Evidence of antibody production in the rat cervical lymph nodes after antigen administration into the cerebrospinal fluid*

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Summary. We previously showed histologically that, in the rat, the cerebrospinal fluid drains from the subarachnoid space along the olfactory nerves to the nasal lymphatics and empties into the superficial and deep cervical lymph nodes. The present study was performed to investigate whether these lymph nodes play a role in the immune response of the central nervous system. For this purpose, keyhole limpet hemocyanin conjugated with fluorescein isothiocyanate (KLH-FITC) was administered into the subarachnoid space of the rat brain, and the time-kinetics and location of FITC and anti-FITC antibody forming cells in the cervical lymph nodes were studied histologically and immunohistochemically. FITC fluorescence was detected in superficial and deep cervical lymph nodes as well as the subarachnoid space and the nasal mucosa 2 h after FITC-KLH injection into the subarachnoid space. The specific antibody-forming cells first appeared in both the superficial and deep cervical lymph nodes on the 4th day after antigen administration although the reaction was more intense in the

deep than in the superficial cervical lymph nodes. These cells were located in the medullary cords of the cervical lymph nodes. The number of antibody forming cells increased thereafter, reached a peak around the day 6, and then declined on day 10. These findings indicate that antigens introduced in the cerebrospinal fluid are drained into the cervical lymph nodes through the nasal lymphatics and initiate the antigen-specific immune response there. Thus, the cervical lymph nodes probably act as a monitoring site for cerebrospinal fluid and play a major role in the central nervous system immune response.

Introduction

The consensus concerning the cerebrospinal fluid drainage holds that the fluid is absorbed through the arachnoid villi, while previous investigators have also discussed the presence of alternative routes for the fluid drainage into the peripheral lymphatic system, especially into the nasal lymphatics (Johnston *et al.*, 2004; Koh *et al.*, 2005). We have also paid attention to these routes and recently demonstrated by light and electron microscopy that India ink administered into the subarachnoid space spreads along the olfactory nerves and drains into the nasal mucosa, where the ink is collected into the lymphatics (Walter *et al.*, 2006). We further showed that ink collected in the nasal lymphatics empties into the superficial and deep cervical lymph nodes, as has been also indicated by some other investigators (Bradbury and Westrop, 1983; Erlich *et al.*, 1986; Kida *et al.*, 1993). These findings indicate that the olfactory pathway acts not only as an alternative route for cerebrospinal fluid absorption but also as the route for antigen drainage from the subarachnoid space to the cervical lymph nodes, where antigen-specific immune

Received October 18, 2005

*This work was supported in part by a research grant from the Society for the Promotion of International Oto-Rhino-Laryngology (SPIO) to Beatriz A. Walter.

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responses may be induced (Weller *et al.*, 1992; Thorne *et al.*, 2004; Hatterer *et al.*, 2006).

Some previous investigators have indeed stressed the importance of the cerebrospinal fluid absorption through the olfactory pathway in terms of the central nervous system immune response (Harling-Berg *et al.*, 1999). Immunological analyses by enzyme-linked immunosorbent assays (ELISA) have revealed that specific antibodies are produced in the cervical lymph nodes following antigen injection in the subarachnoid space in the rat (Harling-Berg *et al.*, 1989; Cserr *et al.*, 1992b). However, no direct histological observation has been made of the formation of the specific antibody-forming cells in the cervical lymph nodes in relation to the central nervous system immune response.

Thus, the aim of the present study is to demonstrate directly the specific antibody forming cells in lymphoid tissues after antigen administration into the subarachnoid space. For this purpose, we used keyhole limpet hemocyanin conjugated with fluorescein isothiocyanate (KLH-FITC) as an antigen to be administered into the subarachnoid space. Since FITC is recognized as a hapten, the specific antibody-forming cells, if present, can be detected in tissue sections by using an indirect immunoalkaline phosphatase method (Matsuno *et al.*, 1989) for staining antibodies to the FITC. We administered the antigen into the subarachnoid space of the rat brain, and the time-kinetics and location of FITC and anti-FITC antibody forming cells in the cervical lymph nodes were studied histologically and immunohistochemically. The present study offers evidence that the cervical lymph nodes produce antibody forming cells in response to antigen administration into the subarachnoid space. The results strongly suggest the importance of the cervical lymph nodes to the central nervous system immune response.

