DIVISION OF IL-2 RECEPTOR EXPRESSING LYMPHOCYTES IN MURINE EMBRYONIC THYMUS

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Upon activation with antigens or lectins, a transient expression of mature T lymphocytes cell surface molecules specifically binding to interleukin-2 (IL-2), one of the growth factors, is induced (2, 5, 20, 23). The molecules are designated as interleukin-2 receptors (IL-2R). Interaction of the receptor with IL-2 provides an universal signal for T cell proliferation, resulting in clonal expansion of the activated T cells. In the physiological condition without specific antigen stimulation, proliferation of lymphocytes is prominent in the thymus, particularly in the embryonic thymus, in which we have previously found IL-2R expression of immature lymphocytes (4, 10, 22). Is the IL-2R expressing lymphocytes responsible for the cell proliferation in the thymus as is for the peripheral T lymphocytes? Based on the finding that during the period of gestation, the proportion of IL-2R positive (IL-2R⁺) cells in the thymus decreases but the cell number of IL-2R⁺ cells moderately increases, one may postulate that the IL-2R⁺ cells may be slow growing lymphocytes. Alternatively, it may be postulated that the IL-2R⁺ cells die in the thymus (27) and that the IL-2R expression of newly arrived lymphocytes, which is induced immediately after their migration into the thymus, contributes to the increase in the number of IL-2R⁺ cells *in vivo*. In this paper, proliferation of IL-2R⁺ cells within the embryonic thymus was examined in the organ culture system in which the mouse embryonic thymus lobes were cultured on a floating membrane to prevent cell migration from extrathymic sites.

3-day culture of BALB/c thymic lobes developed the 15-day embryonic thymus to a further advanced gestational stage: in the cultured thymus, lobular structures composed of medulla and cortex became distinguishable with an increase in cell number and with appearance of differentiation antigens such as Lyt-2 and L3T4 on the lymphocytes. In the fluorescence staining study with monoclonal antibody against interleukin-2 receptor (IL-2R) of dissociated cells from the thymic lobes, the proportion of IL-2R expressing cells (IL-2R⁺) decreased from 61% to 16% in the 3-day culture but the cell number of IL-2R⁺ cells did not decrease. The decline of IL-2R⁺ cell proportion in the 3 day culture was prevented by the addition of 2 mM thymidine in the culture media which arrests the cell cycle by inhibiting DNA synthesis. Using anti-IL-2R antibody in immunohistochemical and immunoelectron microscopic studies, mitotic figures of the nucleus were found in both the IL-2R⁺ and IL-2R⁻ cells of cultured and fresh thymus. Collectively, these observations suggest that IL-2R⁻ embryonic thymocytes proliferate within the thymus and this proliferation probably is related to the disappearance of IL-2R molecules.

MATERIALS AND METHODS

1. Animals

Pregnant BALB/c mice maintained under specific pathogen-free conditions were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan).

2. Organ culture

Thymic lobes were removed from 15-day embryos and each lobe was cultured on the surface of 0.8 μ m Nucleopore filters (Nucleopore Corp., Pleasanton, CA) in a petri dish containing PRMI supplemented with 10% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES buffer, penicillin (100 U/ml) and streptomycin (100 μ g/ml) (4).

3. Antibodies

Monoclonal antibodies (mAb) used in this study were anti-mouse IL-2R (3C7) (21), anti-Lyt-2 (53-6.7) (15), anti-L3T4 (GK 1.5) (7) and anti-Thy-1 (HO-13) (18). These mAbs were conjugated with fluorescein (mAb-f1) for fluorescence study of the dissociated cells. The second-step reagent, anti-rat-Igs, for 3C7 was labeled with horseradish peroxidase (HRP) for immunohistochemical and immunoelectron microscopic examination.

4. Immunohistochemical examination of thymus tissue

Thymic lobes were embedded in OCT compound (Lab Tek Division, Miles Labs, Naperville, IL) and were immediately frozen at -80°C . Acetone-fixed 10 μ m frozen sections of the thymic lobes were incubated with 3C7 for 30 min at room temperature. After washings 3 times with cold phosphate-buffered saline, the tissue sections were incubated with anti-rat-Ig-HRP using the same procedure described in 3. The reacted sections were dipped in Karnovsky's solution and then stained with methyl green solution.

