MORPHOLOGY AND PATHOMORPHOLOGY

Expression of Neuronal and Inducible Nitric Oxide Synthases in the Thymus under Normal Conditions and after Administration of Bacterial Endotoxin

I. V. Titova, V. G. Sergeev, A. A. Yarilin*, and I. G. Akmaev

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 134, No. 10, pp. 470-473, October, 2002
Original article submitted October 30, 2001

The topography of thymocytes expressing neuronal and inducible nitric oxide synthases and changes in the content of luminescent immunoreactive products in these cells after intraperitoneal injection of bacterial lipopolysaccharide were studied by double immunohistochemical labeling. Under normal conditions neuronal nitric oxide synthase-immunopositive cells formed a wide network in thymus medulla (except for perivascular regions). Inducible nitric oxide synthase was expressed in single cells at the corticomedullary boundary. Lipopolysaccharide markedly increased the intensity of luminescence and number of inducible nitric oxide synthase-immunoreactive cells. However, this agent sharply decreased the intensity of luminescence in neuronal nitric oxide synthase-immunopositive cells of the stroma. Our results indicate that neuronal and inducible nitric oxide synthases are synthesized in various stromal cells of the thymus. Expression of these enzyme isoforms undergoes opposite changes during inflammation.

Key Words: *thymus; nitric oxide; nitric oxide synthase; lipopolysaccharide*

Thymus is the major organ of lymphopoiesis. Proliferation and differentiation of thymocytes are controlled by the microenvironment presented by a spatial network of epithelial and dendrite cells, macrophages, and fibroblasts [2]. This microenvironment of the thymus regulates differentiation and migration of thymocytes by secretion of various factors, including thymic hormones, interleukins, and neuropeptides [4,6,12]. Stromal cells of the thymus secrete free radical molecules of soluble gas (nitric oxide, NO) that acts as a nontraditional mediator of intercellular communication and easily diffuses through biological membranes [1].

Institute of Experimental Endocrinology, Research Center for Endocrinology, Russian Academy of Medical Sciences; *Institute of Immunology, Russian Ministry of Health, Moscow. Address for correspondence: sergval@mail.ru. Sergeev V. G.

NO synthase (NOS) catalyzes conversion of L-arginine into citrulline in various tissues, which is followed by the release of NO [9]. There are 3 isoforms of NOS. By the mechanism of activation, NOS isoforms are divided into 2 classes. Neuronal and endothelial NOS (nNOS and eNOS, respectively) are constitutively expressed enzymes, whose activity depends on intracellular Ca^{2+} concentration. Inducible NOS (iNOS) is synthesized in macrophages after binding of bacterial endotoxin to receptors or formation of cytokines during the immune response [5,7]. iNOS is expressed in dendrite cells and macrophages of the thymus under conditions of experimental inflammation. NO produced by these cells is involved in apoptosis in thymocytes [8].

NOS revealed in thymocytes by a nonspecific histochemical assay for NADPH diaphorase is not necessarily colocalized with iNOS-immunoreactive

0007-4888/02/1344-0407$27.00

stromal cells in the thymus [3]. The stroma of this organ contains the neuroectodermal epithelium. These data suggest that the thymus produces not only iNOS, but also nNOS. In the present work nNOS synthesis in the thymus and immunoreactivity of iNOS and nNOS in stromal cells under normal conditions and after administration of bacterial endotoxin were studied by the method of double immunohistochemical labeling.

MATERIALS AND METHODS

Experiments were performed on 8 male Sprague—Dawley rats weighing 270-300 g, kept at 22°C under the natural light/dark regimen and having free access to water and food. Experimental rats (n=4) intraperitoneally received 1 ml sterile 0.9% NaCl containing *Salmonella abortus equi* (Sigma) lipopolysaccharide (LPS) in a dose of 125 mg/kg. Control animals were injected with 1 ml 0.9% NaCl. The rats were anesthetized with sodium pentobarbital (60 mg/ml intraperitoneally) 4 h after treatment and intracardially perfused with 50 ml 4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer (pH 6.9). The thymus was removed, washed with phosphate buffer (pH 7.4) containing 10% sucrose for 24 h, and frozen on dry ice.

