Immunohistochemical Studies on the Antigen Localization and the Germinal Center Formation in Mouse Lymph Nodes

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The antigen, 2,4-dinitrophenylated and alum-precipitated bovine serum albumin (DNP-AP-BSA), was inoculated from the mouse foot pads into popliteal lymph nodes. The DNP-BSA-bearing phagocytes were first found in the paracortex, which was the thymus (T)-dependent area, at 3 hr after inoculation. Subsequently, at 1 week after inoculation, germinal centers containing a few T cells were formed within the lymphoid follicles in close contact with the T-dependent area. At 2 weeks after inoculation, crescent-shaped networks of DNP-BSA accompanying the IgG were observed in the peripheral region of germinal centers. These findings show that the cooperation of phagocytes, T cells and B cells against thymus dependent antigens may be indispensable in the formation of germinal centers and in the retention of their function in lymphocyte proliferation.—*Key words:* BSA, Mouse, Lymph node.

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The participation of macrophages is required in immunoregulation in the presentation of antigenic information to lymphocytes [8, 21], and besides the cooperation of T cells and B cells is essential in antibody production against thymus dependent antigens [11].

As the functional aspect of immunocompetent cells has been studied in detail, immunohistochemical research on antigen localization or the distribution of antigen-bearing phagocytes should also include a work on the behavior of lymphocytes after antigenic stimulation into lymphoid tissues.

This paper describes the intranodal localization of thymus dependent antigen or immunoglobulin and the process of the cooperation of B cells with T cells and phagocytes in the mouse lymph nodes during the early stages of immune response. As a thymus dependent antigen, 2, 4-dinitrophenylated bovine serum albumin (DNP-BSA) was used in this study to compare with the results in the challenges of thymus independent antigen i.e.

DNP-dextran [13] or other carrier proteins. Furthermore, considering the participation and the function of the phagocytes, the antigen was aggregated in the form easy to be phagocytized by them [10].

MATERIALS AND METHODS

Mice: Fifty five male and female BALB/c mice, aged 6 to 10 weeks, were used.

Preparation and administration of antigen: 2, 4-dinitrophenylated bovine serum albumin (DNP-BSA) was prepared with 2, 4-dinitrobenzenesulfonic acid sodium salt (Tokyo Kasei Kogyo Co., Ltd.) and crystalline BSA (Sigma Chemical Co., U.S.A.) by the method of Eisen *et al.* [3]. DNP and BSA in the prepared DNP-BSA were in the molar ratio 3–6:1. Then DNP-BSA was precipitated with alum (DNP-AP-BSA) as described by Katsura [5]. A half mg of DNP-AP-BSA in 0.05 ml of physiological saline was inoculated subcutaneously into each foot pad of mice. Thereafter, 3 to 6 mice were sacrificed in

each time sequence at 10, 30 min, 1, 3, 6, 12 hr, 1, 3 days, 1, 2, 4 and 6 weeks after inoculation.

Immunohistochemistry: Popliteal lymph nodes were fixed in periodatelysine-paraformaldehyde (PLP) and embedded in paraffin. Four μm thick sections were made and exposed to rabbit anti-DNP-BSA serum, which was prepared in our laboratory, rabbit anti-mouse IgG serum (Cappel Lab. Inc., U.S.A.) or rabbit anti-mouse T cell serum (Cedarlane Lab. Ltd., U. S. A.) to detect the respective antigens or cells, according to the enzyme linked antibody method.

Electron microscopy: Tissues were fixed in phosphate buffered 3% glutaraldehyde or PLP at 4°C, postfixed with 1% osmium tetroxide and embedded in epoxy resin. Observations were made with a HU-12A electron microscope (Hitachi).

RESULTS

In the present immunohistochemical studies on the popliteal lymph nodes, DNP-BSA-bearing phagocytes first appeared in the region of the marginal sinuses at 3 hr after inoculation. Some of the cells gathered loosely at the junction to the medullary sinuses; however, they were not observed in the medullary sinuses. The wandering antigen-bearing phagocytes lined up in tight proximity to the endothelial cells of the marginal sinuses of the nodes.

The antigenic material was also observed to be captured by the phagocytes in the paracortex first at 3 hr after inoculation. At 1 day after inoculation, they were dispersed throughout the paracortex (Fig. 1), where the small lymphocytes were loosely packed, in contrast with their tightly packed appearance

Fig. 1. Anti-DNP-BSA serum positive cells diffusely located in the paracortex (arrows) at one day after inoculation. Counter stained with hematoxylin. $\times 720$.

Fig. 2. A germinal center (GC) in close contact with the area rich in the anti-T cell serum positive cells at one week after inoculation; Paracortex (PC). Counter stained with hematoxylin. $\times 230$.

