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Antibodies to an Intracellular Antigen Penetrate Neuronal Cells and Cause Deleterious Effects

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Abstract

Multiple sclerosis (MS) is an autoimmune disease that is increasingly being recognized as a neurodegenerative disorder. Patients with MS produce autoantibodies to heterogenous nuclear ribonucleoprotein A1 (hnRNP A1). A multitude of studies indicate that T-lymphocytes, B-lymphocytes and macrophages contribute to MS pathogenesis. However, a direct autoantibody impact on neuronal cells has received limited attention. This could be explained by the general belief that autoantibodies lack the ability to penetrate neurons. hnRNP A1 is an intracellular RNA binding protein that exports RNA from the nucleus to the cytoplasm. In this study, we investigated possible mechanisms of antibody penetration into neuronal cells. Our results show that anti-hnRNP A1 antibodies and control IgG penetrate SK-N-SH neuronal cells through clathrin-mediated endocytosis. In contrast to control antibodies, anti-hnRNP A1 antibodies produced deleterious effects on the neuronal cells including altered ATP levels and increased caspase 3/7 levels (leading to apoptosis). Remarkably, anti-hnRNP A1 antibodies that targeted the hnRNP A1 M9 domain (its nuclear export/localization sequence) caused redistribution of endogenous hnRNP A1 protein in neuronal cells. These findings indicate that anti-hnRNP A1 antibodies might contribute to the pathogenesis of MS.

Keywords: Multiple sclerosis; Antibodies; hnRNP A1; Apoptosis; Cytotoxicity; ATP levels

Introduction

Antibodies have been implicated in the pathogenesis of a number of autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), paraneoplastic syndromes, multiple sclerosis (MS), and human T-lymphotropic virus type 1(HTLV-1) associated myelopathy/tropical spastic paraparesis (HAM/TSP) (a viral model of MS) [1-9]. The dominant theory asserts that upon cellular injury intracellular antigens are exposed to the adaptive immune response allowing for antibody response to the target antigen. It is generally believed that transected axons are vulnerable to an autoimmune response. However, healthy neurons are considered protected from a direct pathogenic effect. The method by which antibodies cause disease is an ongoing area of research, however, current data proposes that interactions between antibodies and various intracellular molecules cause inflammation, altered cellular messaging, and apoptosis [10]. The antibody response to hnRNPs seen in SLE patients provides some insight into this process of antibody-mediated disease. SLE patients produce antibodies to hnRNP P2 [11]. Following apoptosis of target cells, hnRNP P2 is transported to the cell surface and therefore is available to generate an autoimmune response [11]. In neurological disease previous data suggested that only neuronal surface antigens were reliable targets for pathogenic autoimmune responses [12,13]. This concept is now being challenged. Current data suggest that antibodies have the ability to enter neurons in an epitope specific manner. In autoimmune retinopathy, autoantibodies to enolase, a cytoplasmic glycolytic enzyme, were shown to penetrate neurons and alter the function of enolase [14]. Another example is present in a model of stiff man syndrome in which anti-amphiphysin antibodies entered neurons and co-localized with presynaptic markers and altered gamma-aminobutyric acid (GABA) release *in vivo* [15]. Such studies address important issues. However, to better understand the contribution of immune responses to intra-neuronal antigens in the pathogenesis of immune-mediated neurological diseases, more information is required.

MS is a neurodegenerative disorder resulting in demyelination of neurons, which is increasingly associated with grey matter and neuronal degeneration. MS and HAM/TSP patients have been shown to produce autoantibodies to the intracellular RNA binding protein heterogenous nuclear ribonucleoprotein A1 (hnRNP A1) [1,5-7,9,16]. IgG from MS and HAM/TSP patients reacted specifically with an epitope within the M9 region of hnRNP A1, its nuclear localization/export sequence (NLS/NES). These antibodies decreased neuronal firing and contributed to the death of neuronal cultures [5,9]. However, until now it remained unknown if anti-hnRNP A1 antibodies have the ability to penetrate neuronal cells. Because autoantibodies to the M9 region of hnRNP A1 appear unique to MS and HAM/TSP patients and are not seen in healthy individuals, we decided to investigate whether antibodies that target the hnRNP A1-M9 epitope can penetrate healthy neuronal cells and examine the possible deleterious effects that they might induce.

Methods

Cells

SK-N-SH cell line (ATCC, HTB-11) an immortalized human neuroblastoma cell line was maintained in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics.

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Antibodies

Anti-hnRNP A1 antibodies were obtained from Abcam (ab4791-rabbit polyclonal, ab5832-mouse monoclonal) and rabbit IgG were obtained from Abcam (ab107866) and Millipore (12-370). Anti-clathrin antibodies (Novus Biologicals NBP1-05991).