Cryostat sections of the thymus (14 μ) were mounted on glasses treated with gelatin and chromium alum. For double immunohistochemical labeling these sections were placed on gelatin-coated glasses and incubated with mouse monoclonal antibodies to nNOS (1:1000, Peninsula) dissolved in isotonic phosphate buffer at -4°C for 1 night. Antibodies were catalytically revealed using DuPont commercial kit (New England Nuclear). The sections were washed with Tris buffer containing 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20 for 10 min, preincubated in this buffer and 0.05% blocking reagent (DuPont Blocking Reagent) at room temperature for 30 min, incubated with anti-rabbit porcine antibodies conjugated with horseradish peroxidase (Dako A/S) and dissolved in Tris buffer (1:100), and washed 2 times with Tris buffer for 10 min. The procedure was followed by tyramine amplification of the label signal. The sections were incubated with biotinylated tyramine (1:50) at room temperature for 10 min and washed 3 times in Tris buffer. The chromogenic signal was detected by incubation with avidin conjugated with fluorescein 5(6)-isothiocyanate (FITC, Amersham) and diluted in Tris buffer (1:200) at room temperature for 30 min. The sections were incubated with guinea pig antibodies to iNOS (1:100, Peninsula) in isotonic phosphate buffer at -4°C for 12 h and washed with this buffer. Primary antibodies were detected with rabbit rhodamine-conjugated antibodies (1:100) at 37°C for 30 min. Then the sections were washed in phosphate buffer and covered with a mixture of glycerol and buffer containing 0.1% *p*-phenylenediamine (Sigma).

The sections were examined under a Nikon fluorescence microscope. We used transmitting and blocking filters for FITC (480±10 and 520-550 nm, respectively) and rhodamine (546±5 and 590 nm, respectively).

Counting of cells and measurements of luminescence were performed by a computerized video image analysis using Image Pro software (Medical Kibernetics). The results were analyzed by Student's t test.

RESULTS

In the thymus from control animals nNOS-immunopositive cells formed a wide network at the corticome-

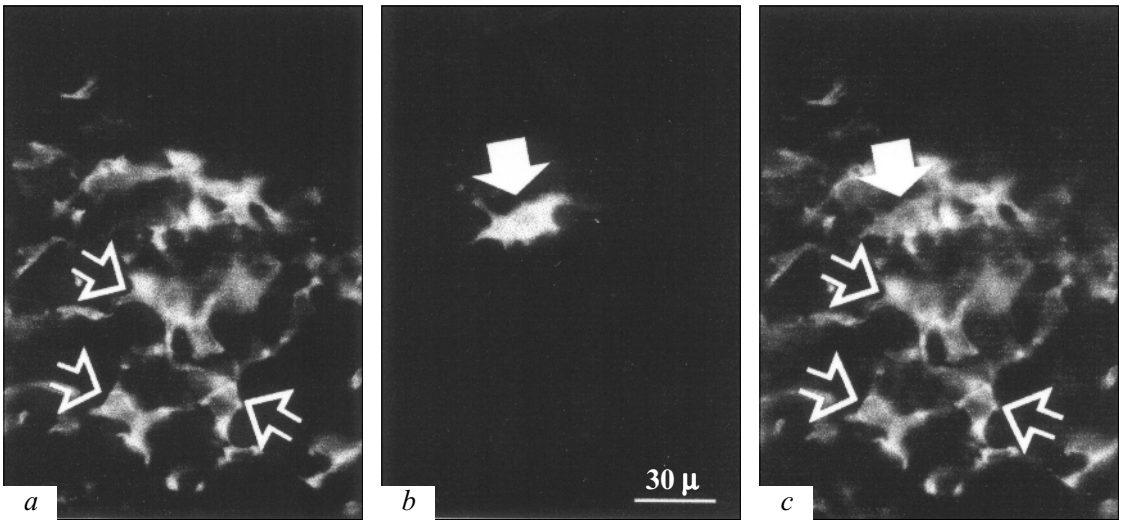

Fig. 1. Corticomedullary region in rat thymus. Immunofluorescence staining with antibodies to neuronal (*a*, arrows), inducible (*b*, full arrow), and both isoforms of nitric oxide synthase (*c*).

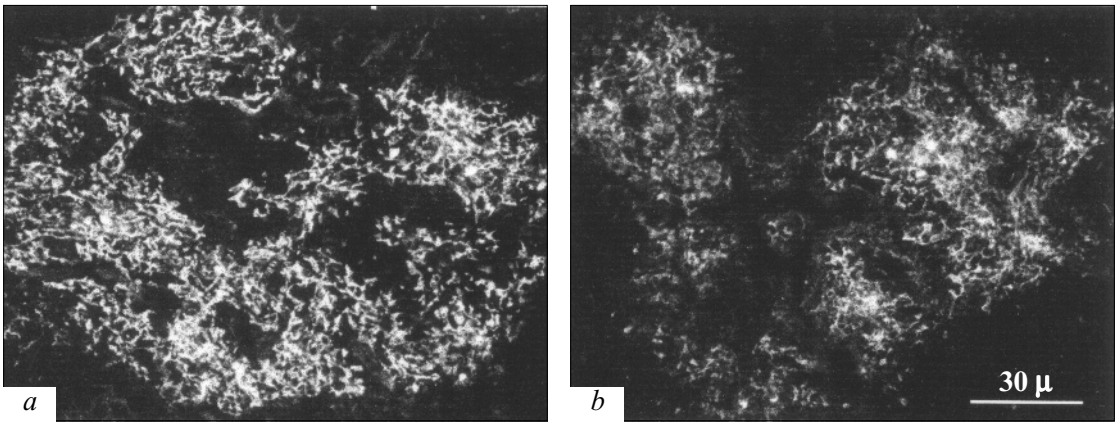

Fig. 2. Luminescence in medullary cells of rat thymus incubated with antibodies to neuronal nitric oxide synthase under control conditions (*a*) and after LPS administration (*b*).

