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RESEARCH ARTICLE

Internalization of Anti-DNA Antibodies by Rat Brain Cells: A Possible Pathogenetic Mechanism of Neuropsychiatric Lupus

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ABSTRACT

Accumulating evidence suggests that DNA-anti-DNA antibody immune complexes are endocytosed by immune cells, where they ligate intracellular nucleic acid sensors. This results in production of type I interferon or other cytokines, which may be a significant pathogenetic mechanism in systemic lupus erythematosus (SLE). Most studies thus far have been performed using cell-lines or peripheral blood mononuclear cells, but it remains unclear whether cells from the brain can take up such immune complexes. Because permeability of the blood-brain interfaces is suggested to be increased and autoantibodies are detected in cerebrospinal fluid of SLE patients with neuropsychiatric manifestations, we hypothesize that DNA-anti-DNA immune complexes may be internalized by cells in the central nervous system and cause dysregulation of neural networks. As a first step to test this hypothesis, here we investigated whether anti-DNA antibodies can be internalized by live brain cells *in vitro*.

Primary culture rat astrocytes were incubated for 1 h at 37°C with or without anti-DNA monoclonal antibodies. After washing, fixation, permeabilization and blocking, internalized anti-DNA antibodies were detected by a fluorescent labeled second antibody. We observed that a dsDNA-specific monoclonal antibody entered the nucleus, while a monoclonal antibody cross-reactive with phospholipid and DNA entered the cytoplasm. Isotype-matched control IgG did not enter the cells, suggesting that they were viable and the cell membrane and nuclear membrane were intact at that time. When the cells were incubated at 4°C, internalization of the anti-DNA antibodies was almost completely abolished. This suggests that antibody internalization was not by passive transfer but is an energy-dependent process. Given that astrocytes play indispensable roles in maintaining the function of neural networks, these results may provide a novel perspective on the pathogenetic mechanisms of the neuropsychiatric manifestations often presented in SLE.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease which preferentially affects women at 20-40 years of age. It presents with varied symptoms including fever, skin rash, arthritis, serositis, glomerulonephritis and neuropsychiatric manifestations (NPSLE). Multiple genetic and environmental factors are thought to contribute to a loss of immunological tolerance resulting in prolonged production of different autoantibodies, the pathogenetic roles of which, however, remain insufficiently understood.[1,2]

More than four decades ago it was reported that anti-DNA and anti-RNP autoantibodies could enter live cells.[3] The term "penetrate" was used at that time, which may have contributed to the skepticism of many researchers regarding this phenomenon. Thereafter, however, many anti-DNA antibodies have been reported to enter live cells; some of them into the nucleus, others remaining in the cytoplasm, depending on which antibodies, cell-types, and assay conditions.[4-7] Suggested mechanisms for this internalization were also heterogeneous, some being Fc-receptor mediated, others not. In any case, progress in the understanding of innate immunity has resulted in the hypothesis that DNA or RNA bound to the antibodies can be recognized by nucleic acid sensors in the endosome or cytoplasm, and stimulate intracellular signaling pathways relevant to the pathogenesis of SLE.[8,9]

Previously, we reported that the monoclonal anti-phospholipid antibody WB-6, which is cross-reactive with DNA, enters the cytoplasm of monocytes. There, it induces expression of tissue factor via activation of the TLR9 pathway, leading to a pro-thrombotic state in an *in vivo* model.[10-12] In another study, we observed that dsDNA-specific monoclonal antibody 2C10 enters the nucleus of monocytes. There, it induces peripheral blood mononuclear cells to secrete multiple cytokines including IFN-α, IFN-γ, TNF-α, which are known to be implicated in lupus pathogenesis.[13] These results suggest that anti-DNA antibodies create a vicious circle in the pathogenesis of SLE.

Among the symptoms of SLE, NPSLE is one of the most challenging for rheumatology researchers because of its complexity and the limited access to tissue. In order to explore the possible involvement of anti-DNA antibodies also in the pathogenesis of NPSLE, the current study aimed to determine whether these antibodies enter live cells of the central nervous system (CNS). It is noteworthy that Stamou et al.[14] have shown internalization of IgG-anti-IgG immune complexes by newborn rat hippocampal cells via Fcγ receptors, but little is known so far about internalization of anti-DNA antibodies into cells of the CNS.

Methods

Animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (A2021-100C).