Fig. 3. An anti-T cell serum positive cell (arrow) in a germinal center at one week after inoculation. Counter stained with hematoxylin. $\times 720$.

Fig. 4. Crescent-shaped network of antigen accumulation (arrow) in the peripheral region of a germinal center (GC) at two weeks after inoculation. Treated with anti-DNP-BSA serum and stained with hematoxylin. $\times 460$.

in the lymphatic nodules.

At 1 week after inoculation, the active formation of the germinal centers were observed within the lymphatic nodules, and the medullary sides of the germinal centers were in close contact with the paracortex. Immunocytochemical analysis revealed that most of the lymphocytes in the paracortex were immunoreactive to the anti-T cell serum (Fig. 2). A few T cells were also observed to be contained within the germinal centers (Fig. 3).

At 2 weeks after inoculation, the intranodal DNP-BSA was restrictively detected within the peripheral region of the germinal centers (Fig. 4). These antigen accumulations were crescent in shape and arranged in fine networks, whose convex sides always arched along the peripheral border of the germinal center. In the sections exposed to the anti-

IgG serum, IgG was also detected within the regions of the antigen accumulations (Fig. 5).

The germinal centers increased in volume by this time. They consisted commonly of large or medium-sized lymphocytes, a few lymphoid cells in mitosis and reticular cells. The so-called tingible body macrophages, containing the highly piknotic nuclei and the condensed cytoplasm of lymphocytes (Fig. 6), were also observed in every germinal center.

No germinal centers were observed to face the marginal sinuses directly, and there was a narrow cortical zone of small lymphocytes between them and the endothelial cells of the marginal sinuses. At 2 weeks after inoculation, it was clearly confirmed that the antigen-bearing phagocytes in the marginal sinuses migrated directly into the cortical zone. Elec-

Fig. 5. IgG detected in the network (arrow) at two weeks after inoculation. Treated with anti-IgG serum and stained with hematoxylin. $\times 460$.

Fig. 6. A tingible body macrophage engulfing lymphocytes (Ly) in a germinal center at two weeks after inoculation. $\times 3530$.

tron microscopically, the phagocytes seemed to migrate through the endothelium of the marginal sinuses into the cortical zone. (Fig. 7).

At 4 weeks after inoculation, although the germinal centers were still present, the crescent-shaped networks of the antigen accumulation in the germinal centers were no longer observed. However, a few wandering phagocytes with rich cytoplasms were found in groups throughout the paracortex to possess only a slight amount of DNP-BSA in their cytoplasms (Fig. 8).

DISCUSSION

The distribution pattern of antigens in the lymph nodes has long been disputed. The process of antigen distribution might depend on the nature of the antigens. Nossal *et al.* [16] showed that the antigen, ^{125}I -labeled flagella of *Salmonella adelaide*, permeated

into the cortex over the lymphoid follicles; otherwise, the great bulk of the same antigen was observed in the macrophages of the medulla, and not in the cortical lymphoid follicles [15]. Mandel *et al.* [9] observed that, although many cells in the subcapsular sinus and the paracortex are labeled, there is already a high concentration of radiolabel in the follicles after the second challenge with ^{125}I -human serum albumin. Kelly *et al.* [6] found that the labeled large mononuclear cells were located predominantly in the paracortical zone in the response to antigen. Kotani *et al.* [7] reported that the free phagocytes engulfing colloidal carbon first appeared in the deep cortex at the corticomedullary junction, or in the medullary cords near the lymph follicles and finally entered the germinal centers through their medullary poles. It is inferred from their reports that the antigens were accumulated in the paracortex or medulla by

Fig. 7. A phagocyte extending the cytoplasmic process between the neighboring lymphocytes in the cortex just beneath the marginal sinus (MS) at two weeks after inoculation. $\times 6860$.

Inset: An anti-DNP-BSA serum positive cell in the same place. Counter stained with hematoxylin. $\times 1010$.

phagocytes, but the antigens themselves percolated into the follicles without participation of phagocytes.

From the histological observation of the basic structure of lymph nodes, the paracortex, which consists predominantly of small lymphocytes, is less densely packed than the cortex, and occasionally faces the marginal sinuses directly. Analogous observation on the paracortex facing the marginal sinuses in human lymph nodes were also delineated by Robb-Smith and Taylor [19].

Therefore considering the structures of the paracortex, the wandering free phagocytes enter more easily the paracortex from the marginal sinus than the cortex. As the antigen-bearing phagocytes pass through the paracortex, they may play a role in presenting

Fig. 8. Phagocyte clusters (arrows) with slight DNP-BSA in the paracortex at four weeks after inoculation. Treated with anti-DNP-BSA serum and stained with hematoxylin. $\times 230$.

the antigen to the lymphocytes, mainly T cells, in this area.