Materials and Methods

Experimental design

Adult male Wistar rats (7 to 9 weeks old) were used according to the Guidelines of the Animal Care, Niigata University, Japan. The animals were anesthetized by an intramuscular injection of 100 mg ketamine and 50 mg xylazine per kg of body weight. They were placed in a stereotactic frame in a prone position, and the parietal and interparietal bones were exposed by a midsagittal incision of the scalp. A twist-drill hole was made in the rostral end of the interparietal bone (two mm lateral to the midline), and the dura mater and the arachnoid membrane were perforated to insert a polyethylene cannula into

the subarachnoid space. After the proper placement of the cannula was confirmed by free back-flow of clear cerebrospinal fluid, the tube was fixed with cyanoacrylate glue and 400 μ g of keyhole limpet hemocyanin conjugated with fluorescein isothiocyanate (KLH-FITC) antigen in a volume of 40 μ l was injected through the cannula, which was heat-sealed followed by closure of the skin. KLH-FITC was prepared after Rhodes and Lind (1968). In brief, 2 mg of FITC (Isomer I, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 5 ml of a 1 M carbonate buffer (pH 9.5), while KLH (Calbiochem, Merck KGaA, Darmstadt, Germany) was dissolved in 5 ml saline. Both solutions were mixed gently for 2 h at room temperature and then dialyzed against multiple changes of phosphate-buffer saline (PBS) for 2 days. The fluorescence protein ratio was approximately 100. After the antigen injection, the animals were allowed to recover from the anesthesia.

The animals were killed by an overdose of the anesthesia at 1 h, 2 h, or 1, 2, 4, 6 or 10 days, and superficial and deep cervical lymph nodes, axillary lymph nodes, and the spleen were removed from the body. The brain and nasal mucosa were also removed from the animals killed 2 h after the antigen administration. All the samples were embedded in OCT compound (Tissue-Tek, CA, USA) and snap-frozen in liquid nitrogen. Cryosections of 5 μ m thickness were cut in a cryostat (Leica, CM 1850, TX, USA), air dried overnight, and stored at -20°C until use.

Localization of the FITC antigen

Cryosections of the superficial cervical lymph nodes, deep cervical lymph nodes, axillary lymph nodes, spleen as well as the brain tissue with the nasal mucosa were examined in a fluorescence microscope (TE 2000-U Nikon, Tokyo). The FITC fluorescence was identified using a filter of EX-450-490 nm.

Specific anti-FITC antibody staining by the indirect immunoalkaline phosphatase technique (AFC staining)

Cryosections of the superficial cervical lymph nodes, deep cervical lymph nodes, axillary lymph nodes or spleen — of 5 μ m thickness prepared as above — were fixed in acetone for 10 min. Samples were then rinsed in phosphate-buffer saline (PBS) containing 0.1% Tween 20 for 5 min three times followed by incubation with a blocking solution consisting of normal goat serum in PBS at 1:10 dilution for 30 min. The sections were then incubated with FITC-labeled streptavidin (Zymed, Carlsbad, CA, USA) at 10 μ g/ml in PBS with 0.2% bovine serum albumin (BSA) for 2 h at room temperature. After washing with PBS

Fig. 1. Fluorescent micrographs of the meninges of the brain (**a**), nasal mucosa (**b**), deep cervical lymph node (**c**, **d**), and spleen (**e**, **f**) 2 h after FITC-KLH injection into the subarachnoid space. **a:** The antigen is observed as green spots for the FITC fluorescence in the subarachnoid space (AS). BP: brain parenchyma. Scale bar: 100 μm . **b:** Fluorescence for the antigen is scattered in the nasal mucosa. NM: nasal mucosa, CP: cribriform plate, NE: nasal epithelium. Scale bar: 200 μm . **c** and **d:** The medullary sinuses (MS) of the deep cervical lymph node are filled with a large amount of the fluorescent antigen. There are also fluorescence substances in the subcapsular sinuses (SS). Scale bars: 500 μm (**c**), 200 μm (**d**). **e** and **f:** This section was made through the germinal center (G) of the spleen. Cells uptaking the antigen are present in the white as well as the red pulp. Scale bars: 200 μm (**e**), 100 μm (**f**)

