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IMMUNOHISTOCHEMICAL LOCALIZATION OF NANOCONTAINERS IN MOUSE TISSUES

Abrahamyan S.S.*[1], Broz P.[2], Semerjyan A.B.[3], Sahakyan I.K.[1], Tumasyan N.V.[1], Sisakian H.S.[4], Hunziker P.[2]

[1] H. Buniatian Institute of Biochemistry, National Academy of Sciences, Yerevan, Armenia
[2] Intensive Care Unit, University Hospital of Basel, Basel, Switzerland
[3] Department of Medical Biology, Yerevan State Medical University, Yerevan, Armenia
[4] Department of Cardiology, Yerevan State Medical University, Yerevan, Armenia

Abstract

The aim of this study was to obtain a methodological technique that would allow detecting or excluding the possible toxic side effects of the nanocontainers in vivo. The animal models and the avidin-biotin-peroxidase complex (ABC) immunohistochemical technique were employed to detect nanocontainers localization in vivo in mice intravenously injected with biotinylated nanocontainer (BN).

In the experimental animals, in contrast to the control animals, we found several BN-immunoreactive (BN-Ir) cells in the glomerular layer of adrenal glands and a number of mast cells at the border of the cortical reticular layer and the medulla. In the brain, weak BN-immunoreactivity (BN-IR) was shown in the Bergman glia between the immunonegative cerebellar Purkin'e cells. In the liver, many BN-Ir granules were located in the bile capillaries between the immunonegative hepatocytes; both central veins and sinusoids were dilated in the experimental mice. In the spleen, a few BN-Ir lymphocytes were demonstrated inside several germinal centers. Many granules and varicose fibers resembling the reticular cell fibers surrounded the germinal centers. In the lungs, a number of lymphocytes and macrophages around the alveoli were detected. The structures in the heart, kidney, and thymus did not demonstrate BN-IR.

Keywords: mice; biotinylated nanocontainer (BN); immunohistochemistry; BN-immunoreactivity (BN-IR); BN-immunoreactive (BN-Ir).

INTRODUCTION

Nanotechnology provides elaboration of new technologic artificial material construction in prosthesis of biologic structures in medicine [*Long H. et al., 2002; Silva G., 2007*]. One of the promising directions in the development of nanotechnology is application of nanocontainers (NCs) for pharmacological delivery. Nanotechnology is expected to have a revolutionary impact on medicine. Among approaches for exploiting nanotechnology developments in medicine, the nanoparticles offer some unique advantages as sensing, delivery, and image enhancement agents. Several types of nanoparticles are available, including: polymeric nanoparticles, metal nanoparticles, liposomes, micelles, dentrimers and nano-assemblies. Flexibility of nanocontainers makes them very convenient for targeted drug delivery [*Sauer M. et al., 2001; Freitas R., 2002; Silva G., 2007*]. These nanoparticles can be targeted to a specific site of the body where they are needed. All of these nanoparticles can play a major role in medicine: in cancer [*Attali P., 2008*], neurodegenerative and Alzheimer's disease [*Nam J. et al., 2003; Georganopoulou D.G., et al., 2005; Seehaas N. et al., 2008; Fluri F., 2010;*], inflammatory and infection diseases, cardiovascular diseases

Address for Correspondence:
* H. Buniatian Institute of Biochemistry
Immunohistochemistry Group
5/1 Paruir Sevak Street, 0014, Yerevan, Armenia
Tel.: (0037491) 605 958
E-mail: silva.abrahamyan@gmail.com

[*Broz P., 2008*] diagnosis and therapy. As a result, this approach has the ability to reduce toxicity and improve efficacy and patient compliance.

In this regard, many questions including those of biocompability, toxicity, and immunogenicity are widely investigated.

The aim of this study was to obtain a methodological technique that would allow detecting or excluding the possible toxic side effects of the NCs *in vivo*, for which we employed the animal models and immunohistochemical approach to detect the intravenously (*i/v*) injected biotinilated NCs in mice.

