Original Article

Cell proliferation in the endolymphatic sac in situ during the immune response of inner ear

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Abstract Background Normally, few immunocompetent cell are present in the endolymphatic sac (ES). During an active immune response in the inner ear, large amount of inflammatory cells, including immunocompetent cells, are seen in the ES. The current study aimed at assessing cellular proliferation within the ES during induced immune response in the inner ear. Methods Fifteen healthy, female SD rats were sensitized systemically with keyhole limpet hemocyanin (KLH), followed by local inoculation in the cochlea through basal turn fenestration with the same antigen. On Days 3, 7 and 14 following inoculation, the animal was sacrificed after intraperitoneal administration of 5-bromo-2’deoxyuridine (BrdUrd), and the temporal bone harvested. Following decalcification, infiltration by BrdUrd- and IgG-positive cells in the ES was studied on frozen sections with H & E and immunohistochemical staining. Results During the secondary immune response in the inner ear against T-dependent antigens, there is increased cellular proliferation in the ES. The proliferated cells may differentiate into immunocompetent cells at the same location. Conclusions These findings indicate that the ES plays an important role in immune response of inner ear.

Key words Endolymphatic sac; Cell proliferation; Immune response

Introduction Studies have repeatedly shown that large number of inflammatory cells including immunocompetent cells are present in the ES when the inner ear is challenged by foreign antigens. This has been considered to represent an important role the ES plays in the immunodefense process in the inner ear. In the present study, cellular proliferation in ES during an inner ear immune response was investigated through cellular marking and immunohistochemical labeling.

Material and methods Twenty healthy adult female SD rats, weighing 180 - 200 g, were divided into an experiment group (n=15) and a control group (n=5). The animals were supplied by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China. Following systemic sensitization and local antigen challenge, animals in the experiment group were randomly sacrificed on Days 3, 7, and 14 and their temporal bones harvested for histological and immunohistochemical studies.

1) Systemic sensitization: For animals in the experiment group, 500 μg KLH (keyhole limpet hemocyanin) (Sigma Chemical Company, USA) in complete Freund adjuvant (CFA) was injected hypodermically at multiple spots in the inner side of fore and hind legs. This was followed 1 week later by a booster of the same dose at the same locations.

2) Inner ear antigen challenge: Inner ear antigen challenge was conducted 2 weeks after initial systemic sensitization. In all animals in the experiment group, the right ear was challenged while the left ear served as self-control. Under general anesthesia induced with intraperitoneal injection of chloral hydrate (300 mg/kg), the bulla was opened via post-aural approach, and a window made in the basal turn of the cochlea under operational microscope. Access to the perilymphatic space was verified via visual identification of perilymph outflow before delivery of 5 μl of KLH.
diluted in phosphate-buffered saline (PBS) (right ear) or Hank's solution (left ear) through a capillary pipette inserted into the perilymphatic space through the bony window. The window was subsequently sealed with bone wax and the wound closed with sutures.

3) Cellular proliferation marker introduction and preparation for histological study: BrdUrd (bromodeoxyuridine) (Sigma, USA) was given intraperitoneally at 50 mg/kg in all animals. General anesthesia was induced 1 hour later and the bulla reopened, as described above. An additional window was made at the apex of the cochlea. The cochlea was then perfused with 4% paraformaldehyde in PBS from round window to oval window for live labyrinthine fixation. The animal was subsequently decapitated and the temporal bone harvested. The temporal bone specimen was fixed in cold PLP for 24 hours before decalcification in 10% ethylene diamine tetraacetic acid (EDTA-Na2) for 2–3 weeks. Air was extracted from specimen under negative pressure for 10 minutes before fixation and decalcification. The specimen was then embedded in OCT compound. The frozen sections of the temporal bone(10 μm) were made at a plane parallel to the long axis of the cochlea and perpendicular to the temporal bone. Sections of the cochlea and ES were collected and stored for later study.

4) Immunohistochemical and H & E staining: Strep avidin-biotin complex (SABC) was used for IgG and KLH staining. After dewaxing in xylene, the specimen was incubated in 0.3% H2O2 in methanol for 15 minutes to neutralize endogenous peroxidases. This was followed by incubation in 10% sheep serum for 30 minutes, and in mouse anti-KLH monoclonal antibody (1:1000, Sigma, USA) or mouse anti-rat IgG (1:1000, Sigma, USA) for 1 to 2 hours. Sections were then incubated with biotinylated sheep anti-mouse IgG antibody and SABC (Boshide Company, Wuhan) respectively for 30 minutes, followed by staining with 3,3′ diamino-benzidine (DAB) for 3 to 5 minutes. Staining was terminated with distilled water. Some sections were double stained with hematoxylin for 1 min. Sections were then dehydrated and sealed in neutral balata. Under light microscope, chocolate brown color was seen on the cell membrane for IgG staining and inside cell and in other tissues for KLH staining.