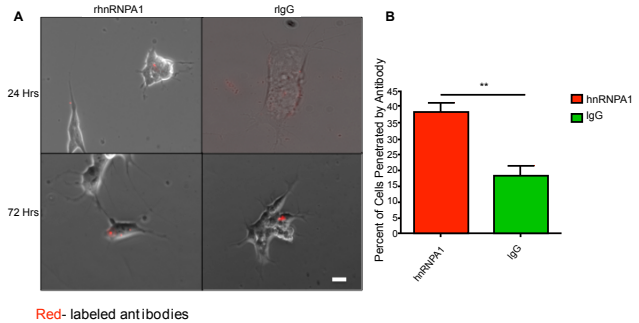
Atto550 NHS labeling of antibodies

A 0.1M $NaHCO_3$ buffer was made (8.4 g $NaHCO_3$, 22.9 g NaCl, 1 liter H_2O) and brought to a pH of 8.4 with the following solution: 10.6 g $Na2CO_3$, 29.2 g NaCl, 1 liter H_2O, 35 µg of antibodies (1µg/ml) were added to 70 µl of Atto550 NHS Ester (Sigma-Aldrich, 92835) and 500 µl of $NaHCO_3$ buffer. The mixture was rotated for 1 hour at room temperature. After the hour-long incubation, the mixture was injected into Slide-a-lyzer dialysis cassette (Thermo Fisher Scientific) and dialyzed overnight in PBS. The next day the labeled antibodies were concentrated by centrifugation in amicon filters (Millipore) and concentration was determined with NanoDrop spectrophotometer (Thermo Fisher Scientific).

All experiments outlined below were repeated at least three times with triplicates for each experimental condition.

Antibody penetration tracing

150,000 SK-N-SH cells were seeded per chamber into four chamber slides. Atto550 labeled anti-hnRNP A1 and rabbit IgG were added at a concentration of 2 µg/ml directly into DMEM/F12. An untouched control cell group was employed as a negative control. At 24 and 72 hours after antibody and IgG addition, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were then washed 4 times 5 min each with PBS, mounted, and imaged using a Zeiss Axio Observer A1 fluorescent microscope (Carl Zeiss). Quantification of antibody penetration percentage between the anti-hnRNP A1 treated and control rabbit IgG treated cells was performed using AxioVision Software. Five images were used per treatment. An example of one of the five field view images from the IgG and anti-hnRNP A1 treated cells used to perform the antibody penetration percentage calculations is shown (Supplement 1). Antibody penetrated cells were divided by the total number of cells to give a percentage of antibody penetration (Figure 1b). (Cells with antibodies per image/Total cells per image)× 100=Penetration Percentage.



Red- labeled antibodies

A. Following addition of antibodies to the media, Atto-550-NHS labeled anti-hnRNP A1 and control antibodies (red) were found to penetrate neuronal cells. **B.** Quantification of antibodies found inside the cells. Five images were used per treatment. Antibody penetrated cells were divided by the total number of cells to give a percentage of antibody penetration. (Cells with antibodies per image/Total cells per image)×100=Penetration Percentage.

Figure 1: Localization of antibodies by immunocytochemistry in SK-N-SH cells.

Anti-hnRNP A1 and early endosomes co-localization

100,000 SK-N-SH cells were seeded per chamber into 8 chamber slides. Atto550 labeled antibodies were added directly into DMEM/F12 at a concentration of 2 µg/ml. At 24 hours prior to antibody addition, 20 µl of Cell Light Early Endosome GFP (Invitrogen) was added to label endosomes. At 6, 24, and 48 hours after antibody addition, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were then washed, mounted, and observed as described above. Quantification of the endosome penetration for the three time points was performed in AxioVision Software. Total co-localization events per cell was determined for 5 cells at the 6hr, 24 hr, and 48hr time points respectively (Figure 3).

Determination of clathrin or caveolar mediated endocytosis

2.5 million SK-N-SH cells were seeded into 35 mm glass-bottom dishes (MatTek Corp). Three plates were designated per each clathrin or caveolar mediated endocytosis. Pitstop 2 reagent (Cellagen tech, C7487-25) was added to three dishes at a 10 nM concentration for 30 minutes into DMEM/F12 media to block clathrin mediated endocytosis. Also, nystatin (24 µl/mL) solution (Sigma-Aldrich, N1638) was added into another three dishes for 30 minutes to block caveolar mediated endocytosis. After the 30-minute incubation, anti-hnRNP A1 and IgG antibodies were added at a concentration of 4 µg/mL. Live imaging of the cells was performed at 6,12, and 24 hours after antibody addition in order to determine antibody penetration. Co-immunoprecipitations were performed in order to confirm the clathrin pathway as the method of penetration. Protein A beads were blocked with 6% Milk-TBS for an hour at room temperature. Beads were then washed 4 x 5 minutes with IP buffer. Anti-hnRNP A1 antibodies and control rabbit IgG were attached to protein A beads overnight at 4°C. Beads were then washed 4×5 min with IP buffer. SK-N-SH cell lysate was added over beads without antibodies, hnRNP A1 antibody beads, and control IgG beads overnight at 4°C. Beads were washed 4 x 5 minutes with IP buffer. Beads were spun down 5 minutes at 5000 rpm. 50 µl of 2x buffer was added per tube and heated 5 minutes at 95°C. Tubes were then cooled while spinning 5000rpm for 5 min at 4°C. 150 µg of each sample was loaded onto a 10% Tris gel and run at 100V for 90 minutes. The gel was then transferred to a PVDF membrane at 100V for 90 minutes. The membrane was blocked for 30 minutes at room temperature with 6% milk-TBS. Membrane was then washed 4 x 5 minutes with TBS-T. Rabbit Clathrin Interactor-1 antibodies (NBP1-05991) were added at a concentration of 1:2000 in 6% milk-TBS overnight at 4°C. Membrane was then washed 4 x 5 minutes in TBS-T. Secondary anti-rabbit HRP antibodies were added for 50 minutes at room temperature at a concentration of 1:10000. Membrane was then washed 4 x 5 minutes. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was then added for 5 minutes. Membrane was then imaged.