5. Cell preparation and reaction with antibodies

Thymic lobes on the fifteenth gestational day were removed using cataract knives under a stereoscopic microscope and pressed between glass slides to make dissociated cell suspensions. For immunofluorescence staining, the dissociated cell suspensions were incubated with mAb-f1 for 30 min at 4°C , and then these stained cells were examined under a fluorescence microscope.

Alternatively, the dissociated cells reacted with 3C7 were incubated with Fab fragments of anti-rat-Ig-HRP. After washings 3 times, the cells were fixed in a mixture of 0.5% glutaraldehyde and 2% paraformaldehyde (GA-PA) at 4°C for 20 min and then, incubated with 3,3-diaminobenzidine (DAB) using Karnovsky's method (8). These cells were submitted to either osmification for immunoelectron microscopic study or nuclear staining by methyl green for light microscopic examination.

6. Block of cell cycle in G1 and M phase

In order to inhibit DNA synthesis of lymphocytes in the cultured thymic lobe, the thymic lobes on the filter were incubated with the culture media containing 2 mM thymidine for 48 hr. For chromosome analysis of mitotic cells, dissociated thymocytes were cultured with 2×10^{-5} g/ml Colcemid for 2 hr. The cells were then washed and submitted to cell surface staining with 3C7.

RESULTS AND DISCUSSION

During the period from 15 to 18 gestational days, anti-IL-2R reactive cells (IL-2R⁺ cells) in the thymus of BALB/c mice showed about a 280-fold increase in the lymphocyte cell number but the proportion of IL-2R⁺ cells decreased from 61% to 17% (Table 1). Considering the length of the entire cell cycle in the thymus (1), the increase in the cell number in the embryonic thymus can be attributed to not only cell division but also to the numerous cell import into the thymus from extra-thymic sites. To determine whether or not IL-2R⁺ cells proliferate in the thymus, resulting in the increase in the IL-2R⁺ cell number *in vivo*, the thymic lobes of 15-day-old embryo were used for organ culture preventing lymphocyte migration. Of the thymic lobes removed from the 15-day-old embryo, the left lobes were cultured on a Nucleopore filter for 3 days and the right lobes without culture were submitted to cell number measurement, immunohistochemical and fluorescence study.

Firstly, the thymic tissues were histologically examined in order to confirm whether the thymic lobes in 3-day culture develop as well as the thymus *in situ*. The cultured thymic lobe was composed of lobules with the distinct demarcation between the medulla and cortex. This was undistinguishable in the precultured thymus of 15 gestational days (Fig. 1). The IL-2R⁺ cells which were stained with HRP-labeled anti-IL-2R antibody, were diffused throughout the thymus parenchyma of 15 gestational days, but they were scattered predominantly in the marginal zones of the cortex in the cultured thymic lobes. At the same time, the expression of the differentiation antigens was examined using the relevant antibodies for the dissociated lymphocytes from fresh and cultured thymus. Although Ly-2 and L3T4 antigens, which are useful antigens for studying thymocyte differentiation (7, 12, 19, 24), were undetectable on the lymphocytes of 15 gestational days, they were observed on 45% to 72% of the lymphocytes in the cultured thymus. The proportion of Thy-1 expressing cells was 67% in the fresh thymus of 15 gestational days and 89% in the cultured thymus (Table 1). These findings were almost equivalent to the properties observed in the *in situ* thymus of 16 to 17 gestational

TABLE 1. *Development of embryonic thymocytes in 3-day culture and the in vivo*

gestation	culture duration ^{a)}	Total cells	IL-2R ⁺ cells		proportion (%) of antigen ⁺ cells ^{b)}		
		cell number per lobe ($\times 10^4$) ^{b)}	percent ^{b)}	cell number per lobe ($\times 10^5$) ^{c)}	Ly-2	L3T4	Thy-1
15 days	0 day (right lobes)	0.3 $\pm$ 0.07	61 $\pm$ 8	0.18	<1	<1	67
	3 days (left lobes)	2.0 $\pm$ 0.12	16 $\pm$ 3	0.32	45	72	89
18 days	0 day	76.2 $\pm$ 5.1	5 $\pm$ 0.6	3.81	75	83	<95

a) Right and left lobes of embryonic thymus were separately removed from 10 pregnant BALB/c mice and left lobes were cultured on a Nucleopore filters for 3 days.

b) The dissociated lymphocytes obtained from pre-cultured (right) and post-cultured (left) thymic lobes were counted in the cell number and were stained with monoclonal antibodies against IL-2R, Ly-2, L3T4 and Thy-1.

c) The number of IL-2R⁺ cells per lobe was estimated by the figures of total cell number and proportion of IL-2R⁺ cells.

