Brief Definitive Report

L3T4 EFFECTOR CELLS IN MULTIPLE ORGAN-LOCALIZED AUTOIMMUNE DISEASE IN NUDE MICE GRAFTED WITH EMBRYONIC RAT THYMUS

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The most important function of the thymus is education of T cells that properly recognize self and nonself. The basis of this function is dependent on interactions between the T cell precursors and nonlymphoid components, such as thymic epithelial cells. Recently we demonstrated that the T cell function of congenitally athymic nude (*nu/nu*) mice can be corrected by implantation of xenogeneic thymic rudiments from embryonic rat (1). Namely, rat thymic rudiments transplanted under renal subcapsule of nude mice developed well and formed a proper thymic structure composed of donor epithelia and host lymphocytes. Skin grafts from syngeneic mice and thymic donor rat strains were perfectly accepted in the rat thymus-grafted nude (TG nude) mice, whereas those from the third party were vigorously rejected. Considerable antibody responses to SRBC antigen were also observed. Histological and immunological studies, however, showed severe development of multiple organ-localized autoimmune diseases, especially in endocrine organs such as thyroid and ovary (1).

In the present experiments, we attempted to characterize the effector cell population in autoimmune diseases in TG nude mice by transfer of a selected population of spleen cells into syngeneic BALB/c nude mice. The selection was carried out using the cytotoxicity method and FACS.

Materials and Methods

Thymic Rudiment Transplantation. The rudiments of the thymuses were aseptically dissected from 15-d-old F344/DuCcj (Clea Japan Inc., Tokyo, Japan) rat embryos (observation of vaginal plug, 0 d). Female BALB/c *nu/nu* mice, 5 wk of age, (Clea Japan Inc.) were grafted under each kidney subcapsule with two lobes of thymic rudiments. These TG nude mice developed multiple organ-localized autoimmune diseases as reported previously (1). Incidence of the diseases assessed by histological examination in 109 TG nude mice, which were used as donors for the transfer experiments, was as follows: ovary, 92%; thyroid gland, 72%; stomach, 72%; adrenal gland, 44%; salivary gland, 98%. No lesions were observed in pancreas, kidney, liver, and lung as examined so far.

Adoptive Transfer System. The spleen was removed from the TG nude mouse 4–7 mo after grafting. Viable spleen cells were collected by Ficoll centrifugation. 5×10^4 to 1×10^7 cells

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were injected intraperitoneally into 4–5-wk-old female BALB/c *nu/nu* mice. 1–4 wk later, the mice were killed and examined.

Treatment of Spleen Cells with Antibodies and C. Polyclonal antiserum against rat cell antigen was prepared by immunization of female BALB/c mice with spleen cells prepared from F344 rats. Rat mAbs, anti-Lyt-2 (IgG2a, 53.6.72) and anti-L3T4 (IgG2b, GK1.5), were kindly provided by Dr. N. Shinohara, National Cancer Institute, Bethesda, MD, while mouse mAb to Thy-1.2 (IgM) was kindly given by Dr. F.-W. Shen, Memorial Sloan-Kettering Cancer Center, New York, NY. The titer of three mAbs was $\sim 1:10^5$ by C-dependent cytotoxicity assay. Bulk treatment of cells was carried out as previously described (2). $1\text{–}5 \times 10^6$ viable cells in 0.5 ml were injected intraperitoneally into BALB/c *nu/nu* mice.

Immunofluorescence Staining and Collection of Antigen-positive and -negative Cells by FACS. Cell sorting was performed in a FACS (FACS IV, Becton Dickinson & Co., Mountain View, CA) using a logarithmic scale. Approximately 10^8 spleen cells from each donor TG nude mouse were stained with 1.0 ml diluted FITC-labeled anti-L3T4 or anti-Lyt-2 mAb (50 μg/ml) at 4°C for 30 min. Cells stained with FITC-labeled anti-L3T4 or anti-Lyt-2 were sorted into $L3T4^+$ and $L3T4^-$ or $Lyt\text{-}2^+$ and $Lyt\text{-}2^-$ fractions, respectively. Antigen-positive and -negative fractions from each donor TG nude mouse were injected intraperitoneally into one BALB/c *nu/nu* mouse, respectively. Some recipients received the $Lyt\text{-}2^+$ fraction, obtained from four donors, for adjustment of the cell number comparable with the $L3T4^+$ fraction.