Measurement of ATP levels

ATP levels were determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). 50,000 SK-N-SH cells were seeded per well into a 96 well plate. Anti-hnRNP A1 antibodies produced in rabbit and normal rabbit IgGs were added at a concentration of 8 µg/ml directly into 100 µl of DMEM/F12 media. An untouched control cell group also served as a control. At 48 and 72-hour points after antibody addition 100 µL of CellTiter-Glo Reagent (Promega) was added. After one hour of incubation, the plate was read on a spectrophotometer between wavelengths 560Ex/590Em.

Determination of antibodies effect on Caspase 3/7 Levels

50,000 SK-N-SH cells were seeded per well into a 96 well plate. Anti-hnRNP A1 antibodies and normal rabbit IgGs were added at a concentration of 8 μg/ml and an untouched control cell group served as an additional control. At 24, 48, and 72 hours after antibody addition 100 μl of ApoOne Homogenous Caspase 3/7 reagent (Promega) was added per well. After one hour of incubation the plate was read on a spectrophotometer at 485Ex/530Em wavelengths.

Measurement of cytotoxicity

Cytotoxicity was determined using the Cytotox-ONE Cytotoxicity Assay (Promega). 100,000 SK-N-SH cells were seeded per well in triplicates into a 96 well plate. Anti-hnRNP A1 antibodies and normal rabbit IgGs were added at a concentration of 8 μg/ml and an untouched control cells were used as normal control (cytotoxicity percent) and a group with only culture media served as a background control. At 24, 48, and 72 hours plates were taken out of the incubator and lysis solution (Promega) was added for 30 minutes at room temperature. Cytotox-ONE reagent was added to all chambers and plates were incubated at room temperature for 10 minutes. Stop solution was then added to all chambers and readings were taken with a spectrophotometer at wavelengths 560Ex/590Em. To determine cytotoxicity the following equation was used (Experimental group fluorescence–Background fluorescence from media group)/(Control cell group fluorescence–Background fluorescence from media group)×100.

Double labeling to image redistribution of endogenous hnRNP A1

2.5 million SK-N-SH cells were seeded into 35mm MatTek dishes with a glass coverslip bottom. Normal rabbit IgG and rabbit produced anti-hnRNP A1 antibodies were added into DMEM/F12 media at a concentration of 4 μg/mL. 24 hours after antibody addition, cells were fixed for 15 minutes with 4% paraformaldehyde. Cells were then blocked and permeablized for 30 minutes with 6% Milk-PBS+0.4% Triton. Cells were then washed 4 x 5min with PBS. Mouse monoclonal hnRNP A1 antibodies were added at a dilution factor of 1:1000 for 1 hour at room temperature in PBS to label endogenous hnRNP A1. Cells were washed 4 x 5min with PBS. Anti-mouse Fluorescein labeled secondary antibody was added at 1:1000 for 50 min at RT in PBS. Cells were then washed and imaged in PBS. Quantification of endogenous hnRNP A1 redistribution was performed by comparing the densitometric mean of the FITC fluorescence expression of the nucleus vs. the cytoplasm of 10 cells per group using AxioVision Software (Figure 7).

Nuclear/Cytosol fractionation of SK-N-SH cells

To determine hnRNP A1 presence in the nucleus and the cytoplasm of SK-N-SH cells fractionation kit (BioVision) was used. Briefly, cells in three groups were collected by centrifugation at 600xg for 5 minutes at 4°C. Ice-cold cytosol extraction buffer, containing protease inhibitors and DTT was added. Cells were vortexed, centrifuged (16,000 xg) and cytoplasmic fraction was collected. The pellet was treated with ice-cold nuclear extraction buffer, vortexed on the highest setting multiple times and then centrifuged (16,000 xg) to collect nuclear fractions. Protein concentration was determined using BCA assay (BioRad). Equal amount of each fraction per condition was loaded onto 10% SDS gel. The gel was transferred onto nitrocellulose membrane and probed with anti-hnRNP A1 antibody (Abcam). Band quantitation was performed in Image J program using gel analysis method (Figure 7b).

Statistics

Statistical analyses were performed in GraphPad Prism software using either student t-test or one-or two-way ANOVA with post-tests. Significant differences depicted as *<0.05, **<0.01, ***<0.001, ns=not significant.

