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Immunological Effects of Lumin-A, a Fluorescent Antioxidant Drug, on T Cell Subsets in Various Immune Organs of Mice

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Summary. **It is important to determine how fluorescent antioxidant drugs act as immunomodulators. Since a new T-cell population, namely, extrathymic T cells, has recently been identified in the liver and other immune organs, our attention was focused on how one of the above-mentioned drugs (Lumin-A in this study) affects the distribution of extrathymic T cells and thymus-derived T cells in various immune organs. When Lumin-A was administered into mice daily (10 μg/mouse/day) with intraperitoneal or oral route for 3 wks, its unique effect was observed, depending on the routes. Especially, the oral *ad libitum* administration of Lumin-A induced a prominent induction of thymus-derived T cells, but not extrathymic T cells, in the peripheral immune organs, the spleen and the liver. The present results suggest that a fluorescent antioxidant drug, Lumin-A, has an ability to induce the thymus-derived T cells, i.e., usual T cells for processing foreign antigens, into the periphery.**

Key words—lumin-A, antioxidant drug, extrathymic T cell, thymus-derived T cell.

INTRODUCTION

It is well established that antioxidant substances absorb free radicals and active oxidants in the body. In this regard, such substances protect the body from the oxidation of molecules.[1] These drugs are, therefore, very useful for the treatment of suppurative diseases, trauma, burn, aging, etc.

In the present study, we investigated how such antioxidant drugs modulate the immune systems in mice. Since recent studies have revealed that T cell populations are comprised of both thymus-derived T cells and extrathymically differentiated T cells,[2-4] we here applied a method to identify simultaneously both subsets in mice. Two-color staining for CD3 and IL-2Rβ identifies extrathymic T cells as CD3-intermediate (int)$^+$IL-2Rβ^+, thymus-derived T cells as CD3-high$^+$IL-2Rβ^-, and NK cells as CD3$^-$IL-2Rβ^+. There are some specific roles in the variation of these extrathymic T cells and thymus-derived T cells. Namely, extrathymic T cells tend to increase under conditions of infection of the intracellular pathogens[5] and with aging[6] and inversely, thymus-derived T cells decrease under such conditions. Since these conditions are accompanied with elevated levels of the oxidation of molecules in the body, this reciprocal variation of T cell subsets might be induced as a result of the oxidation under such conditions. Therefore, our working hypothesis was that antioxidant drugs induce the reduction of molecules in the body and result in certain immunomodulatory effects.

When Lumin-A, one of the fluorescent antioxidants[7] was administered into mice daily for 3 wks, its unique effects on T cell subsets in the peripheral immune organs were observed, depending on the administration routes. Namely, the oral *ad libutum* administrations induced a prominent increase in the levels of thymus-derived T cell in the liver and spleen. On the other hand, extrathymic T cells were activated only when Lumin-A was administered intraperitoneally. The present results suggest that antioxidant drugs have an ability to induce thymus-derived T cells into the peripheral immune organs.

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METERIALS AND METHODS

Mice and drug administration

C3H/He mice at the age of 8 wks, which were maintained under specific pathogen-free conditions, were used. Lumin-A is one of the fluorescent antioxidant drugs (Japan Create Co., Tokyo, Japan) and is commercially available from drug stores. One tablet contains 100 μg of Lumin-A (Cript cyanine O.A. complex).[7] To administer Lumin-A into mice, the tablet was disruped and suspended in saline or water (i.e., 100 μg/ml in saline or 300 μg/200 ml in water). Finally, approximately 10 μg of Lumin-A was administered into mice daily for 3 wks, via the intraperitoneal or oral route.

Cell preparations

Hepatic mononuclear cells (MNC) were isolated as previously described.[8] Briefly, mice anesthetized with ether were killed by total exsanguination by cardiac puncture. To obtain MNC, the liver was removed, pressed through 200-gauge stainless steel mesh, and suspended in saline. After one washing with saline, MNC were isolated from hepatocytes and hepatocyte nuclei by Ficoll-Isopaque density (1.090 g/ml) gradient centrifugation. To avoid a selective cell loss due to gradient centrifugation, sufficient dilution of mashed liver samples with a medium (e.g., 30 ml for two livers) was necessary before application to the gradient cushion. MNC collected from the interface were then suspended in MEM supplemented with 2% heat-inactivated newborn calf serum. The preparations of hepatic MNC contained less than 4% Kupffer cells. Spleen cells were also collected by the Ficoll-Isopaque method, and thymocytes were obtained by forcing the thymus through 200-gauge steel mesh.