Results and Discussion

The previous study (1) showed the development of multiple organ-localized autoimmune disease in TG nude mice. To characterize the effector cells in these autoimmune diseases, passive transfer of the lesions was attempted. Oophoritis, thyroiditis, and gastritis were focused on in the present study, because the incidence of these three lesions were high in TG nude mice. Spleen cells (10^6) from female TG nude mice with autoimmune diseases were transferred into syngeneic female nude mice, and then the recipient mice were killed at 7-d intervals after the transfer to assess the degree of organ injury. Oophoritis was found to develop in the recipients at 2 wk after cell transfer, while thyroiditis and gastritis were induced only at 21 d after transfer (data not shown). Effective doses of spleen cells for induction of the development of autoimmune diseases were studied. Spleen cells (5.0×10^4 to 1.0×10^7) from TG nude mice were transferred intraperitoneally and the recipient nude mice were killed 28 d later. Table I shows that oophoritis developed at a high incidence, when the mice received injection of $>5 \times 10^5$ spleen cells.

There are two possibilities as to the origin of effector cells of autoimmune diseases in TG nude mice. One is the rat T cell population that was present in the thymic grafts and migrated into peripheral lymphoid tissues, while the other is mouse T cell population educated in the grafts. In the mouse, it is reported that lymphoid precursor cells first enter the fetal thymus at 10.5 d of gestation (3). The developmental stage of rat thymus is almost the same as that of mouse, or slightly behind. In the 15-d-old embryonic thymuses that were used for grafting, first generation of thymocytes has been identified as the first wave of lymphoid cells from bone marrow (4). In the previous study, however, we did not detect a rat T cell population in the thymic grafts or peripheral lymphoid tissues at 8 wk after transplantation. The possibility, however, still remains that a very minor population of rat T cells may peripheralize to be effector cells shortly after transplantation. Thus, the identification of species of effector cells was carried out.

Pretreatment of donor spleen cells with the antiserum against rat cell antigen and C did not abolish the capacity to induce the lesions (oophoritis, 5:5; thyroiditis, 3:5;

TABLE I

Effect of Spleen Cell Doses on Induction of Autoimmune Diseases in BALB/c nu/nu Mice

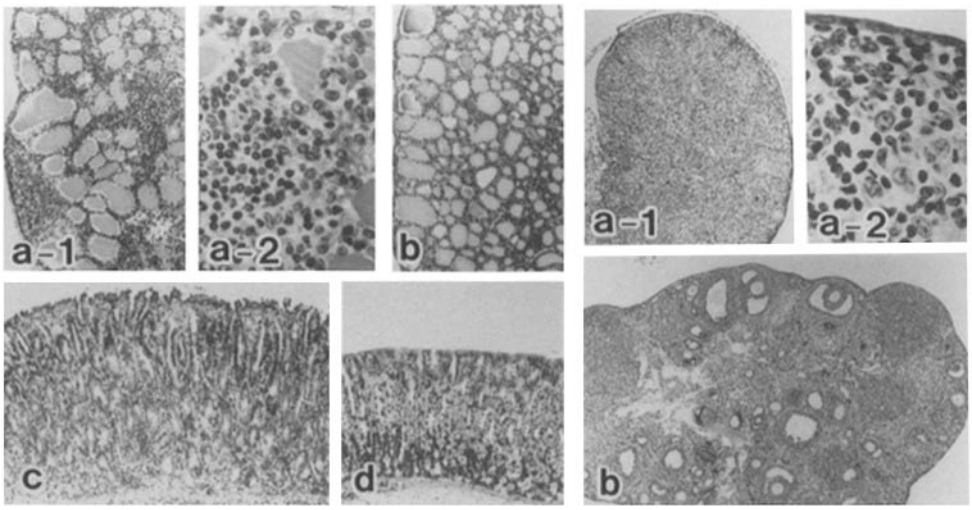
No. spleen cells transferred ($\times 10^5$)	No. recipient mice	No. mice with autoimmune diseases*		
		Ovary	Thyroid	Stomach
		%	%	%
100	2	2 (100)	1 (50)	2 (100)
50	3	2 (67)	0 (0)	2 (67)
10	5	4 (80)	1 (20)	2 (40)
5	6	5 (83)	2 (33)	2 (33)
1	7	1 (14)	0 (0)	1 (14)
0.5	4	0 (0)	0 (0)	0 (0)

Incidence of oophoritis, thyroiditis, and gastritis in 16 TG nude mice that were used as donors was 88%, 69%, and 69%, respectively.

* The recipient BALB/c *nu/nu* mice were killed at 28 d after transfer and examined. Incidence of the diseases was assessed by histological examination.

gastritis, 4:5). When Thy-1.2$^+$ cells were removed, no lesion was induced in any recipient mice (three lesions, 0:10). The present findings denied the possibility of rat lymphoid cells as the effector cells in TG nude mice, and clearly indicated that a certain population in mouse T cell becomes effector cells after having been educated in xenogeneic rat thymic grafts. Pathogenesis of autoimmune diseases has long been associated with disorder of educational cell-to-cell interactions in the thymus and our TG nude mouse model may be the case.