MATERIAL AND METHODS

Normal outbred male mice (body weight: 30 *g*) were used in the present study. The animals were divided into two groups: 1) control mice (n=3), *i/v* injected with 0.05 *mL* physiological saline; and 2) experimental mice (n=3), *i/v* injected with 0.05 *mL* of 10% biotinylated nanocontainer (BN, biotinylated PMOXA-PDMS-PMOXA copolymer building blocks) per mouse. Following the anesthesia with Nembutal (40-50 *mg/kg*), the non-perfused animals were decapitated in 1 *h* after injection. The brain, adrenal glands, spleen, liver, thymus, heart, and lungs were rapidly removed and fixed in the fixative [4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS), pH 7.4] for 24 *h* at 4°C. Tissues were washed and cryoprotected overnight in 0.1M PBS containing 15% sucrose.

Free-floating freezing microtome sections (50-μm) were used for immunohistochemistry with the Avidin-Biotin-Peroxidase Complex (ABC) technique [*Hsu S. et al., 1981*]. Several variants were tested to figure out the best recipe for the immunohistochemistry:

- Protocol omitting the primary antibody;
- Protocol with normal goat serum (NGS) omitting the primary antibody and secondary biotinylated antibody;
- Protocol with secondary biotinylated antibody omitting the primary antibody and NGS;
- Protocol without NGS, primary and secondary antibodies.

The best results were obtained with the complete protocol omitting the primary antibody, which is described below.

The study procedure has been approved by the Ethic Committee of the Yerevan State Medical University and the Subcommittee of the Animal Research. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Immunohistochemistry

After rinsing the tissue sections in phosphate-buffered saline (PBS) several times, the sections were treated with: a) 0.3% hydrogen peroxide (H_2O_2) for 45 *min* to suppress the background peroxidase activity; b) NGS (1:30) for 45 *min* to block non-specific binding of antibodies; c) biotinylated anti-rabbit immunoglobulin (1:200) for 1 *h;* and (d) a 1:100 dilution of avidin-biotin-peroxidase complex (ABC) (Vector Labs, Burlingame, CA, USA) for 1 *h*. The bound tissue antibody was visualized by the use of 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) as a chromogen and 0.6% nickel-ammonium-sulfate diluted in 50 mM Tris-HCl buffer (pH=7.6) in the presence of 0.03% H_2O_2 as an oxidant. The incubations were performed at room temperature. The secondary antiserum was diluted in PBS containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide. All incubations, except the incubation with NGS, were separated with washes in PBS. After washing and dehydrating, the sections were mounted on gelatin-coated slides and coverslipped in D.P.X. mounting medium for immunohistochemistry. Results of the immunochemistry were analyzed and documented with light microscope.

RESULTS AND DISCUSSION

Our data demonstrated BN-immunoreactivity (BN-IR) in several tissue structures of the mice injected with BN. In the experimental animals, in contrast to the control, some BN-immunoreactive (BN-Ir) cells were found in the glomerular layer of the adrenal cortex. Many mast cells situated mainly at the border of the cortical reticular layer and the medulla were immunopositive.

In the brain of mice injected with BN, weak BN-IR was shown in the Bergman glia between the immunonegative cerebellar Purkin'e cells (Figure 1B; C). In the control animals, no BN-IR was detected in the brain structures (Figure 1A).

In the lungs of BN-injected mice, abundant lym-

phocytes scattered everywhere and a number of macrophages around the alveoli were detected (Figure 1 E; F), compared to the diffuse staining in the control animals (Figure 1 A).

Many granules in the liver of experimental mice showed immunoreactivity to BN in the bile capillaries between the immunonegative hepatocytes (Figure 2 C; D). Both central veins and sinusoids were dilated in the experimental mice in contrast to the control animals (Figure 2 A; B). Besides, in control animals, the sinusoids of liver were filled in with blood only (Figure 2 B).