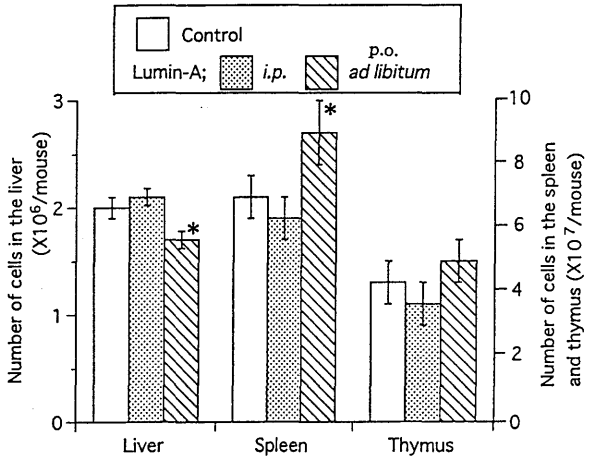

Fig. 1. Variations in the number of MNC yielded by various immune organs in mice administered with Lumin-A. Lumin-A was administered to mice by the intraperitoneal (i.p.) or oral *ad libitum* (p.o.) route (10 μg/mouse/day) for 3 wks. The numbers of MNC yielded by the liver, spleen, and thymus are enumerated. Control mice were examined in parallel. * $p<0.05$

Immunofluorescence tests

The surface phenotype of cells was analyzed using mAb in conjunction with two-color immunofluorescence tests.[4] FITC-conjugated aliquots of anti-CD3 (145-2C11), anti-TCR-$\alpha\beta$(H57-597), and anti-CD4 (L3T4) mAbs were obtained from PharMingen, San Diego, CA. Biotin-conjugated aliquots of anti-IL-2Rβ (TM-β1), anti-TCR-$\gamma\delta$ (GL3), and anti-CD8(Lyt-2) mAbs were also used (PharMingen). Biotin-conjugated reagents were developed with phycoerythrin (PE)-conjugated avidin (Caltag, San Francisco, CA, U.S.A.). The fluorescence-positive cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, U.S.A.). For each figure, 10,000 cells were analyzed.

RESULTS

Variation in the number of cells yielded by the liver, spleen, and thymus in mice administered with Lumin-A

Lumin-A was administered into mice by two different routes, and the numbers of cells yielded by various immune organs in control mice and treated mice were enumerated (Fig. 1). When the i.p. injection was carried out, the numbers of cells remained almost stable in all tested organs. On the other hand, the numbers of cells yielded varied when Lumin-A was administered orally *ad libitum*. It was demonstrated that the number of liver MNC decreased whereas those of splenic MNC increased ($p<0.05$). The values were obtained from repeated experiments using five mice.

Phenotypic characterization of MNC in various immune organs of control mice and treated mice

Since there was a tendency for the oral administration of Lumin-A to increase the number of cells yielded by the spleen, it was investigated as to how lymphocyte subsets varied at that time. The effects of both administration routes were examined (Fig. 2). To simultaneously identify NK cells, int TCR cells