The above process differs from that of avian lymph nodes. According to the results on duck lymph nodes [14], the phagocytes ingesting foreign materials appeared first in the lymphatic cords, where small and medium-sized lymphocytes were loosely packed, and became located then in the lymphatic nodules, where small lymphocytes were densely packed. Sugimura *et al.* [22] elucidated that the lymphatic cords, and possibly the germinal centers, were the bursa of Fabricius (BF)-dependent area, while the deep area of lymphatic nodules were the thymus (T)-dependent one. Therefore, these phagocytes first entered the BF-dependent area and then T-dependent one. Moreover, the germinal centers were reported to be formed exclusively in the T-dependent area [22].

As the germinal centers in the duck and mouse lymph nodes appeared in close contact with the T-dependent area, T cells were possibly associated with the initiation of B cell blastogenesis to form new germinal centers. Concerning the relationship between the germinal centers and T cells, van Ewijk *et al.* [23] and Rouse *et al.* [20] observed mouse helper T cells [1] in the follicles and/or the germinal centers of lymph nodes. Poppema *et al.* [17] also reported that human helper/inducer T cells [18] were found in a crescentic pattern at the border of the mantle zone and germinal center facing the capsular sinus.

The thymus independent antigens are known to cause the blastogenesis of B cells without the overt cooperation with T cells [13]. Therefore, on the supposition that the germinal centers were formed with the assistance of T cells in the paracortex or the other areas, the analysis on the lymph node response to the thymus independent antigens might be an aid for the ascertainment of T cell involvement in the formation of the germinal center.

As to the tingible body macrophages (TBM), Flidner *et al.* [4] suggested that the tingible bodies in TBM were formed primarily from dying cells of germinoblasts, and that they were less frequent in "small" young-looking germinal centers than in the larger germinal centers. In the present study, TBM appeared frequently in every germinal center, and some of the tingible bodies were formed with nuclei of degenerated lymphocytes. TBM, therefore, seemed to play some kind of role against the germinal centers to phagocytize the over-proliferated lymphocytes. There is, however, a question as to what role TBM play exactly and which lymphocytes were destroyed by TBM.

The crescent-shaped network of antigen in the germinal centers was also reported by others [9, 12]. Miller and Nossal [12] reported that the web of antigen, which extended first from the peripheral sinus, occupied all of

the primary follicles and finally was compressed in a semilunar area at the superficial aspect of the germinal centers. As Chen *et al.* [2] also found that the administered horseradish peroxidase (HRP)-anti-HRP complexes became located in the white pulp nodules of the mouse spleen, it was considered in the present study that the antigen and antibody might exist as antigen-antibody complexes at the crescent-shaped network of the germinal centers. The authors, however, did not detect any accumulation of antigen-antibody complexes before the formation of the germinal center. Furthermore, the antigen and/or complexes facing the network after their formation were not observed.

Further studies on the function and fate of germinal centers are needed to elucidate the mechanism of antigen retention and following process of antibody production.

REFERENCES

1. Cantor, H. and Boyse, E. A. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* 141: 1376-1389.
2. Chen, L. L., Frank, A. M., Adams, J. C. and Steinman, R. M., 1978. Distribution of horseradish peroxidase (HRP)-anti-HRP immune complexes in mouse spleen with special reference to follicular dendritic cells. *J. Cell Biol.* 79: 184-199.
3. Eisen, H. N., Carsten, M. E. and Belman, S. 1954. Studies of hypersensitivity to low molecular weight substances. III. The 2,4-dinitrophenyl group as a determinant in the precipitin reaction. *J. Immunol.* 73: 296-308.
4. Flidner, T. M., Kesse, M., Cronkite, E. P. and Robertson, J. S. 1964. Cell proliferation in germinal centers of the rat spleen. *Ann. New York Acad. Sci.* 113: 578-594.
5. Katsura, Y. 1972. Studies on γ M and γ G antibody response of mice to bovine serum albumin. I. A systematic survey of immunizing conditions. *Jpn. J. Microbiol.* 16: 223-232.
6. Kelly, R. H., Balfour, B. M., Armstrong, J. A. and Griffiths, S. 1978. Functional anatomy of lymph nodes. II. Peripheral lymph-borne mononuclear cells. *Anat. Rec.* 190: 5-22.
7. Kotani, m., Okada, K., Fujii, H., Tsuchiya, H., Matsuno, K., Ekino, S. and Fukuda, S. 1977.