Results

Direct antibody addition into growth media results in antibody penetration into neuronal cells

In order to investigate the role of antibodies to hnRNP A1 on neuronal cells, we determined whether the antibodies had the ability to penetrate SK-N-SH neuronal cells. In previous work MS and HAM/TSP patients have been shown to produce autoantibodies to hnRNP A1 [9]. Specifically, MS and HAM/TSP patients produced antibody to a small region within the nuclear export sequence/nuclear localization sequence (NES/NLS) of hnRNP A1, called M9 [6,9]. We utilized commercially available anti-hnRNP A1 antibodies that recognize the M9 region of hnRNP A1 and control IgG antibodies to determine the antibodies' ability to penetrate neuronal cells. To visualize antibody penetration, we labeled the antibodies with a red fluorescent dye –Atto-550-NHS dye as previously described [17]. Atto-550-NHS is a new label with high molecular absorption and quantum yield. Excitation and emission for Atto-550-NHS (Ex.556/Em.578 nm) is similar to a well-known Cy3 dye. Atto-550-NHS labeled antibodies were added directly to the cell growth media at different concentrations (2, 4, 6, and 8 μg/mL) without any additional reagents. Cells were examined under Zeiss Axio Observer1 microscope in phase and fluorescence modes 24, 48 and 72 hours following treatment. Cells treated with fluorescent label itself did not uptake the dye, indicating that Atto-550-NHS did not allow for antibody penetration (data not shown). At the 24-hour point, the anti-hnRNP A1 and control IgG antibodies were found inside neuronal cells (Figure 1a). At 72 hours, penetration was seen in both groups. Interestingly, quantification of the percentage of cells penetrated by antibodies reveals that the anti-hnRNP A1 antibodies penetrate the neuronal cells with higher affinity than control IgG (Figure 1b). These findings suggest that antibodies possess the ability to penetrate neuronal cells and that the anti-hnRNP A1 antibodies penetrated more readily than control antibodies.

Antibodies to hnRNP A1 penetrate neuronal cells and co-localize with early endosomes

Next, we sought to examine the mechanisms of cellular penetration. Since endocytosis is involved in the uptake of numerous molecules from the extracellular environment, we set out to determine whether endosomes were involved in antibody entry into neurons. To accomplish this task, we performed localization studies and tested whether antibodies co-localized with early endosomes. Early endosomes were pre-labeled with GFP BacMam reagent (green) and antibodies with Atto-550-NHS (red). As early as 6 hours after antibody addition, anti-hnRNP A1-M9 antibodies co-localized with early endosomes (Figure 2). Maximum co-localization was seen at 24 hours after antibody addition (Figures 2 and 3). At 48 hours, antibody co-localization with early endosomes was still present (Figure 3) however, the number of endosomes that contained antibodies was significantly less than at 24 hours. The fact that co-localization is seen at multiple time points rather than just a single window of time, suggests that antibodies continually penetrate neuronal cells as long as there are still free antibodies in the extracellular media. These results indicate that endocytosis appears to be the mechanism by which antibodies penetrate neuronal cells.

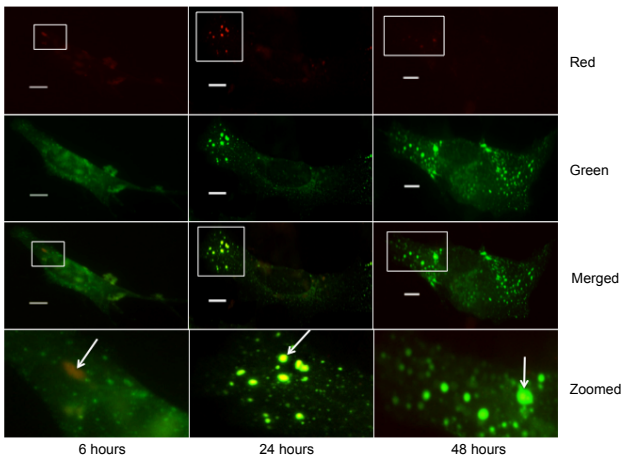


Figure 2: Antibodies co-localize with early endosomes. Early endosomes labeled with BacMam particles GFP (green), antibodies with Atto550-NHS (red). Examples of co-localization (yellow) are shown in boxes. Arrows in zoomed images point to colocalized antibody example. Scale bar=10 µm.

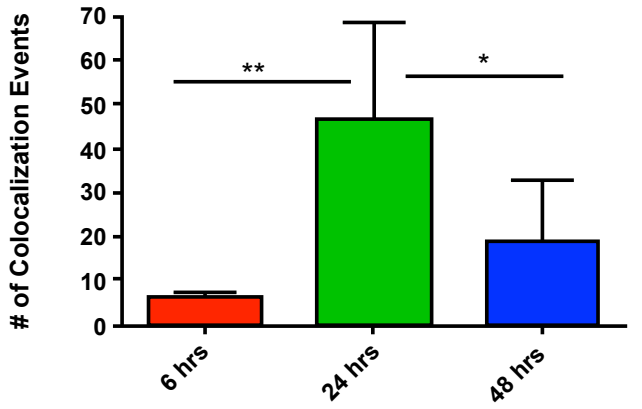


Figure 3: Quantification of antibodies co-localized to early endosomes at 6, 24 and 48 hours after antibody addition to the media. Images were taken on fixed cells and number of co-localized antibodies was calculated per cell at a given time point. Events were calculated in 5 cells per time point. Groups were analyzed with one-way ANOVA with Newman-Keuls multiple comparison test. *P<0.05, **P<0.01.