The dsDNA-specific monoclonal antibody 2C10 (IgG2b,κ) originated from an MRL/*lpr* mouse; the fine specificity and amino acid sequence of the variable regions has been reported previously.[15,16] Monoclonal antibody WB-6 (IgG2b,κ) was derived from an (NZW × BXSB) F1 mouse, and cross-reacts with ssDNA, dsDNA and cardiolipin-β_2 glycoprotein I.[10] Both antibodies were purified from hybridoma culture supernatant by salting out with half-saturated ammonium sulfate followed by column chromatography on Protein G HP Spin Trap (Cytiva). We did not intentionally add DNA into the assay. However, anti-DNA antibodies inevitably bind a certain amount of DNA both *in vivo* and *in vitro*.[17] Therefore, the antibodies we used in this study can be regarded as immune complexes with DNA. The hybridomas producing 2C10 and WB-6 are available from the Cell Bank, RIKEN BioResource Research Center (Tsukuba, Japan).

An enriched astrocyte culture was obtained from the cortex of embryonic day 20 Wistar rat embryos essentially by following the protocol of Schildge et al.[18] The cells were incubated with 2 or 5 μg/mL 2C10, WB-6 or isotype control mouse IgG (R&D Systems) for 1 h in a CO_2 incubator. After washing with cold PBS, fixation with 4% paraformaldehyde, permeabilization with 0.1% Triton X-100, and blocking with 1% BSA-PBS, antibodies were detected by Alexa Fluor 555-labeled goat anti-mouse IgG (Invitrogen). Purity of the astrocytes was confirmed by staining with Alexa Fluor 488-labeled anti-glial fibrillary acidic protein (GFAP) antibody (BioLegend). After the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), cells were inspected using a fluorescence microscope (Keyence, BZ-X800).

Results

Incorporation of 2C10 and WB-6 is not a cell-type specific phenomenon but the efficiency varies depending on the cell type. Monocytes and vascular endothelial cells incorporate these antibodies prominently,[10] while lymphocytes barely do so.[13] Here, we tested cells in the CNS

for the first time and found that astrocytes were high incorporators. When rat primary astrocytes were incubated for 1 h at 37°C with 5 µg/mL 2C10, but not with the isotype control, Alexa Fluor *555*-anti-mouse IgG stained the nuclei of most of the cells red (Figure 1). Because 2C10 binds to the dA-dT base pairs in the minor groove of dsDNA,[15] it colocalized with DAPI and appeared purple in the merged image. Despite a few speckles outside the nucleus in some cells, when incubated at 4°C, 2C10 did not enter the nucleus, nor did it accumulate in the cytoplasm (Figure 2). This suggests that the internalization of 2C10 is not by passive transfer but is an energy-dependent dynamic process. In contrast, WB-6 was retained in endosome-like speckles in the cytosol and did not enter the nucleus at 37°C (Figure 3). These different intracellular localizations of 2C10 and WB-6 are similar to the observations previously made using monocytic cells.[10]

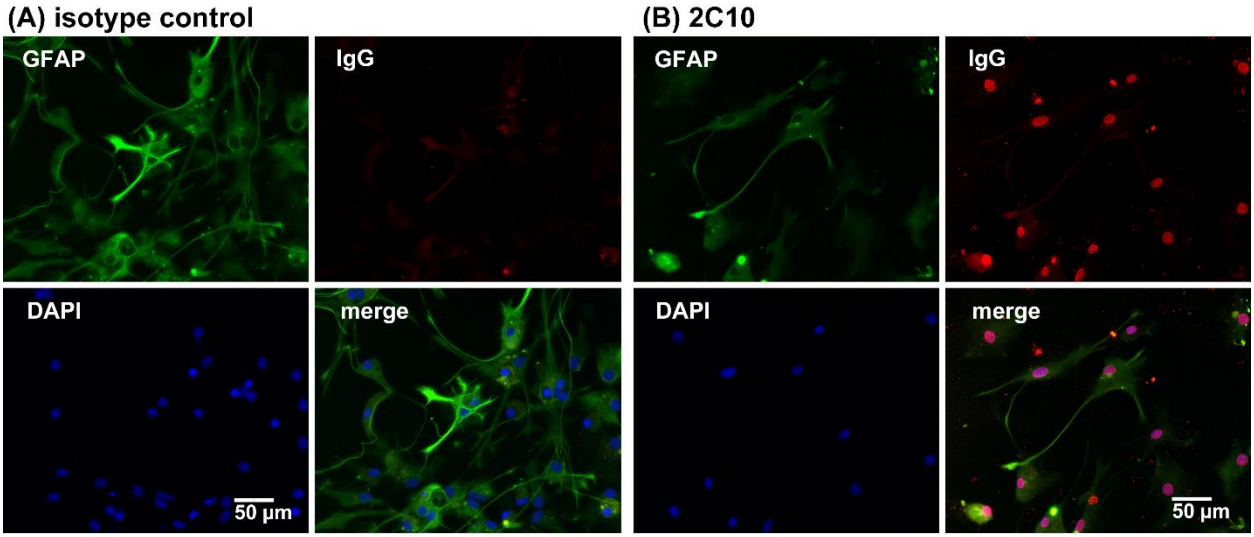

Figure 1. Internalization of anti-dsDNA antibody 2C10 by astrocytes. Rat primary astrocytes were incubated for 1 h at 37°C with 5 µg/mL 2C10 (B) or isotype control IgG (A). After washing, fixation and permeabilization, cells were stained with Alexa Fluor 488-labeled anti-GFAP (green) and Alexa Fluor *555*-labeled anti-mouse IgG (red). The nuclei were stained with DAPI (blue).