- Lymph macrophages enter the germinal center of lymph nodes of guinea pigs. *Acta Anat.* 99: 391-402.
8. Lipsky, P. E. and Rosenthal, A. S. 1975. Macrophage-lymphocyte interaction. II. Antigen-mediated physical interaction between immune guinea pig lymph node lymphocytes and syngeneic macrophages. *J. Exp. Med.* 141: 138-154.
 9. Mandel, T. E., Phipps, R. P., Abbot, A. and Tew, J. G. 1980. The follicular dendritic cell: Long term antigen retention during immunity. *Immunol. Rev.* 53: 29-59.
 10. Mehl, T. D. and Lagunoff, D. 1975. Uptake of aggregated albumin by rat macrophages *in vitro*: Affinities of cells for monomeric and aggregated bovine serum albumin. *J. Reticuloendothelial Soc.* 18: 125-135.
 11. Miller, J. F. A. P. and Mitchell, G. F. 1967. The thymus and the precursors of antigen reactive cells. *Nature (Lond.)* 216: 659-663.
 12. Miller, J. J., III and Nossal, G. J. V. 1964. Antigens in immunity. VI. The phagocytic reticulum of lymph node follicles. *J. Exp. Med.* 120: 1075-1086.
 13. Mosier, D. E. and Subbarao, B. 1982. Thymus-independent antigens: Complexity of B-lymphocyte activation revealed. In: *B Lymphocytes Today* (Inglis, J. R. ed.), 70-75. Elsevier Biomedical Press, Cambridge.
 14. Nakamura, K., Hashimoto, Y., Kitagawa, H. and Kudo, N. 1982. Intranodal pathway of phagocytes ingesting foreign materials in ducks. *Jpn. J. Vet. Sci.* 44: 735-742.
 15. Nossal, G. J. V., Abbot, A. and Mitchell, J. 1968. Antigens in immunity. XIV. Electron microscopic radioautographic studies of antigen capture in the lymph node medulla. *J. Exp. Med.* 127: 263-276.
 16. Nossal, G. J. V., Abbot, A., Mitchell, J. and Lummus, Z. 1968. Antigens in immunity. XV. Ultrastructural features of antigen capture in primary and secondary lymphoid follicles. *J. Exp. Med.* 127: 277-290.
 17. Poppema, S., Bhan, A. K., Reinherz, E. L., McCluskey, R. T. and Schlossman, S. F. 1981. Distribution of T cell subsets in human lymph nodes. *J. Exp. Med.* 153: 30-41.
 18. Reinherz, E. L., Kung, P. C., Goldstein, G. and Schlossman, S. F. 1979. Further characterization of the human inducer T cell subset defined by a monoclonal antibody. *J. Immunol.* 123: 2894-2896.
 19. Robb-Smith, A. H. T. and Taylor, C. R. 1981. General structure of the lymph node. In: *Lymph Node Biopsy*, 13-41. Miller Heyden Ltd., London.
 20. Rouse, R. V., Ledbetter, J. A. and Wissman, I. L. 1982. Mouse lymph node germinal centers contain a selected subset of T cells-the helper phenotype. *J. Immunol.* 128: 2243-2246.
 21. Seeger, R. C. and Oppenheim, J. J. 1970. Synergistic interaction of macrophages and lymphocytes in antigen-induced transformation of lymphocytes. *J. Exp. Med.* 132: 44-65.
 22. Sugimura, M., Hahsimoto, Y. and Nakanishi, Y. H. 1977. Thymus and bursa-dependent areas in duck lymph nodes. *Jpn. J. Vet. Res.* 25: 7-16.
 23. Van Ewijk, W., Van Soest, P. L. and Van den Engh, G. L. 1981. Fluorescence analysis and anatomic distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2 and T-200. *J. Immunol.* 127: 2594-2604.

要 約

マウスリンパ節内における抗原物質の分布および胚中心形成に関する免疫組織化学的研究：中村和市・橋本善春・北川 浩・工藤規雄（北海道大学獣医学部家畜解剖学教室）——抗原としてジニトロフェニール基結合明ばん沈澱ウシ血清アルブミンをマウス肉球皮下に投与後3時間で、リンパ節内傍皮質（胸腺依存領域）において抗原摂食細胞が観察された。投与後1週間には、皮質リンパ小節において胸腺依存領域に接して胚中心が形成され、内部には少数のTリンパ球を含んでいた。投与後2週間では、胚中心皮質側にIgGを伴った抗原が細網状にみとめられた。これらの所見から、リンパ節胚中心の形成と機能の保持には、食細胞を介する傍皮質内への抗原の侵入と、それに続くTリンパ球、Bリンパ球の協調が重要であることが示唆された。