Fig. 2. Staining of anti-FITC antibody forming cells in the deep cervical lymph nodes (dcln), superficial cervical lymph nodes (scln) and the spleen. Each column shows sections taken at 2 (a, e, i), 4 (b, f, j), 6 (c, g, k), and 10 (d, h, l) days after FITC-KLH injection into the subarachnoid space. Note the change in location and number of the antibody forming cells with the time course. Scale bar: 200 μ m

for 15 min, biotinylated alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA) in 20 μ g/ml PBS with 0.2% BSA was applied onto the sections for 40 min at room temperature. The samples were fixed with 1% glutaraldehyde in PBS for 30 sec and rinsed in PBS. The alkaline phosphatase activity was developed with

the Vector Blue Alkaline Phosphatase Substrate Kit III, (Vector Laboratories), with 0.2 mM of Levamisol (Dakocytomation, Carpinteria, CA, USA) to block the activity of the endogenous alkaline phosphatase. The samples were lightly counterstained with 2% methyl green or hematoxylin and mounted using an aqueous medium

(Mount-Quick "Aqueous", Daido Sangyo Co, Ltd, Tokyo).

In order to examine the number of antibody forming cells, the numbers of AFC-FITC positive cells on sections were counted in three portions of each section from day 2 up to day 10, and a statistical analysis was performed using ANOVA and Kruskal-Wallis methods (SPSS 9.0 Chicago, IL, USA).

Double staining of anti-FITC antibody forming cells and type IV collagen

The cryosections of the deep cervical lymph nodes were first stained for anti-FITC antibody forming cells as described above. The slices were fixed with 1% glutaraldehyde for 10 min at room temperature and rinsed with distilled water. They were treated with a 0.001% trypsin solution (Sigma) for 2 min at 37°C, washed with PBS, and incubated with 10% normal goat serum as blocking solution for 30 min. The sections were reacted with a rabbit anti-human type IV collagen antibody (LSL Co, Tokyo) at 1:500 dilution with 0.2% bovine serum albumin in PBS for either 2 h at room temperature or overnight at 4°C. The sections were then rinsed with PBS and incubated with goat anti-rabbit IgG (Dakocytomation, Glostrup, Denmark) at 1:100 dilution with 0.2% bovine serum albumin in PBS for 45 minutes at room temperature. The sections were rinsed with PBS and immersed in 0.02% diaminobenzidine tetrahydrochloride (DAB) in Tris-buffer, with 0.006% H₂O₂ for visualization of the immunohistochemical reaction. They were rinsed in distilled water and mounted using an aqueous medium for observation by light microscopy.

Results

Antigen localization

Two hours after FITC-KLH administration into the subarachnoid space, FITC fluorescence was found in the subarachnoid space, especially on the surface of the brain parenchyma (Fig. 1a). The fluorescence was also detected in the nasal mucosa, around the olfactory nerves, and in some lymphatics (Fig. 1b). As for the lymph nodes examined, FITC fluorescence was observed in the superficial and deep cervical lymph nodes but was not found in the axillary lymph nodes. In longitudinal sections of both the superficial and deep cervical lymph nodes, FITC was present in the subcapsular and medullary sinuses—though the amount was the most abundant in the medullary sinus (Fig. 1c, d). At high magnification, FITC fluorescence was observed as green spots, suggesting that the antigen was phagocytosed

Fig. 3. Time kinetics of specific antibody-forming cells in the deep cervical lymph nodes after antigen injection into the subarachnoid space. Values represent the mean (SE) of the number of antibody forming cells in three samples taken from each group. There was a statistically significant increase in the number of antibody forming cells that reached a peak at day 6 and declined progressively thereafter.

by macrophages in the medullary sinus. FITC fluorescence was also detected in the spleen; the fluorescent substances were scattered in the white and red pulp though they were scanty (Fig. 1e, f).

Antibody forming cells in the cervical lymph nodes and spleen

The indirect immunoalkaline phosphatase technique for detecting the specific anti-FITC antibodies visualized the presence of antibody forming cells and immune complexes that had been induced by the injected KLH-FITC antigen as blue spots. In the lymph nodes examined, the specific antibody-forming cells first clearly appeared in both the superficial and deep cervical lymph nodes at 4 days after KLH-FITC administration into the subarachnoid space although the number of the antibody forming cells was larger in the deep cervical lymph node than in the superficial one (Fig 2a, h). The antibody forming cells were scattered in the medulla of the lymph nodes, whereas they rarely were found in the cortex. These cells gradually increased in number thereafter, reached a peak from the 6th to 8th day, and then decreased gradually in number afterwards (Fig. 3). No antibody forming cells specific for