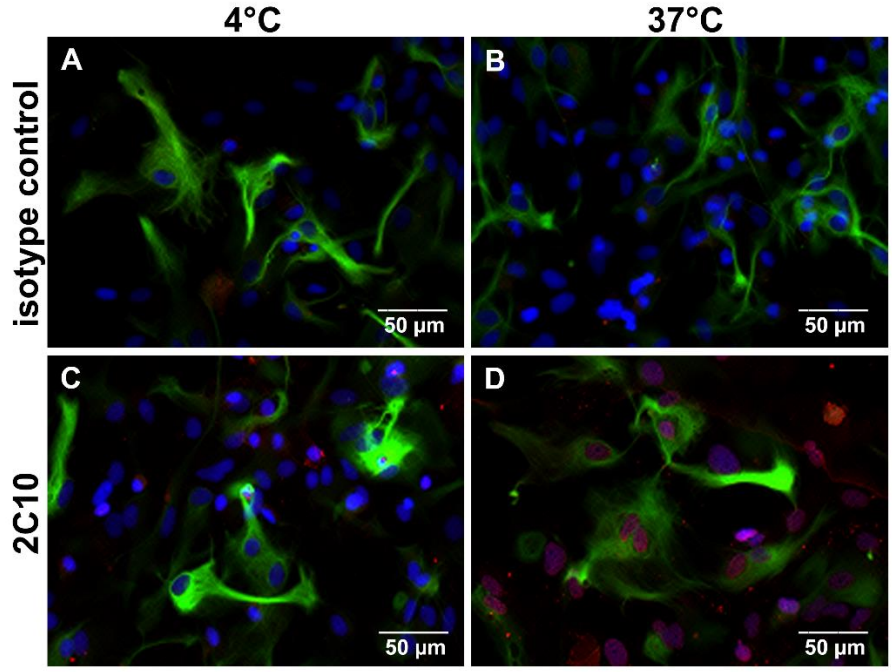

Figure 2. Internalization of anti-dsDNA antibody 2C10 is temperature-dependent. Rat primary astrocytes were incubated for 1 h at 4°C with 2 µg/mL 2C10 (C) or isotype control IgG (A); or at 37°C with 2C10 (D) or isotype control IgG (B). Thereafter, cells were stained as described in Figure 1. Only merged images are shown.

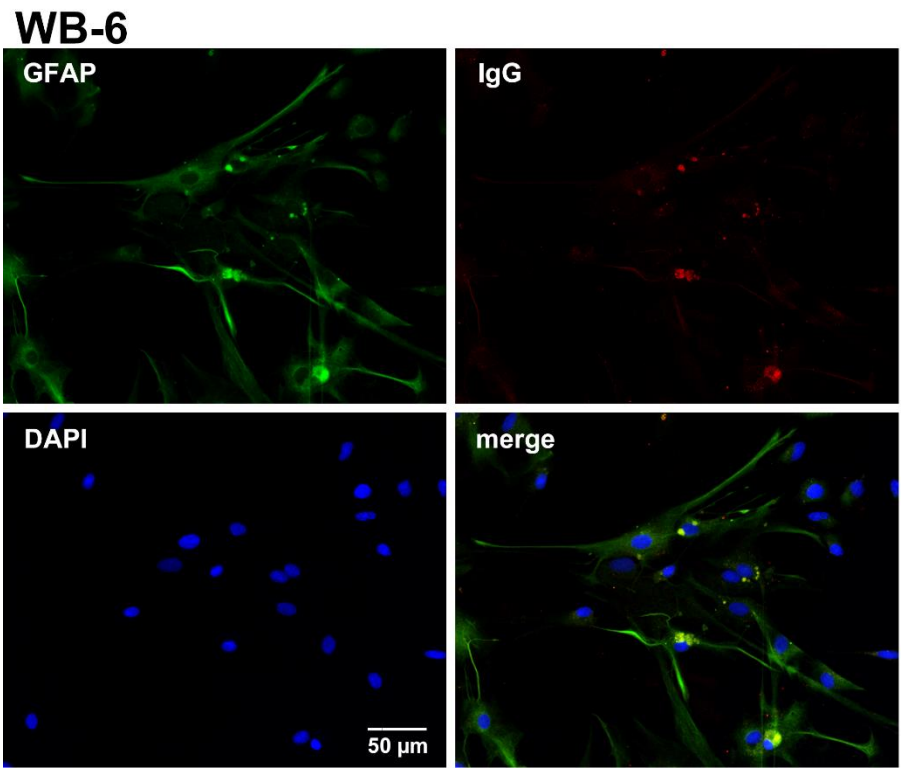

Figure 3. Internalization of anti-phospholipid antibody WB-6 by astrocytes. Rat primary astrocytes were incubated for 1 h at 37°C with 2 µg/mL WB-6. Thereafter, internalized IgG was detected as described in Figure 1.