FIG. 1. Frozen section of mouse thymic lobe from BALB/c embryo, reacted with a monoclonal antibody, 3C7, against mouse IL-2R. Binding of the antibody was visualized with HRP-labeled F(ab')₂ fragments of rabbit anti-rat Ig. Distinct lobules (L) with medulla and cortex were shown in the 3-day cultured thymus of 15-day-old embryo (1a). Such lobulation was not distinguishable in the pre-cultured thymic lobe of the same age (1b). Note the distribution of IL-2R⁺ cells which were isolated near the subcapsular area (arrow) of the cultured thymus. $\times 48$

days (10, 24), indicating that culture of the thymic lobes on the filter paper for 3 days is an appropriate system for studying the intrathymic development of lymphocytes.

For the 3-day culture, the cell number in the 15-day embryonic thymus increased from 3×10^4 to 2×10^5 (Table 1). Although the 7-fold increase in the cell number of the cultured thymus is lower than the *in situ* increase from 15 to 18 gestational days, it is not inconsistent with the figure predicted from the mitotic

TABLE 2. Proportion of IL-2R⁺/IL-2R⁻ cells expressing nuclear division^{a)}

thymocytes	culture duration	mitotic index (%) ^{b)}		
		in total cells	in IL-2R ⁻ cells	in IL-2R ⁺ cells
15-day				
embryo	0 day	9.1	9.8	8.4
//	2 day	8.1	8.8	7.4
4W. adult	0 day	4.7	ND	ND

a) After incubation with Colcemid (0.06 µg/ml) for 2 hr, the dissociated thymocytes were stained with anti-IL-2R antibody and methyl green for visualization of IL-2R molecules and the nucleus as described in Materials and Methods.

b) The proportion of the IL-2R⁺ and IL-2R⁻ cells showing nuclear division was calculated under the microscope.

index obtained in 15-day embryonic thymocytes (Table 2). In addition, the increase in the cell number in the *in situ* thymus is composed of intrathymic cell proliferation and imported cells from the extrathymic sites, as previously described. The fluorescence study of dissociated cells showed that the proportion of IL-2R⁺ cells detected by anti-IL-2R antibody decreased from 61% to 16% in the 3-day-culture. In contrast to the decreased proportion, the number of IL-2R⁺ cells never decreased, slightly increased. This dynamics of IL-2R⁺ cells corresponds to that observed in the embryonic thymus *in vivo* (4, 10, 22).

In order to determine whether or not IL-2R⁺ cells divide within the thymus, the nuclear division of IL-2R⁺ cells was morphologically examined in the 15-day embryonic thymocytes. The lymphocytes which were arrested in the mitotic (M) phase of the cell cycle by Colcemid treatment for 2 hr were incubated with anti-IL-2R antibody and subsequently with anti-rat-Ig-HRP, and then the reaction products with the antibody on the cell surface and nuclear structures were examined as described in the Materials and Methods section. The structural changes in the nucleus at the M phase were morphologically identified as the rearranged chromosomes with the spindle shaped arms radiating out into the surrounding cytoplasm (Figs. 2, 3) or in a coiled shape without a nuclear envelope. Such nuclear structures were found in both the IL-2R⁻ cells and the IL-2R⁺ cells, which were characterized as the brown ringed cells. Immunoelectron microscopic study demonstrated a typical mitotic figure in the IL-2R⁺ cells (Fig. 5). The proportion of IL-2R⁺ and IL-2R⁻ cells in the M phase (mitotic index) was 8.4% and 9.8% respectively in the 15-day embryonic thymus and was 7.4% and 8.8% in the cultured thymus for 2 days (Table 2). This finding indicated that IL-2R⁺ cells in embryonic thymocytes are lymphocytes undergoing division. However, the increase in number of the cells identified as IL-2R⁺ during the culture was much less than the figures estimated from the mitotic index. The most acceptable explanation for this discrepancy is that IL-2R⁺ cells lose IL-2R molecules during the process of their proliferation. This explanation was supported by our experimental result showing that in the lymphocytes in which DNA synthesis was inhibited by thymidine treatment, the proportion of IL-2R⁺ cells decreased slightly but the cell number was lower than in control lymphocytes of the thymic lobes cultured without thymidine for 2 days (Table 3). There is evidence that in physiological condition, some cell surface molecules are