Antibodies to hnRNP A1 and IgG penetrate neuronal cells via clathrin mediated endocytosis and not via caveolar mediated endocytosis

Two main types of endocytosis occur in neuronal cells, clathrin and caveolar mediated endocytosis [18,19]. Molecules endocytosed into cells can traffic through one or both pathways. We examined whether neuronal cells uptake antibodies via one or both of these pathways. To answer this question, we used competitive inhibitors of the individual pathways to determine whether antibody penetration was blocked. To evaluate clathrin-mediated endocytosis we used Pitstop2 reagent, which selectively inhibits clathrin via inhibition of the clathrin terminal domain [19,20]. With clathrin-mediated endocytosis inhibited, we witnessed complete blockage of the penetration of the antibodies, thus revealing that clathrin-mediated endocytosis is at least one mechanism by which antibodies penetrate neuronal cells (Figure 4a). To assess whether caveolar-mediated endocytosis was also involved in antibody entry into the cell we used nystatin, a lipid raft inhibiting agent, to block caveolar-mediated endocytosis [21]. When caveolar-mediated endocytosis was blocked, we observed a standard amount of antibodies inside neuronal cells (Figure 4a). Cells with nystatin did not differ from cells without any blocking reagent. This suggests that caveolar-mediated endocytosis is not involved in the intracellular penetration of antibodies (Figure 4a). To confirm our observation biochemically, we performed co-immunoprecipitation experiments. In these experiments, anti-hnRNP A1-M9 antibodies or control rabbit IgG were attached to agarose-A beads. The whole cell lysate was incubated with the beads overnight, complexes were eluted, immobilized onto PVDF membranes and probed with anti-clathrin antibodies. Beads without antibodies (B) served as negative control (Figure 4b). A clathrin band is seen in the whole cell lysate lane (L) confirming its presence in SK-N-SH cells. Signal is enriched in both the control IgG and anti-hnRNP A1-M9 lanes, but not in the blank lane (B), indicative of antibody binding to clathrin, thus confirming the specificity of observation seen by immunocytochemistry. Taken together, these results demonstrate that antibodies penetrate neuronal cells via clathrin-mediated endocytosis.

Anti-hnRNP A1 antibodies deplete cellular ATP

Since antibodies possess the ability to penetrate neuronal cells, we sought to determine what cellular events might be compromised due to the presence of antibodies inside the cells. Previously, we showed that transfection of anti-hnRNP A1-M9 antibodies into dNT-2 neurons resulted in expression of neurodegenerative markers and cell death within 7 days [9]. Transfection of normal rabbit IgG did not have the same effect. Thus, anti-hnRNP A1-M9 antibodies play a role in neuronal degeneration. In this study, we carried out all experiments without transfection and examined the possible effects of these antibodies on SK-N-SH cells. Since ATP is essential to cell health and function, we determined if cellular ATP levels, a measure of cellular viability, were altered by anti-hnRNP A1-M9 antibodies. We performed the CellTiter-Glo Luminescent Cell Viability Assay to determine the ATP levels of the neuronal cells with and without the antibodies. In these experiments we used unlabeled anti-hnRNP A1-M9 or IgG. Our results reveal that ATP levels were compromised by as early as 48 hours after anti-hnRNP A1-M9 antibody addition (Figure 5a).

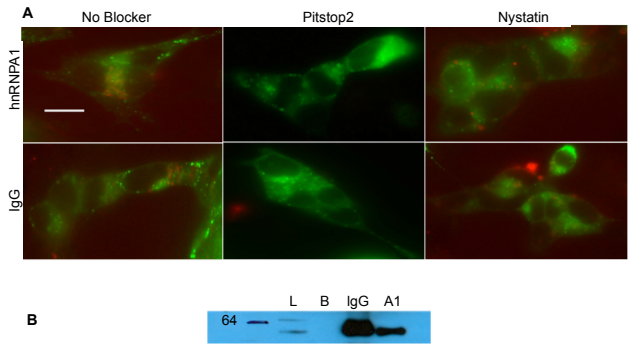


Figure 4: Antibodies penetrate SK-N-SH cells via clathrin-mediated endocytosis. **A.** Addition of Pitstop2 reagent prevented clathrin-mediated endocytosis of both anti-hnRNP A1 and IgG into SK-N-SH cells. Addition of nystatin did not block penetration. **B.** Clathrin immunoprecipitation on agarose A-linked anti-hnRNP A1 and control IgG. L=SK-N-SH cell lysate. B=Agarose A beads, IgG=control IgG attached to agarose A beads, A1=anti-hnRNP A1 antibodies attached to agarose A beads. Both control IgG and anti-hnRNP A1 antibodies bound clathrin, confirming the observation by immunocytochemistry in figure 4A.