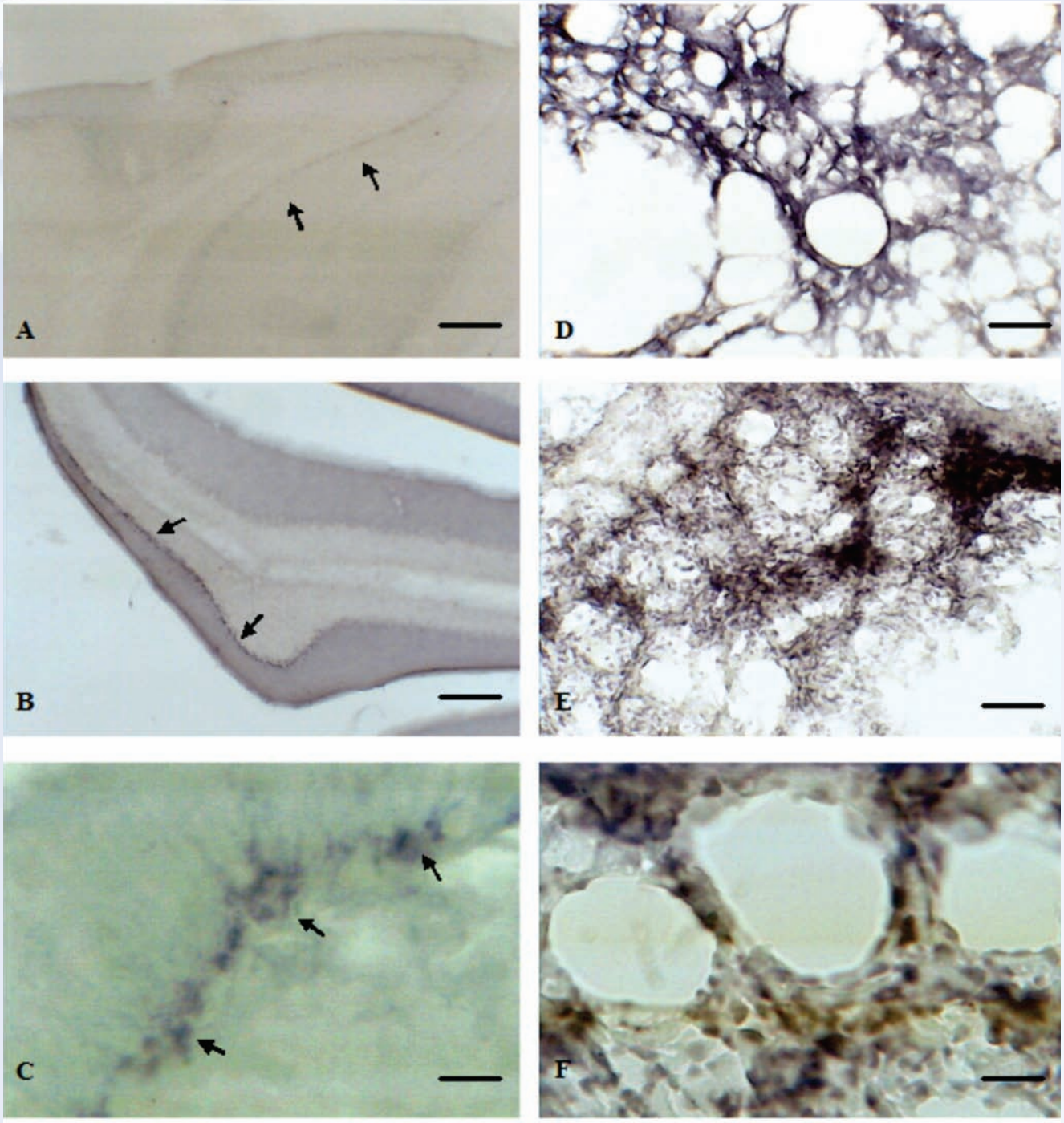

Figure 1. *Brain and lung structures of control and injected with biotinylated nanocontainer mice. Immunonegativity in the brain structures of control mice (A) injected with physiological saline. (B,C): BN-immunoreactive (BN-Ir) Bergman glia (arrows) with the long processes in the experimental mice injected with BN. (D): diffuse staining in the control lungs. (E,F): BN-Ir lymphocytes and macrophages in the lungs of the experimental animals. ABC immunohistochemical method. Bar: 100 μ (A, B, D, E); 50 μ (F); 25 μ (C).*