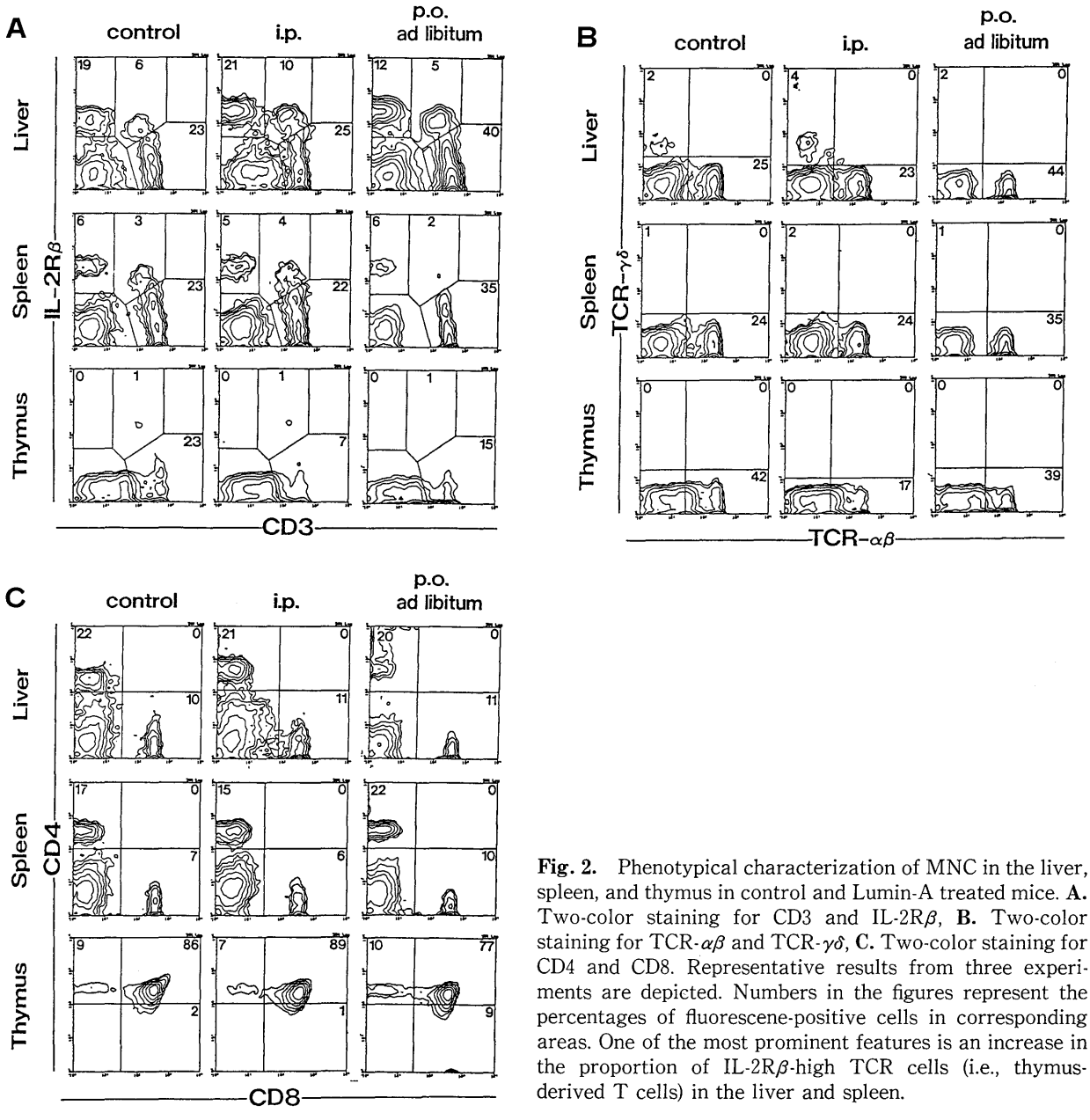

Fig. 2. Phenotypical characterization of MNC in the liver, spleen, and thymus in control and Lumin-A treated mice. **A.** Two-color staining for CD3 and IL-2Rβ, **B.** Two-color staining for TCR-$\alpha\beta$ and TCR-$\gamma\delta$, **C.** Two-color staining for CD4 and CD8. Representative results from three experiments are depicted. Numbers in the figures represent the percentages of fluorescene-positive cells in corresponding areas. One of the most prominent features is an increase in the proportion of IL-2Rβ-high TCR cells (i.e., thymus-derived T cells) in the liver and spleen.