Fig. 4. Deep cervical lymph node 4 days after antigen injection into the subarachnoid space. Anti-FITC antibody-forming cells are stained in blue, and the collagen is immunostained in brown with anti-collagen IV antibody. **a:** The antibody-forming cells are mainly present in the medullary region of the lymph nodes. Arrowheads: medullary cords. Scale bar: 500 μm . **b:** A closer view of a part of **a**. The antibody forming cells are scattered in the medullary cords. Scale bar: 200 μm . **c:** At a higher magnification, antibody forming cells are indicated by arrows. GC: germinal center; MS: medullary sinus. Scale bar: 50 μm

Fig. 5. Deep cervical lymph node 6 days after FITC-KLH injection into the subarachnoid space. **a:** Antibody forming cells are present mainly in the medullary region of the lymph node. Arrowheads: medullary cords. Scale bar: 500 μ m. **b:** Germinal centers are lightly stained in blue, with some positive cells present inside. In the medullary cords, antibody forming cells are strongly positive. Scale bar: 200 μ m. **c:** Higher magnification of a part of **b**. Antibody forming cells with blue cytoplasm are indicated by arrows. GC: germinal center; MS: medullary sinus. Scale bar: 50 μ m

Fig. 6. Deep cervical lymph node 10 days after immunization of the antigen into the subarachnoid space. **a:** Antibody forming cells are present mainly in the medullary region. Scale bar: 500 μm . Arrowheads: medullary cords. **b:** Antibody forming cells are observed inside the medullary cords. Scale bar: 200 μm . **c:** High magnification of a part of **b**. Antibody forming cells are present within the medullary cords. GC: germinal center; MS: medullary sinus. Scale bar: 50 μm .

KLH-FITC were detected at either period in the axillary lymph nodes (micrographs are not shown).

In the spleen, the antibody forming cells specific for KLH-FITC were observed in the outer periarterial lymphoid sheath in the same fashion as most of the antibody-forming cell reactions (Matsuno *et al.*, 1989) from 2 days after the antigen administration into the subarachnoid space. These cells were most numerous at 4 days and decreased rapidly in number at 6 days (Fig. 2 i–l).

Localization of antibody forming cells in the deep cervical lymph nodes.

To visualize the reticular framework and to identify anatomical compartments in the cervical lymph nodes, immunostaining for type IV collagen was performed in sections stained for the antibody forming cells (Matsuno *et al.*, 1996). Immunoreactivity for type IV collagen was strongly detected around the blood vessels due to the presence of the basal lamina while it appeared weak and discontinuous beneath the sinus-lining cells and in the reticular fibers, resulting in the visualization of a reticular framework throughout the lymphoid tissue: two or three deep cortical units with some germinal centers were clearly identified, and subcapsular, intermediate and marginal sinuses as well as blood vessels were clearly recognized in these sections. Thus, the localization of the antibody-forming cells was able to be studied clearly and precisely in relation to the structure of the deep cervical lymph nodes. On the 4th day after KLH-FITC administration into the subarachnoid space, the specific antibody-forming cells were present predominantly in the medulla, while very few or even none of these cells were detected in the cortex, including germinal centers (Fig. 4a). At higher magnification, the antibody forming cells with a strong reactivity were restricted in the medullary cords but not in the medullary sinuses (Fig. 4b,c). Around the 6th day, the antibody-forming cells were also present in the medullary cords throughout the medullary region (Fig. 5a–c). The deep cortex in each deep cortical unit appeared to increase in volume, but there were no antibody forming cells in this region. In germinal centers, specific antibody forming cells were occasionally found—although their numbers were very limited at this period. On the 10th day, the antibody forming cells were accumulated more clearly in the medullary cords (Fig. 6a–c). There were also some antibody forming cells in the germinal center in the reacting lymph node.

Discussion

The present study has clearly shown that the cervical lymph nodes, both superficial and deep, play an important role in the antigen-specific immune response to antigen injected into the subarachnoid space in the rat brain. We have recently investigated the pathway of the cerebrospinal fluid drainage through the olfactory nerve into the lymphatics anatomically and histologically (Walter *et al.*, 2006); we demonstrated by light and electron microscopy that India ink injected into the subarachnoid space of the rat brain was drained along the olfactory nerves to the nasal mucosa, where the ink was collected into the nasal lymphatics to finally empty into the superficial and deep cervical lymph nodes. We then suggested the importance of this route for the movement of antigens from the subarachnoid space to the extracranial lymphatic vessels. To clarify this point, in the present study we injected KLH-FITC as an antigen into the subarachnoid space and succeeded in obtaining the evidence that this antigen also appears in the nasal mucosa, which suggests the drainage of this antigen through the olfactory route. Thus, the abundance of the antigen appearing in both the superficial and deep cervical lymph nodes in this study can be explained by a collection of the antigen from the lymphatics in the nasal mucosa.