Discussion

In this study, we observed that monoclonal anti-DNA antibodies entered the cytoplasm or the nucleus of rat live astrocytes. To the best of our knowledge, this is the first report to show the internalization of anti-DNA antibodies by cells from the brain, and suggests the possible involvement of this phenomenon in the pathogenesis of NPSLE.

Clinical phenotypes of NPSLE are diverse and are classified into neurological syndromes (including headache, seizure disorders, cerebrovascular disease) and diffuse psychiatric or neuropsychological syndromes (including cognitive impairment, mood disorder, anxiety disorder, psychosis).[19-21] A significant proportion of the neurological syndromes may be ascribed to the pathogenetic effects of anti-phospholipid antibodies. On the other hand, although some autoantibodies such as anti-DNA antibodies cross-reactive with the NR2 glutamate receptor,[22] and anti-ribosomal P protein antibodies[23] are hypothesized to be involved, the pathogenetic role of autoantibodies in diffuse psychiatric or neuropsychological syndromes has remained unclear[24]. In addition to the blood-brain barrier, however, several other interfaces may serve as sites of antibody transfer into the CNS, such as the meningeal barrier, the glymphatic pathway and the blood-cerebrospinal fluid (CSF) barrier, the permeability of which is proposed to be altered under pathological conditions.[19] Indeed, autoantibodies are detected in the CSF of patients with NPSLE.[25] Therefore, there is a basis for the hypothesis that anti-DNA antibodies play a significant role in the pathogenesis of NPSLE by means of entering cells in the CNS.

Because anti-DNA antibodies generally do not recognize specific DNA sequences that are as long as those recognized by restriction enzymes or transcription factors, binding of the antibodies to DNA in the nucleus probably causes non-specific effects on the cells. These may be harmful, but it is also possible that cells are activated before their death, because anti-DNA antibodies usually accompany DNA and stimulate endosomal or cytoplasmic nucleic acid sensors such as Toll-like receptors.[1,9,12,26] Consistent with this, Jang Y-J and colleagues have used anti-DNA antibodies including 2C10 and observed induction of proinflammatory cytokine expression in some experiments or apoptosis in others.[27]

The mechanism responsible for the internalization of 2C10/WB-6 by astrocytes has not been determined. In a previous study, internalization of 2C10 by monocytes was partially but significantly suppressed by the macropinocytosis inhibitor cytochalasin D, while an

Fc receptor blocker was not effective.[13] In addition, the recombinant single chain fragment (scFv) of 2C10 which lacks the Fc region was reported to enter live mesangial cells.[27] Although astrocytes are known to express Fc receptors[14] and their involvement is undeniable, they might be dispensable for the uptake of 2C10. Currently, we have preliminary results which show that $F(ab')_2$ fragments of 2C10 do enter astrocytes, but more experiments are needed to validate this. It is to be noted that mechanisms responsible for internalization into the nucleus remain unknown for any anti-DNA antibodies described in the literature.

In the current study, thus far we have examined only astrocytes. Given that these cells account for 20% of human brain cells and regulate various brain activities physiologically and pathologically,[28-30] it would be intriguing to study the fate of the astrocytes harboring 2C10 or WB-6. In addition, internalization of anti-DNA antibodies by microglia may also occur because they show similar, albeit not identical, characteristics to monocytes which were previously shown to internalize 2C10 and WB-6.[10] Activated microglia may contribute to neurodegeneration directly or indirectly via activation of astrocytes.[31-33] Furthermore, anti-DNA antibodies may contribute to increased permeability of the above-mentioned blood-brain interfaces by entering vascular endothelial cells. Thus, once anti-DNA antibodies enter the CNS, we propose that they will be internalized by different cell-types and affect their function, resulting in dysregulation of the neural network.

Conclusion

Herein, we showed that two different anti-DNA antibodies enter the cytoplasm or nucleus of rat astrocytes. Additionally, microglia and vascular endothelial cells in the CNS might be good candidates for high incorporators. Therefore, investigation of anti-DNA antibody internalization by brain cells may open an exciting research field that could lead to a better understanding of the pathogenesis of NPSLE.

Conflicts of Interest Statement:
The authors have no conflicts of interest to declare.

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Author Contributions:
K.I. and O.H. performed experiments. T.K. wrote the manuscript. All authors approved the final version of the manuscript.

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