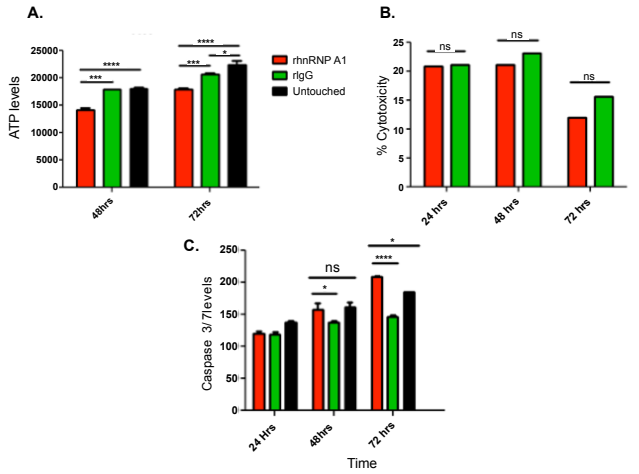


Figure 5: Anti-hnRNP A1 antibodies caused an increase in apoptosis and depletion of ATP levels. **A.** Cellular ATP levels are significantly reduced when SK-N-SH cells were exposed to anti-hnRNP A1 antibodies. **B.** Antibodies were not cytotoxic to the SK-N-SH cells (Percent cytotoxicity was normalized based on the numbers detected in untouched cells). **C.** In contrast to control antibodies, reduced levels of caspase 3/7 were seen at 48 and 72 hours after anti-hnRNP A1 antibodies were added to the media. Results were analyzed with two-way ANOVA with Bonferroni multiple comparison test.

Addition of normal rabbit IgG did not significantly affect ATP levels in these cells. The alteration of cellular ATP levels induced by anti-hnRNP A1-M9 antibodies could lead to a multitude of deleterious effects on the neuronal cells.

Anti-hnRNP A1 antibodies are not cytotoxic

Considering that anti-hnRNP A1-M9 antibodies diminished the viability of neuronal cells, we performed the Cytotox-ONE Cytotoxicity Assay to determine the possible cytotoxic effects of anti-hnRNP A1-M9 antibodies on neuronal cells. SK-N-SH cells were seeded in 96-well plate in triplicate for each group: 1) untouched, 2) cells with control rabbit IgG, 3) cells with anti-hnRNP A1-M9 antibodies. All groups were normalized based on relative fluorescence of untouched cells. Plates were evaluated at 24, 48, and 72 hours after antibody addition. There were no statistical differences in cytotoxicity between the anti-hnRNP A1 and control antibodies, suggesting that neither control rabbit IgG or anti-hnRNP A1-M9 antibodies were cytotoxic to SK-N-SH cells (Figure 5b).

Anti-hnRNP A1 antibodies increased apoptosis in neuronal cells

Considering the effects of anti-hnRNP A1-M9 on neuronal viability (ATP levels), we hypothesized that the anti-hnRNP A1-M9 treated cells would show an increase in apoptosis compared to control antibodies. Caspases 3 and 7 are the executioner apoptosis caspases. In order to examine the levels of these caspases we used the ApoOne Homogenous Caspase 3/7 Kit (Promega). Cells treated with anti-hnRNP A1-M9 antibodies were compared to control IgG and untouched cells at 24, 48 and 72 hours after antibody addition. No differences were detected between the groups at 24h. However at 48h, cells treated with anti-hnRNP A1-M9 antibodies showed increased Caspase 3/7 levels in comparison to control IgG. This trend continued at the 72-hour time point showing a significant increase in Caspase 3/7 activity in the anti-hnRNP A1-M9 antibody treated group compared to controls (Figure 5c). These results reveal that one mechanism by which anti-hnRNP A1-M9 antibodies alter cell viability in neuronal cells is due to an increase in Caspase 3/7 activity leading to apoptosis of neuronal cells.

Anti-hnRNP A1 antibodies cause redistribution of endogenous hnRNP A1 protein

Considering the specificity of an antibody-antigen interaction, we hypothesized that the anti-hnRNP A1-M9 antibodies not only penetrated neurons and localized to early endosomes, but also targeted endogenous hnRNP A1 protein. In order to resolve this question, we performed double labeling experiments in which we added rabbit Atto550 labeled anti-hnRNP A1-M9 and control antibodies to neuronal cells, fixed and permeabilized the cells and then labeled endogenous hnRNP A1 with mouse monoclonal anti-hnRNP A1 followed by secondary anti-mouse FITC antibodies (Figure 6). In contrast to control antibodies, our results reveal that following anti-hnRNP A1-M9 antibody addition there is a redistribution of endogenous hnRNP A1 from being primarily nuclear to being equally distributed in the nucleus and in cytoplasm (Figures 6 and 7). Total densitometric means of FITC expression were quantified for both the nuclear and cytoplasmic portions of the cells in all experimental groups using Axiovision software. The densitometric mean of FITC expression of the nuclear and cytoplasmic cellular portions was used to quantify a nuclear to cytoplasmic ratio for each experimental group (Figure 7a). In addition to microscopy experiments, we separated nuclear and cytoplasmic fractions of untreated and treated cells. These fractions were subjected to SDS-PAGE and quantitated in Image J software (Figure 7b). In untouched cells, hnRNP A1 is primarily nuclear. However in cells, treated with control antibody a small portion of hnRNP A1 is present in cytoplasm. SK-N-SH cells treated with anti-hnRNP A1 antibody have almost equal presence of hnRNP A1 protein in the nucleus and in the cytoplasm. Thus, presence of anti-hnRNP A1 antibodies affected endogenous distribution of hnRNP A1 (Figure 7b).