Spleen cells were obtained from TG nude mice with autoimmune lesions. The incidence of oophoritis, thyroiditis, and gastritis in 20 donors was 95%, 65%, and 65%, respectively. The cells were depleted of L3T4$^+$ cells by mAb and C treatment and transferred to *nu/nu* recipients at doses of 1 or 5 $\times$ 10^6/recipient (five animals each). Oophoritis was not observed in any recipient mice except one (not shown). Neither thyroiditis nor gastritis was transferred (Fig. 1 *b* and *d*). On the other hand, spleen cells of the same source depleted of Lyt-2$^+$ cells by mAb and C treatment were able to induce these three lesions (Fig. 1, *a-1*, *a-2*, and *c*; histology of oophoritis, see Fig. 2). 28 d after adoptive transfer, 9:10 recipients of Ly-2$^-$ cells had oophoritis and 3:10 each had thyroiditis and gastritis. In order to demonstrate more directly that the L3T4$^+$ subset contains the effector cells, positive selection of the antigen-positive cells was attempted by FACS. In this experiment, antigen-positive and -negative populations were obtained from each donor TG nude mouse and were transferred intraperitoneally into a recipient BALB/c *nu/nu* mouse, respectively. As shown in Table II, L3T4$^+$ cells were 1.5–6.8 $\times$ 10^5 per mouse, while Lyt-2$^+$ cells were 0.5–2.7 $\times$ 10^5, being smaller than L3T4$^+$ cells, reflecting the ratio between these T subsets. L3T4$^+$ cells were able to transfer the oophoritis (Fig. 2, *a-1* and *a-2*), while L3T4$^-$ cells induced the lesion to only one of eight recipient mice. As little as 1.5 $\times$ 10^5 L3T4$^+$ cells were found to have the effector activity. Lyt-2$^+$ cells could not transfer the oophoritis, while Lyt-2$^-$ cells did. No lesion (Fig. 2 *b*) was found, even in the recipients that were injected higher doses (2.5–7.0 $\times$ 10^5) of Lyt-2$^+$ cells harvested from four donors. Thus, the results altogether showed that the effector cell population is present in the L3T4$^+$ subset. To our knowledge, this experiment is the first



FIGURES 1 and 2. (*Fig. 1, left*) Histology of thyroid and stomach from recipient BALB/c *nu/nu* mice that had received single injection of spleen cells from TG nude mice after elimination of Lyt-2$^+$ or L3T4$^+$ cells. A cytotoxicity method with Lyt-2 or L3T4 mAb plus C was used for elimination of the cells. (*a*) A lesion found in the thyroid of a recipient that had received 10^6 spleen cells after elimination of Lyt-2$^+$ cells. Destruction of follicular architecture and extensive infiltration of mononuclear cells were noticed. (*b*) A thyroid from recipient mouse that had received 5×10^6 spleen cells after elimination of L3T4$^+$ cells. No lesion was found. (*c*) A lesion found in the stomach of a recipient mouse that had received 10^6 spleen cells after elimination of Lyt-2$^+$ cells. Depletion of parietal and chief cells and hyperplasia of endocrine cells were noticed with mononuclear cell infiltration around the muscularis mucosa. (*d*) A stomach from a recipient mouse that had received 5×10^6 spleen cells after elimination of L3T4$^+$ cells. No lesion was found. (*a-1*, *b*, *c*, and *d*) × 50, (*a-2*) × 260. (*Fig. 2, right*) Histology of ovary of recipient BALB/c *nu/nu* mice that had received a single injection of L3T4$^+$ or Lyt-2$^+$ cells from TG nude mice after positive selection by FACS. (*a*) An ovary from a recipient mouse, which was transferred with positively selected 3.5×10^5 L3T4$^+$ cells, shows no follicle with diffuse infiltration of mononuclear cells (donor 3 in Table II). (*b*) An ovary from a recipient mouse, which was transferred with positively selected 7.0×10^5 Lyt-2$^+$ cells, is histologically intact (donor 19 in Table II). (*a-1*, and *b*) × 50. (*a-2*) × 300.

one to demonstrate that the cells sorted by FACS still maintained effector cell activity in vivo after transfer.