TABLE 1. Count of NOS-Immunoreactive Stromal Thymocytes and Intensity of Their Luminescence in Rats Receiving LPS (% of the Control, *M*±*m*)

Parameter	iNOS		nNOS	
	control	LPS	control	LPS
Count of immunoreactive cells	100.0±6.2	146.8±11.4*	100.0±5.8	106.0±7.3
Intensity of luminescence	100.0±11.3	137.7±15.2*	100±12	41.6±6.1*

Note. *$p<0.05$ compared to the control.

dullary boundary and in the medulla (except for perivascular regions, Fig. 1, *a*, Fig. 2, *a*). iNOS-expressing cells were mainly localized at the corticomedullary boundary in the thymus (Fig. 1, *b*). Double immunohistochemical labeling did not reveal cells simultaneously labeled with antibodies to iNOS and nNOS (Fig. 1, *c*). There results indicate that the stroma in the thymus contains various cell populations differing in the specificity of expression of NOS isoforms.

The count of iNOS-expressing cells and intensity of their luminescence markedly increased 4 h after LPS administration (Table 1). The count of nNOS-immunoreactive cells remained unchanged, but their luminescence sharply decreased (Table 1).

Previous studied showed that iNOS is synthesized in dendrite cells and macrophages of the thymus during inflammation [1]. NO produced in the thymus causes apoptosis in thymocytes [8]. The data suggest that this molecular mechanism is involved in negative selection of thymocytes induced by inflammation. In the thymus of control animals numerous nNOS-immunoreactive cells were seen. It can be hypothesized that NO synthesized in these cells plays a role in maturation of thymocytes not only during antigenic stimulation, but also in the absence of inflammatory factors.

In our experiments the count of thymocytes expressing iNOS increases after treatment with LPS, which is consistent with published data [11]. Our results indicate that NO synthesis by macrophages is a component of the complex reaction of the thymus to inflammation. However, the functional significance of reduced expression of nNOS during experimental inflammation remains unclear. It can be hypothesized that these changes are a response to increased content of macrophage-derived NO in the thymus aimed at preventing excess death of thymocytes. It should be emphasized that NOS isoforms were localized in various stromal cells, and NO synthesis in these cells underwent opposite changes after LPS administration. Therefore, these thymocytes perform different specific functions. Activation of cells probably determines differences in the dynamics and directionality of thymocyte selection under normal conditions and during inflammation.

Our results suggest that nNOS and iNOS are synthesized in various stromal cells of rat thymus. Bacterial endotoxin induced different changes in expression of these isoforms in thymocytes.

REFERENCES

1. S. Aiello, M. Noris, G. Piccinini, *et al.*, *J. Immunol.*, **158**, No. 10, 4696-4703 (1997).
2. R. L. Boyd, C. L. Tucek, D. I. Godfrey, *et al.*, *Immunol. Today*, **14**, 445-459 (1993).

3. J. G. Downing, L. Virag, and I. W. Jones, *Immunology*, **95**, No. 1, 148-155 (1998).
4. A. G. Farr, S. Hosier, S. C. Braddy, *et al*., *Cell. Immunol*., **119**, 427-433 (1989).
5. U. Forstermann, E. I. Closs, J. S. Pollock, *et al*., *Hypertension*, **23**, 1121-1131 (1994).
6. V. Geenen, H. Martens, E. Vandersmissen, *et al*., *Ann. N. Y. Acad. Sci*., **840**, 328-337 (1998).
7. R. G. Knowles and S. Moncada, *Biochem*., **298**, 249-258 (1994).
8. T. Murayama and Y. Nomura, *J. Immunol*., **164**, No. 9, 4649-4658 (2000).
9. R. M. J. Palmer, D. D. Rees, D. S. Ashton, and S. Moncada, *Biochem. Res. Commun*., **153**, 1251-1256 (1988).
10. K. Sato, K. Miyakawa, M. Takeya, *et al*., *J. Leukoc. Biol*., **57**, No. 1, 36-44 (1995).
11. W. Savino and T. Dardenne, *Eur. J. Immunol*., **14**, 987-991 (1984).