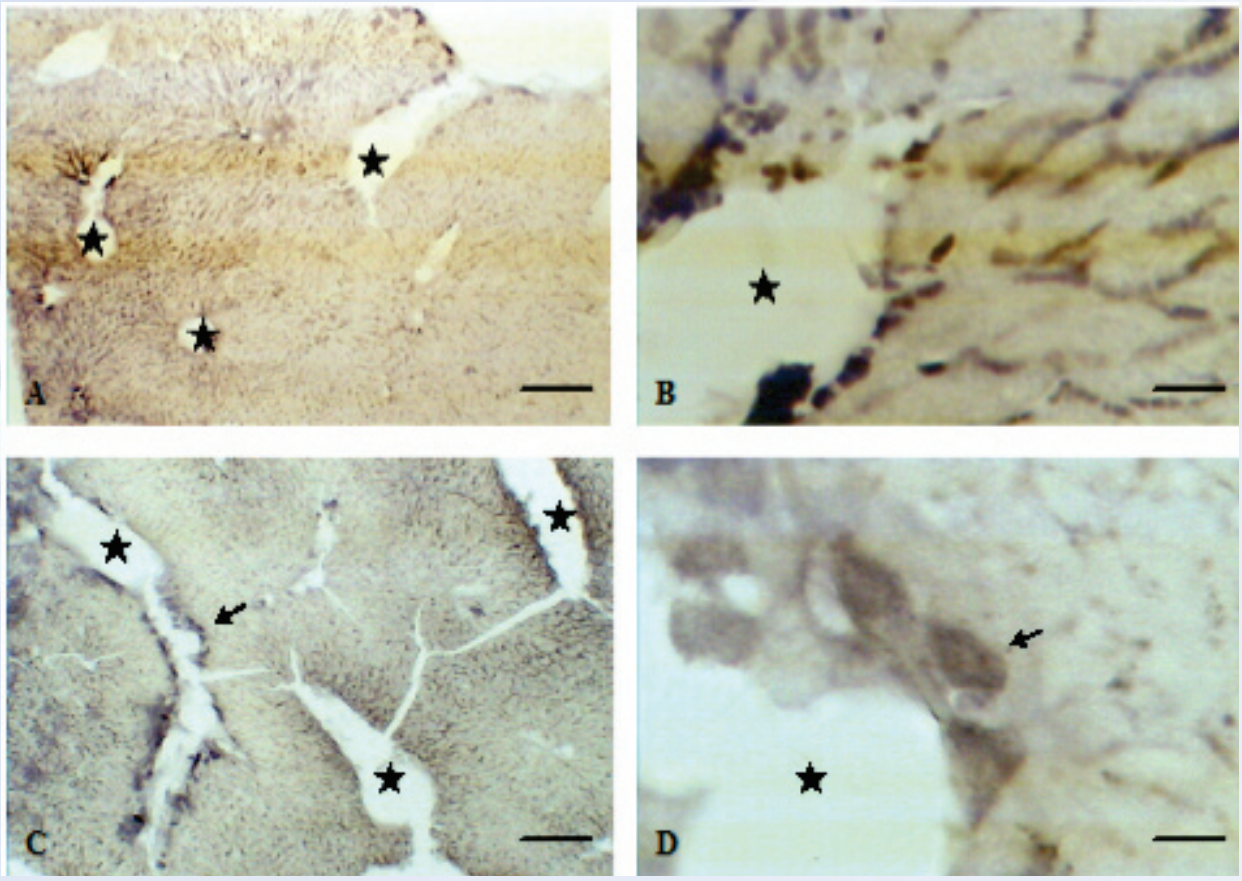

Figure 2. *Liver structures of control and injected with biotinylated nanocontainer mice. Sinusoids filled in with blood in the liver of the control animals (A, B). Diluted central veins (asterisks) in the liver of experimental mice (C,D) and BN-Ir cells resembling the mast cells (arrows) situated in close vicinity to the central vein walls. ABC immunohistochemical method. Bar: 100 μ (A); 50 μ (C); 25 μ (B, D).*