We previously reported another experimental model of multiple organ-localized autoimmune diseases, i.e., lesions with circulating antibodies against specific cells or components were induced in many strains of mice by neonatal thymectomy without any antigen stimulation. In this model, we demonstrated the presence of autoreactive T cells to be effector cells (5), as well as that regulatory T cells suppress the emergence of effector T cells (2), and demonstrated that the effector cells are Lyt-1$^+$, Lyt-2$^-$ (6), which seems to be in agreement with the phenotype of the effector cells in the present TG nude model. The importance of L3T4$^+$ cells as a effector population was also indicated by adoptive transfer of experimental autoimmune encephalomyelitis (EAE) (7). It was further demonstrated that L3T4$^+$ clones alone were sufficient for the induction of EAE (7, 8) or experimental autoimmune thyroditis (9). These clones were reported to have various immunological functions including in vitro (7, 9) helper activity for antibody production and in vivo delayed-type hypersensitivity (DTH) (8), although none have cytotoxic killer cell activity.

TABLE II

Transfer of Autoimmune Diseases by T Cell Subsets Obtained by Selection of Antigen-positive and -negative Cells with FACS

		L3T4$^+$ fraction*		L3T4$^-$ fraction*	
Donor	Lesions‡	No. cells transferred ($\times 10^5$)	Lesions‡	No. cells transferred ($\times 10^5$)	Lesions‡
1	O	1.5	O	3.0	-
2	O,T,G	2.5	O,T,G	110.0	-
3	O,G	3.5	O§	5.0	-
4	O,T,G	4.0	O,T	20.0	-
5	O	4.0	O	6.2	O
6	O,T,G	4.0	O	10.0	-
7	O	5.7	O	8.0	-
8	O,G	6.8	O	8.3	-
		Lyt-2$^+$ fraction		Lyt-2$^-$ fraction	
11	O,G	0.5	-	8.7	O,G
12	O,G	0.8	-	5.4	O,G
13	O,T	1.0	-	18.0	O
14	O,T,G	2.0	-	16.0	O,G
15	O,G	2.7	-	13.0	O,G
16‖		2.5	-	30.0	O,G,T
17‖		3.0	-	47.0	O
18‖		3.2	-	26.0	O
19‖		7.0	-§	55.0	O,T

* Spleen cells from each donor TG nude mouse with autoimmune diseases were separated into T cell subsets (L3T4$^+$ and L3T4$^-$ or Lyt-2$^+$ and Lyt-2$^-$) by FACS. The antigen-positive and -negative populations thus obtained from each donor mouse were transferred into one recipient BALB/c *nu/nu* mouse, respectively (except donor 16–19, see footnote ‖). Incidence of the disease was assessed by histological examination at 28 d after the cell transfer.

‡ The lesions observed in donor TG nude mouse. O, oophoritis; T, thyroiditis; G, gastritis.

§ Histology of the lesions was illustrated in Fig. 2.

‖ Lyt-2$^+$ and Lyt-2$^-$ populations collected from four donors were transferred into one recipient mouse. Oophoritis was observed in all donor mice. At least one of four donors had thyroiditis and gastritis.

Thus, it is difficult at present to speculate which function is directly associated with effector cell activity in autoimmune diseases, although the correlation between DTH and autoimmune lesions was often observed (8). Establishment of L3T4$^+$ class II-restricted CTL clones (10) reactive with autoantigens is also important for understanding the effector cell population, because T cells as such may also act as effector cells in autoimmune diseases. The results described above altogether suggested the importance of L3T4$^+$ effector cells. Bendelac et al. (11) however, recently reported that both L3T4$^+$ and Lyt-2$^+$ T cells were required for syngeneic transfer of the diabetes into newborn mice in the nonobese diabetic mouse. The difference in T cell phenotype of effector cells may be explained by the nature of the disease studied, i.e., the results that their transfer system need >10 times effector cells (2.0×10^7), when compared with ours (10^6), suggested that the development of effector cells cytotoxic for β cells in the pancreas may need another cell population.

Summary

Rat thymic grafts reconstituted T cell functions of BALB/c nude (*nu/nu*) mice to a considerable degree, but multiple organ-localized autoimmune diseases such as oophoritis and thyroiditis generally developed. The effector cell population in this autoimmune model was studied by adoptive transfer of the lesions into syngeneic nude mice. The transfer activity was not diminished when spleen cells were incubated with antiserum against rat cell antigen and C, but the activity was completely vanished by incubation with anti-Thy-1.2 plus C, indicating that the effector cells are T cells of mouse origin. Elimination of the L3T4$^+$ subset virtually abolished the transfer activity, whereas that of the Lyt-2$^+$ subset did not, indicating that the effector cells are L3T4$^+$. Positive selection experiments by FACS also demonstrated that L3T4$^+$ cells, but not Lyt-2$^+$ cells, were capable of inducing the lesion, confirming the results with depletion experiments described above.

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