As described in the Introduction, some previous investigators have indeed pointed out the importance of the cervical lymph nodes in the systemic humoral immune response to cerebrospinal fluid-administered antigens in experimental animals, including the rat (Cserr *et al.*, 1992a; Knopf *et al.*, 1998; Ling *et al.*, 2003). For example, Harling-Berg *et al.*, (1989) injected human serum albumin (HSA) into the cerebrospinal fluid in the rat and examined the formation of antibody titers using an enzyme-linked immunosorbent assay (ELISA); from their findings, they considered that the serum anti-HSA antibody was probably predominantly produced in the superficial and deep cervical lymph nodes by demonstrating a high titer of the antibody in cell cultures of the cervical lymph nodes. However, to our knowledge, no direct morphological observation of antibody forming cells in response to an antigen has been previously performed. In the present study, we are first to show direct histological evidence that both the superficial and deep cervical lymph nodes actually produce antibody-forming cells in response to antigen administration into the subarachnoid space.

It is true that the antibody forming cells for KLH-FITC can also be detected in the spleen, as shown in our study. The production of an antibody against the cerebrospinal fluid-administered antigen in the spleen as well as cervical lymph nodes has been also suggested in

the previous studies (Harling-Berg *et al.*, 1989). However, total antibody (e.g. anti-HSA antibody) production by combined superficial and deep cervical lymph nodes was reported to be greater than that by the spleen (Harling-Berg *et al.*, 1989). Our histological studies also suggest a greater role in the rat central nervous system immune response for cervical lymph nodes than for the spleen.

Another point of interest is the pathway of cerebrospinal fluid-administered antigen to the spleen. Since it is probable that the cerebrospinal fluid also drains into the dural sinus *via* the arachnoid villi, the antigen might be carried into the spleen directly through the blood circulation. Another possibility is that the antigen, which has entered the cervical lymph nodes, may leave the lymph nodes from the efferent lymphatics, come into the blood circulation, and reach the spleen. Although this question was not elucidated in the present study, the primary immune response of cervical lymph nodes and spleen found in the present study may be comparable to that of the popliteal lymph nodes and spleen in the mouse after subcutaneous immunization with KLH-trinitrophenyl (TNP) conjugates in the hind footpad (Delemarre *et al.*, 1989); the antibody forming cells appeared in both the lymph nodes and spleen—although the response was earlier and weaker in the spleen than in the lymph nodes. This may suggest that the immune response to the cerebrospinal fluid-administered antigen in the cervical lymph nodes and spleen is mainly concerned with antigens drained through the lymphatics.

As for the localization of the antibody-forming cells in the lymph node, previous investigators reported that the cells predominantly accumulated in the medullary cords three to five days after the immunization of antigens (Straus, 1970; Geldof *et al.*, 1984; van Rooijen, 1987). The present study has clearly shown that the antibody-forming cells specific to the cerebrospinal fluid-administered antigen also appeared and were accumulated in the medullary cords. The accumulation of the specific antibody-forming cells in the medullary cords is considered a common phenomenon in the reacting lymph nodes in the antigen-specific immune response (Wehrli *et al.*, 2001; Brighenti *et al.*, 2005; Pabst *et al.*, 2005). The medullary sinuses are limited by a thin sheet of lining cells that separates the sinuses from the medullary cords (Ushiki *et al.*, 1995; Gretz *et al.*, 1997; Ohtani *et al.*, 2003). This suggests that the antibody secreted by the antibody-forming cells probably permeates through the lining cell sheet, enters the medullary sinuses, leaves through the efferent lymphatics, and increases the antibody titer in the serum. The relationship of the antibody-forming cells with accessory cells and T-lymphocytes in the lymph nodes is not a subject of this

study, although it would be interesting to investigate the precise mechanism of the initiation of the primary immune response.

In conclusion, we have clearly shown evidence that the superficial and deep cervical lymph nodes have the ability to produce the antibody forming cells specific for an antigen administered into the cerebrospinal fluid. Thus, these lymph nodes are important not only for monitoring but also for regulating the central nervous system immune response in the rat—and probably in some other animals.

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