FIG. 2. Anti-IL-2R reactive embryonic thymocytes showing mitotic figures of nucleus. Dissociated lymphocytes from 15-day old embryonic thymus were indirectly stained with anti-IL-2R (3C7). 3C7 reactive cells were ringed with brown pigments. Mitotic figures of nucleus was observed in both the antibody reactive and non-reactive cells. 2a). The 3C7 reacted cell suspension in GA-PA were submitted to the Cyto-spin (Shandon, USA) for 15 min at 4°C. After incubation with DAB the nucleus was stained with Methy green. $\times 200$ 2b) After centrifugation of the 3C7 reacted cells on the glass slide by Cyto-spin, they were fixed with GA-PA and reacted with DAB. The nucleus was stained with hematoxylin. $\times 250$

FIG. 3. Immunoelectron micrograph of thymocytes from 15-day embryonic mouse. The reaction products with anti-IL-2R antibody (arrow) were observed as dark fine deposits on the cell surface of the lymphocyte with typical chromosomal figures in M phase. $\times 1,000$

TABLE 3. *Effect of DNA synthesis blockade on proportion of IL-2R⁺ cells in embryonic thymus*

embryonic thymus ^{a)} in culture	thymidine	IL-2R ⁺ ^{b)} cells (%)	total cell ^{b)} number
Expr 1. right lobe	+	44.5	3×10^4
left lobe	—	15.0	16×10^4
Expr 2. right lobe	+	40.0	2.5×10^4
left lobe	—	15.5	14.8×10^4

^{a)} Right and left lobes of 15-day embryonic thymus were cultured with or without 2 mM thymidine for 65 hr on the filter papers.

^{b)} The dissociated lymphocytes from post-culture thymic lobes were stained with anti-IL-2R-f1. IL-2R⁺ and IL-2R[—] cells were calculated under the UV microscope.

lost in the thymocytes during their differentiation pathway (11, 24). In addition, it has been reported that the expression of IL-2R is induced transiently on the activated peripheral T cells and is maintained by the presence of both IL-2 and appropriate stimulation (6, 13, 17). If this is the case for thymocytes, it seems likely that IL-2R molecules on embryonic thymocytes disappear after cell division due to the absence of IL-2. In fact, *in vitro* stimulation with lectins failed to induce only detectable IL-2 production for lymphocytes in the thymus prior to 19 gestational days (3). What is the promoter for the IL-2R⁺ cells to progress through the cell cycle? As far as IL-2R⁻ cells do divide in the thymus, there must be activating factors present other than IL-2 although they are remained unclear from the present study (16).

Another question still remained is the fate of the IL-2R⁺ cells in the thymus. IL-2R molecules are expressed on Ly-2⁺L3T4⁻ immature thymocytes immediately after the lymphoid stem cells migrate into the thymus (26). In addition, there is accumulating evidence that Ly-2⁺L3T4⁻ thymocytes are precursor of T cell lineages and develop to express both or either of the antigens in the thymus (25). Thus, it is postulated that IL-2R⁺ cells differentiate towards mature T cells following the cell division and disappearance of IL-2R expression. The hypothesis is supported by the findings that the thymocytes in the scid mouse characterized by disfunction of T and B cells express IL-2R molecules but not express Ly-2 and L3T4 (17) and that the scid thymus contains extremely small number of lymphocytes (manuscript in preparation). To define directly whether or not the cell division of IL-2R⁺ thymocytes results in their advanced maturation stage and proliferation of thymocytes, further studies using a culture system only for the separated IL-2R⁺ cell population in the thymic environment are required and are now under the way.

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