These analyses indicate that the anti-hnRNP A1-M9 treated cells have a significantly altered nuclear to cytoplasmic expression ratio of endogenous hnRNP A1 when compared to untouched control and the IgG treated cells.

Discussion

The role of autoantibodies and their ability to target intracellular antigens is an important focal area of research for autoimmune

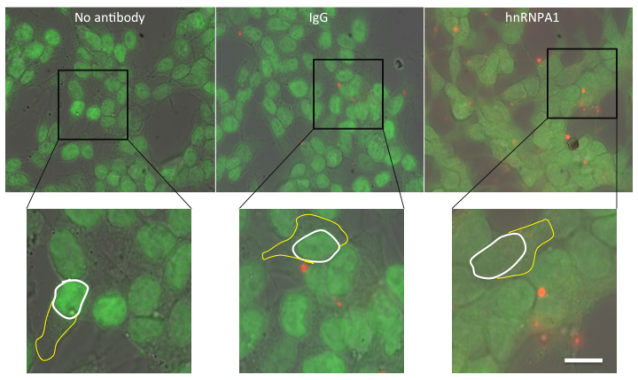


Figure 6: Anti-hnRNP A1 antibodies cause redistribution of endogenous hnRNP A1 in SK-N-SH cells. Atto-550-NHS labeled anti-hnRNP A1 or control antibodies (red) were added to the cells. 24 hours later cells were fixed, washed and permeabilized. Endogenous hnRNP A1 was labeled with primary mouse monoclonal antibodies followed by FITC anti-mouse secondary antibodies. White borders outline nuclei, yellow-cytoplasm.

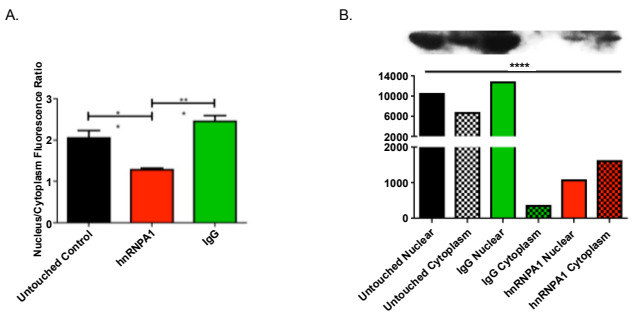


Figure 7: Antibodies cause redistribution of endogenous hnRNP A1. **A.** Quantification of endogenous hnRNP A1 redistribution in comparison to untouched cells and cells with control IgG. Nuclear to cytoplasmic fluorescence ratio calculated using densitometric means, n=5. **B.** Quantification of endogenous hnRNP A1. Nuclear and cytoplasmic fractions were collected from untouched, IgG and hnRNP A1 treated SK-N-SH cells. Equal amount of each fraction per condition was loaded onto 10% SDS gel. The gel was transferred onto nitrocellulose membrane and probed with anti-hnRNP A1 antibody (Abcam). Band quantitation was performed in Image J program using gel analysis method.

disorders. The notion that cellular injury is initiated and swiftly followed by intracellular antigens being exposed to the adaptive immune response resulting in the ability of antibody response to the target antigen prevails as the dominant theory. Autoantibodies produced by patients with autoimmune disorders have been implicated in the pathogenesis of various diseases such as SLE, RA, paraneoplastic syndromes, MS and HAM/TSP [1,5-7,9]. In previous work it has been shown that patients with MS and HAM/TSP produce autoantibodies to the nuclear shuttling proteinhn RNP A1; and specifically to its M9 region [9]. These autoantibodies to hnRNP A1 have been implicated in the pathogenesis of MS and HAM/TSP, however until now it was not clear how these antibodies might cause target cell damage.

