (1994).
- 3) Weston S. A., Parish C. R., *J. Immunol. Methods*, **133**, 87—97 (1990).
- 4) Mathew L. T., Perumal T., Frank W., *Nucl. Med. Biol.*, **14**, 51—58 (1987).
- 5) Friedrich G., Soriano P., *Genes Dev.*, **5**, 1513—1523 (1991).
- 6) Kennedy D. W., Abkowitz J. L., *Blood*, **90**, 986—993 (1997).
- 7) Morgan G. M., McKenzie I. E., Melvold R. W., *Immunogenetics*, **11**, 43—53 (1980).
- 8) Young D. C., Kingsley S. D., Ryan K. A., Dutko F. J., *Anal. Biochem.*, **215**, 24—30 (1994).
- 9) Shaper N. L., Lepers A. H., Shaper J. H., *J. Biol. Chem.*, **269**, 25165—25171 (1994).
- 10) Bronstein I., Edward B., Voyta J. C., *J. Biolumi. Chemilum.*, **4**, 99—111 (1989).
- 11) Jain V. K., Magrath I. T., *Anal. Biochem.*, **199**, 119—124 (1991).
- 12) Yamaoka K., Tanigawara Y., Nakagawa T., Uno T., *J. Pharmacobio-Dyn.*, **4**, 879—885 (1981).
- 13) Castro-Faria-Neto H. C., Penido C. M., Silva A. R., Bozza P. T., *Mem. Inst. Oswald Cruz.*, **92**, 197—200 (1997).
- 14) Kisseberth W. C., Brettingen N. T., Lohse J. K., Sandgren E. P., *Dev. Biol.*, **214**, 128—138 (1999).