(i.e., extrathymic T cells), and high TCR cells (i.e., thymus-derived T cells), two-color staining for CD3 and IL-2Rβ was first carried out (Fig. 2A). Representative results from the three experiments are depicted. The levels of lymphocyte subsets were not significantly changed in the case of the i.p. injection, except for a slight elevation of int TCR cells in the liver and a decrease in the most mature T cells (23% →7%) in the thymus. On the other hand, the oral administration of Lumin-A greatly changed the levels of high TCR cells in the liver and spleen (i.e., 23%→ 40% in the liver, and 23%→35% in the spleen).

Two-color staining for TCR-$\alpha\beta$ and TCR-$\gamma\delta$ was then examined (Fig. 2B). Reflecting the increase in the proportion of int TCR cells in the liver at the i.p. administration, $\gamma\delta$ T cells increased slightly (2% → 4% in the liver). Namely, $\gamma\delta$ T cells belonged to a population of int TCR cells. From the data from the liver and spleen of mice with the oral administration, we determined that all expanding T cells in these

organs were $\alpha\beta$ T cells. Finally, two-color staining for CD4 and CD8 was performed (Fig. 2C). In all tested organs and under all tested conditions, the ratios of $CD4^+$ and $CD8^+$ cells remained almost stationary.

DISCUSSION

The present study clearly demonstrated that Lumin-A, a fluorescent antioxidant drug, increased the number of MNC yielded by the spleen and thymus, and increased the proportion of high TCR cells (i.e., thymus-derived T cells) in the liver and spleen, when administered by the oral *ad libitum* route. Since both the number of splenic MNC and the proportion of high TCR cells in the spleen were elevated, the increase in the alsolute number of high TCR cells was substantial in this organ, it is concluded that Lumin-A has the potential to elevate the level of thymus-derived T cells in the peripheral immune organs.

It has been established that extrathymic T cells play a role in the elimination of abnormal self-cells by means of their autoreactivity.[9] It is speculated that they may exsist at an earlier position of phylogenetic T-cell development.[10] On the other hand, the thymus and thymocytes exsist at the most developed position in phylogeny. In this regard, they eliminate autoreactive clones during the maturational stages, and act as effector cells for the elimination of foreign antigens in the context of self-MHC antigens.[11,12] In such situations, Lumin-A might potentiate the most developed immune system when orally administered.

Lumin-A is known to be a fluorescent antioxidant drug and, therefore, act as a protective agent from the oxidation of molecule in the body. It can be speculated that they have the potential to suppress suppurative diseases (the prominent production of oxidants from granulocytes in this case), trauma, burns, and aging. In this study, we found reason to propose an additional effect of antioxidants as immunomodulators, especially for the potentiation of thymus-derived T cells.

As shown previously, the pathways of extrathymic and intrathymic T-cell differentiation were reciprocally regulated in opposite directions.[13-15] The results in the present study were compatible with such rules. Thus, Lumin-A potentiates only the pathway of intrathymic T-cell differentiation, but not the extrathymic pathways. On the other hand, we have often observed that extrathymic T cells increase under conditions of infection and with aging.[5,6] Since these conditions are suspected to be accompanied by oxidation in the body, the resultant variation of T cell subsets under conditions of infection and with aging moves in the opposite direction of the administration of Lumin-A.

The administration route is also very important to induce the above-mentioned effects. Thus, only the oral route induced such results. In a preliminary study, we have discovered that a high dose of Lumin-A via the intraperitoneal route activates rather phagocytic cells such as macrophages and granulocytes (our unpublished data). A similar result by Lumin-A in macrophages was produced by others using the intraperitoneal route.[16] On the other hand, oral administration of Lumin-A does not have such an effect. A relative small amount of Lumin-A might be important for the induction of T cell activation, and oral administration supports such a notion because of limited absorption from the intestine in this case. We feel that all antioxidant drugs have a similar tendency, in which higher doses activate the phagocytic cell system but lower doses activate the T and B cell systems. The detailed mechanisms of this remain to be further investigated.