The difference in BrdUrd staining was application of 4mol/L hydrochloric acid for 25 min, after blocking endogenous peroxidase, to denature DNA double-helical chains for exposure of BrdUrd. After neutralizing acidity with cold boracic acid buffer, 0.01% protease was added for 5 min for destruction of the cell membrane and karyotheca to improve permeability for antibodies. In addition, the primary antibody was mouse anti-BrdUrd monoclonal antibody (1:1000, Sigma USA). The remaining steps for IgG staining were the same. Under light microscope, chocolate brown color in the nucleus represents positive IgG staining.

For test control, the primary antibody was replaced with PBS.

In addition, routine H & E staining was used for observation of cell proliferation in the cochlea and ES.

Results

1) H & E staining observation: In the 5 ears harvested 3 days after antigen challenge, various degree of mononuclear-phagocyte infiltration was seen in the ES lumen, underneath the epithelium and perisaccular regions. In ears harvested on the 7th and 14th day following antigen challenge, cellular infiltration increased significantly and was dominated by plasma cells and lymphocytes (Fig.1). Similar cellular infiltration was also observed in the scalae tympani and vestibuli. No cellular infiltration was seen in ears that received Hank's solution as self-control.

2) KLH staining observation: In all antigen challenged ears, phagocytization of KLH was seen in the ES lumen and perisaccular regions (Fig.2) on the 3rd and 7th day post-challenge, followed by clearance of KLH from these areas on later days. These changes were absent in control ears.

3) BrdUrd and IgG staining observaion: Sporadic presence of BrdUrd+ cells and a small number of IgG+ cells were seen in the ES lumen, underneath the Fig. 1 Infiltration by lasmocytes and lymphocytes in the ES on Day 7 after inner ear antigen challenge (H & E staining, ×400)
animals, compared to those in the control group (Fig.5). On Days 7 and 14, there was an increase of IgG + cells in the ES lumen, mostly within the epithelium and around the saccule(Fig.6). There were no such changes in control ears treated with Hank's solution.

No chocolate brown staining was seen on any of the control sections.

**Discussion**

Studies have shown that normal ES contains a small amount of immunocompetent cells including various types of T lymphocytes, plasmocytes and macrophagocytes. These are called local resident immunocytes[1]. During inflammatory or immune reactions in the inner ear, large amount of lymphocytes are seen in the ES[2]. These cells may have arrived from the systemic circulation, or they may be a result of epithelium and around the saccule in the control group animals (Fig.3 and 4). On Day 3 following antigen challenge, there was a noticeably increased number of BrdUrd+ cells in the same regions in experiment group
proliferation and differentiation of local resident lymphocytes. Following systemic antigen stimulation, precursors of IgG+ cells are sensitized in the spleen, lymph nodes or other immune organs. When the inner ear is challenged by the same antigen, these precursors enter the ES through a “homing” mechanism mediated by adherent molecules[3], where they proliferate and differentiate into mature cells under the influence of antigen-presenting cells (APC) and cytokines such as IL-2[4]. It is also possible that, during this process, local resident lymphocytes increase in number through in situ proliferation and differentiation to provide abundant antigen-specific lymphocytes, important for the development and maintenance of inner ear immune reactions[5].

The cellular cycle is known to consist of four phases: G1, S, G2 and M. The S phase (DNA synthesize phase) is an important sign of cellular proliferation. BrdUrd is a thymidine analogue that can be specifically incorporated into the nuclei during the S phase. BrdUrd labelling can therefore be used to demonstrate existence of S phase cells in the tissue as an indicator of cellular proliferation. Immunohistochemical staining can be used to reveal the existence of such cells in the tissue, as an indicator of cell proliferation[6]. The increase of BrdUrd+ cells (i.e., S phase cells) in the ES on Day 3 following antigen challenge in this study likely represents elevated cellular proliferation activity in the ES as part of inner ear immune response.

KLH is a T-dependent antigen. It is generally agreed that IgG is the main immunoglobulin type in secondary immune responses to T-dependent antigens. Production of IgG in this response requires interaction between T and B cells. In the current study, the number of IgG+ cells in the ES increased gradually from Day 7 after inner ear antigen challenge, at locations where BrdUrd+ cells were observed. This may indicate in situ differentiation of newly proliferated cells in the ES. In addition, the time difference between the appearance of IgG+ cells and BrdUrd+ cells is consistent with the time needed for S-phase cells to differentiate into IgG+ cells.

Our study also demonstrated existence of mononucleo-phagocyte proliferation in the ES 3 days after inner ear antigen challenge. The timing is consistent with phagocytosis of antigens. The dominant presence of lymphocytes and plasmacytes on Day 7 suggests presentation of the antigen to lymphocytes in the ES by the phagocytes after recognizing and processing the antigen, which in turn stimulates lymphocyte proliferation and then differentiation into a large number of immunocompetent cells to participate in the immune response.

The results in this study indicate that there is increased cellular proliferation in the ES during secondary immune response to T-dependent antigen and that the newly proliferated cells may differentiate into immunocompetent cells locally. These findings suggest that the ES may be an important location for generating immunocompetent cells and play a pivotal role in inner ear immune responses.

References