Anti-recoverin antibodies that are present in patients with Cancer Associated Retinopathy syndrome were previously shown to penetrate E1A. NR3 cells via endocytosis [22]. In this study, we have shown that antibodies to hnRNP A1-M9 are capable of penetrating SK-N-SH neuronal cells through clathrin-mediated endocytosis. Compared to control antibodies, the anti-hnRNP A1 antibodies penetrated neuronal cells more readily and showed a higher level of co-localization within early endosomes. Interestingly, anti-hnRNP A1-M9 antibodies added directly into the media penetrated near the efficiency rate seen previously through transfection methods of anti-hnRNP A1-M9 antibodies into the SK-N-SH neuronal cells [9]. The fact that the method of antibody penetration occurs through endosomes gives great insight because endocytosis is a general method of cellular uptake of various molecules and it occurs in various neuronal cell types. Therefore, it would be important to note that this method of antibody penetration might be taking place in a multitude of neurons throughout the nervous system. Retrograde axonal transport through signaling endosomes has been shown as a growing area of research in neurodegenerative disorders. A link has been formulated between disruption of retrograde transport and a variety of neurodegenerative diseases [23]. Various neurotrophic factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4) have been shown to bind specific Trk receptors at axon terminals and are ultimately transported retrogradely via signaling endosomes by microtubule dependent dynein motors [23-25]. Dynein function disruption has been shown to lead to neurodegeneration in both mice and human disease [23]. Furthermore, it has also been presented that both retrograde and anterograde transport malfunction can result in a "traffic jam" of sorts, displaying that mutations in kinesins as well as dynein can influence signaling endosomes in a negative manner [23,26]. Of specific interest, hereditary spastic paraplegia (HSP) a disease that is clinically indistinguishable from progressive MS and HAM/TSP, exhibits the "dying back phenomena" specific to disruption of retrograde transport as the disease is seen to progress in a distal to proximal progression [23]. Several forms of HSP contain causative mutations of molecular motors or in other proteins that affect axonal transport indirectly [23,27-29]. These studies provide insight into future studies to assess whether autoantibodies to hnRNP A1 produced by MS and HAM/TSP patients have an effect upon retrograde transport, thus providing a possible mechanism for the distal to proximal neurodegeneration seen in patients with MS.

In these studies, we show for the first time that antibodies to hnRNP A1 have the ability to penetrate healthy neuronal cells and cause negative effects on the intracellular environment. Compared to control antibodies, anti-hnRNP A1 antibodies significantly depleted cellular ATP levels and caused significant increases in caspases 3/7 levels leading to apoptosis in SK-N-SH neuronal cells. It is important to note that decreases in cellular ATP often correlates with the initiation of apoptosis leading to cell death [30]. In addition, we showed that anti-hnRNP A1-M9 antibodies caused a redistribution of endogenous hnRNP A1 from being primarily nuclear to being almost equally nuclear and cytoplasmic. The epitope recognized by the anti-hnRNP A1-M9 antibodies is AA 293-304, which contained within hnRNP A1's NLS/NES [6]. The anti-hnRNP A1 antibodies targeted this sequence and altered its nuclear-cytoplasmic transport. Importantly, Michael et al. [31] showed that deletion mutations in this region of M9 result in loss of nuclear import and export of hnRNP A1. Taken together, these data indicate that the anti-hnRNP A1-M9 antibodies specifically targeted M9 and in doing so, caused a change in localization of hnRNP A1, which considering its critical role in cellular function, would likely have deleterious effects on neurons. Because the epitope recognized by the anti-hnRNP A1-M9 antibodies used in these experiments overlaps the epitope of the antibody response in MS and HAM/TSP patients (6,9), we believe that these antibodies might be pathogenic, rather than merely disease markers. Pathologically, MS and HAM/TSP patients develop a robust B-lymphocyte, plasma cell and antibody response within the central nervous system (CNS) [32-36]. Neuronal degeneration and apoptosis are also present [32,33,35]. In our model, anti-hnRNP A1 M9 antibodies might target neurons in the CNS that contain this sequence and enter neurons via clathrin-mediated endocytosis. Next, the antibodies target hnRNP A1 in the cytoplasm and disrupt its ability to shuttle into and out of the nucleus, which in turn results in apoptosis, alterations in ATP levels and ultimately, neuronal degeneration. Importantly, other studies have shown that disruption of hnRNPs results in apoptosis and alteration in ATP levels [37-39]. For example, siRNA knockdown of hnRNP A1/A2 caused apoptosis in human cell lines [38] and depletion of hnRNP K altered ATP levels [37]. hnRNP A1 is an RNA binding protein (RBP). RBPs have recently been found to play critical roles in neurological and autoimmune diseases [40,41]. For example, overexpression of the RBP Fus caused apoptosis and neuronal degeneration in *Drosophila* motor neurons [42]. Like hnRNP A1, Fus has a NLS and its ability to cause apoptosis was related to its localization within neurons [42].

In summary, these experiments showed that anti-hnRNP A1-M9 antibodies penetrate SK-N-SH neuronal cells through clathrin-mediated endocytosis and enter early endosomes. In addition, the antibodies significantly altered ATP levels and increased Caspase 3/7

levels that ultimately lead to apoptosis and cell death. Further, our studies reveal that anti-hnRNP A1-M9 antibodies also have specific effects on hnRNP A1. These findings have implications concerning the pathogenesis of MS and HAM/TSP, in which neuronal damage and neurodegeneration are important features. Future studies are needed to determine the complete effects of antibodies to hnRNP A1 on neuronal cells in order to better understand their role in the pathogenesis and mechanisms of neurodegeneration in MS and HAM/TSP.

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