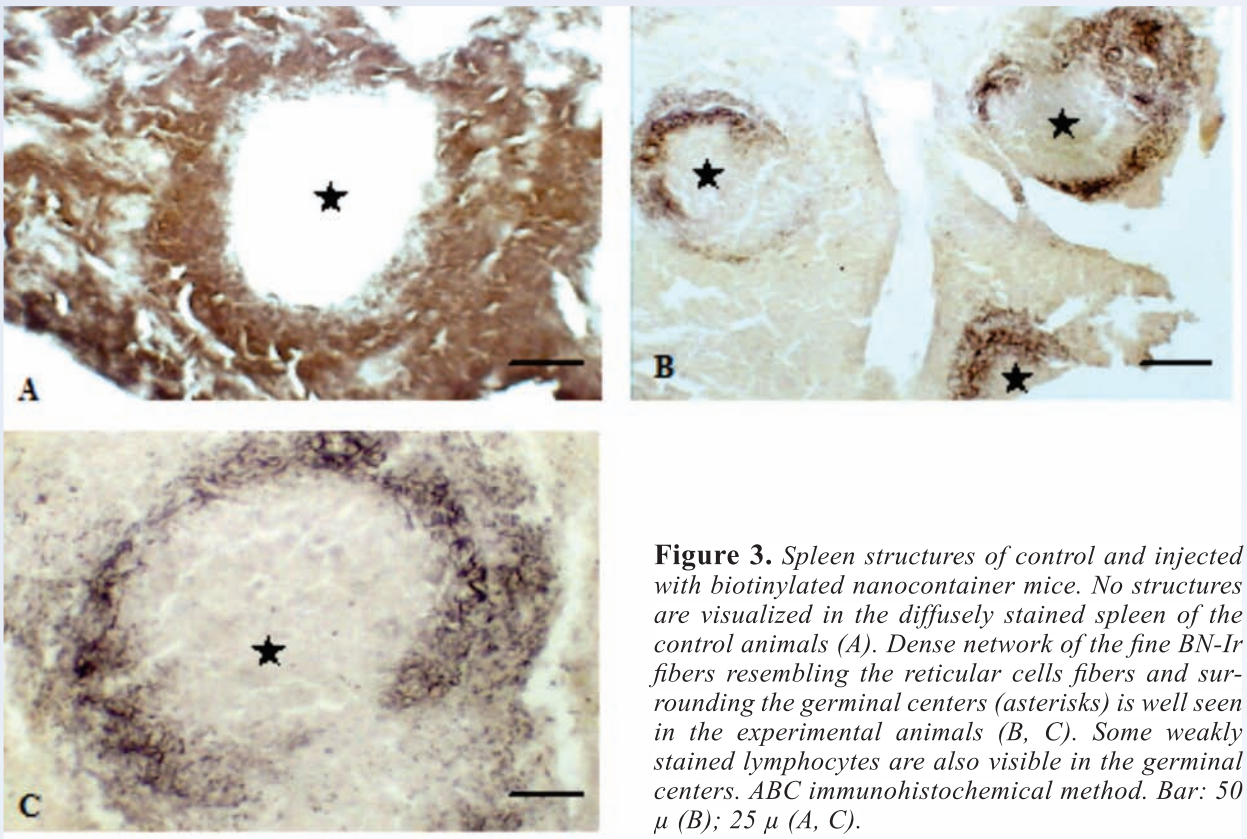

Figure 3. *Spleen structures of control and injected with biotinylated nanocontainer mice. No structures are visualized in the diffusely stained spleen of the control animals (A). Dense network of the fine BN-Ir fibers resembling the reticular cells fibers and surrounding the germinal centers (asterisks) is well seen in the experimental animals (B, C). Some weakly stained lymphocytes are also visible in the germinal centers. ABC immunohistochemical method. Bar: 50 μ (B); 25 μ (A, C).*

In the spleen, a few BN-Ir lymphocytes were demonstrated on the very clear background inside the several germinal centers. Many granules and varicose fibers resembling the reticular cell fibers surrounded the germinal centers (Figure 3 B; C). The staining was diffuse in the control sections of the spleen (Figure 3 A).

No structure demonstrated BN-immunoreactivity in the heart, kidney, and thymus.

CONCLUSION

Thus, the data obtained gave an initial idea on the BN-immunoreactivity in mouse tissues studied. In spite of data regarding the immunonegativity of the structures obtained in the control tissues, which serves as an evidence of differences between the control and experimental mice, the better and more convincing results would be expected with anti-biotin antibody used as the primary antibody.

In respect to the mast cells, the BN-IR in the mast cells in some of the studied tissues is known to be non-immunospecific.

As mentioned in the study design, only one dose (0.05 *mL*) of BN per mouse was applied in our primary study. Therefore, it would be reasonable to conduct a new series of experiments with the injection of different amounts of nanocontainer solution per mouse, e.g. 0.0125 *mL*, 0.025 *mL*, 0.075 *mL*, 0.*1 mL*.

Nevertheless, our results serve as a guide only and further study is needed, using different amounts of BN, various periods following the BN injection, and more targeted immunohistochemical technique.

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