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REFERENCES

1) Buttke TM, Sandstrom PA: Oxidative stress as a mediator of apoptosis. *Immunol Today* **15:** 7-10, 1994.
2) Abo T, Ohteki T, Seki S, Koyamada N, Yoshikai Y, Masuda T, Rikiishi H, Kumagai K: The appearance of T cells bearing self-reactive T cell receptor in the livers of mice injected with bacteria. *J Exp Med* **174:** 417-424, 1991.
3) Seki S, Abo T, Ohteki T, Sugiura K, Kumagai K: Unusual $\alpha\beta$-T cells expanded in autoimmune *lpr* mice are probably a counterpart of normal T cell in the liver. *J Immunol* **147:** 1214-1221, 1991.
4) Iiai T, Watanabe H, Seki S, Sugiura K, Hirokawa K, Utuyama M, Takahashi-Iwanaga H, Iwanaga T, Ohteki T, Abo T: Ontogeny and development of extrathymic T cells in mouse liver. *Immunology* **77:** 556-563, 1992.
5) Ohtsuka K, Sato K, Watanabe H, Kimura M, Asakura H, Abo T: Unique order of the lymphocyte subset induction in the liver and intestine of mice during *Listeria* monocytogens infection. *Cell Immunol* **161:** 112-124, 1995.
6) Ohteki T, Okuyama R, Seki S, Abo T, Sugiura K, Kusumi A, Ohmori T, Watanabe H, Kumagai K: Age-dependent increase of extrathymic T cells in the liver and their appearance in the periphery of

older mice. *J Immunol* **149:** 1562-1570, 1992.
7) Yamamoto M, Homma S, Nakagawa Y, Hayami M, Imanaga H, Kurimoto M, Mitsuhashi M, Kimoto T: Activation of mouse macrophages by *in vivo* and *in vitro* treatment with a cyanine dye, lumin. *J Photochem Photobiol* **13:** 295-306, 1992.
8) Watanabe H, Ohtsuka K, Kimura M, Ikarashi Y, Ohmori K, Kusumi A, Ohteki T, Seki S, Abo T: Details of an isolation method for hepatic lymphocytes in mice. *J Immunol Methods* **146:** 145-154, 1992.
9) Ikarashi Y, Abo T, Kawai K, Iiai T, Watanabe H, Suzuki K, Matsumoto Y, Omata S, Fujiwara M: Expansion of intermediate TCR cells of mice with autoimmune-like graft-versus-host disease. *Immunology* **83:** 205-212, 1994.
10) Abo T, Watanabe H, Iiai T, Kimura M, Ohtsuka K, Sato K, Ogawa M, Hirahara H, Hashimoto S, Sekikawa H, Seki S: Extrathymic pathways of T-cell differentiation in the liver and other organs. *Int Reviews Immunol* **11:** 61-102, 1994.
11) Smith CA, Williams GT, Kingston R, Jenkinson EJ, Owen JJT: Antibodies CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* **337:** 181-184, 1989.
12) Teh HS, Kisielow P, Scott B, Kishi H, Uematsu Y, Blüthmann H von Boehmer H: Thymic major histocompatibility complex antigens and the $\alpha\beta$ T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature* **335:** 229-233, 1988.
13) Seki S, Abo T, Sugiura K, Ohteki T, Kobata T, Yagita H, Okumura K, Rikiishi H, Masuda T, Kumagai K: Reciprocal T cell responses in the liver and thymus of mice injected with syngeneic tumor cells. *Cell Immunol* **137:** 46-60, 1991.
14) Abo T, Kusumi A, Seki S, Ohteki T, Sugiura K, Masuda T, Rikiishi H, Iiai T, Kumagai K: Activation of extrathymic T cells in the liver and reciprocal inactivation of intrathymic T cells by bacterial stimulation. *Cell Immunol* **142:** 125-136, 1992.
15) Kusumi A, Abo T, Masuda T, Sugiura K, Seki S, Ohteki T, Okuyama R, Abo T: Lymphoxin activates hepatic T cells and simultaneously induces profound thymic atrophy. *Immunology* **77:** 177-184, 1992.
16) Yamamoto N, Homma S, Nakagawa Y, Hayami M, Imanaga H, Kurimoto M, Mitsuhashi M, Kimoto T: Activation of mouse macrophages by *in vivo* and *in vitro* treatment with a cyanine dye, lumin. *J Photochem Photobiol* **13:** 295-306, 1992.