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Altered neurological function in mice immunized with early endosome antigen 1

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Abstract

Background: Autoantibodies directed against the 160 kDa endosome protein early endosome antigen 1 (EEA1) are seen in patients with neurological diseases. To determine if antibodies to EEA1 have a neuropathological effect, mice from three major histocompatability haplotype backgrounds ($H2^q$, $H2^b$ and $H2^d$) were immunized with EEA1 (amino acids 82–1411) that was previously shown to contain the target EEA1 epitopes. The mice were then subjected to five neuro-behavioural tests: grid walking, forelimb strength, open field, reaching and rotarod.

Results: The immunized SWR/J mice with sustained anti-EEA1 antibodies had significantly reduced forelimb strength than the control non-immune mice of the same strain, and BALB/CJ immune mice demonstrated significantly more forelimb errors on the grid walk test than the control group.

Conclusions: Antibodies to recombinant EEA1 in mice may mediate neurological deficits that are consistent with clinical features of some humans that spontaneously develop anti-EEA1 autoantibodies.

Background

Autoimmune neurological diseases occur after alterations of immunological tolerance to certain components of the nervous system. The factors that cause the breakdown of tolerance and the subsequent autoantigen-specific activation of self-reactive B and T lymphocytes are not well understood. A small number of autoimmune neurological diseases, such as myasthenia gravis, are well characterized whereas many others are still the subject of intense research [1-3]. The influence of genetic [4] and hormonal [5] factors on autoimmunity are among the best understood co-morbid variables in disease expression.

Autoantibodies to early endosome antigen 1 (EEA1) were reported in the sera of the patients with neurological disorders [6,7], but the pathological significance of EEA1 antibodies is not known. EEA1 is a peripheral endosomal protein expressed in a variety of tissues, including nervous tissues [6,8-10]. Since early endosomes are key functional components of both pre-synaptic and post-synaptic neurons [11,12], the association of EEA1 autoantibodies with neurological diseases suggests a number of interesting clinical and neurobiology studies.

In order to investigate if EEA1 autoantibodies may underlie the clinical expression of disease, studies *in vivo* were

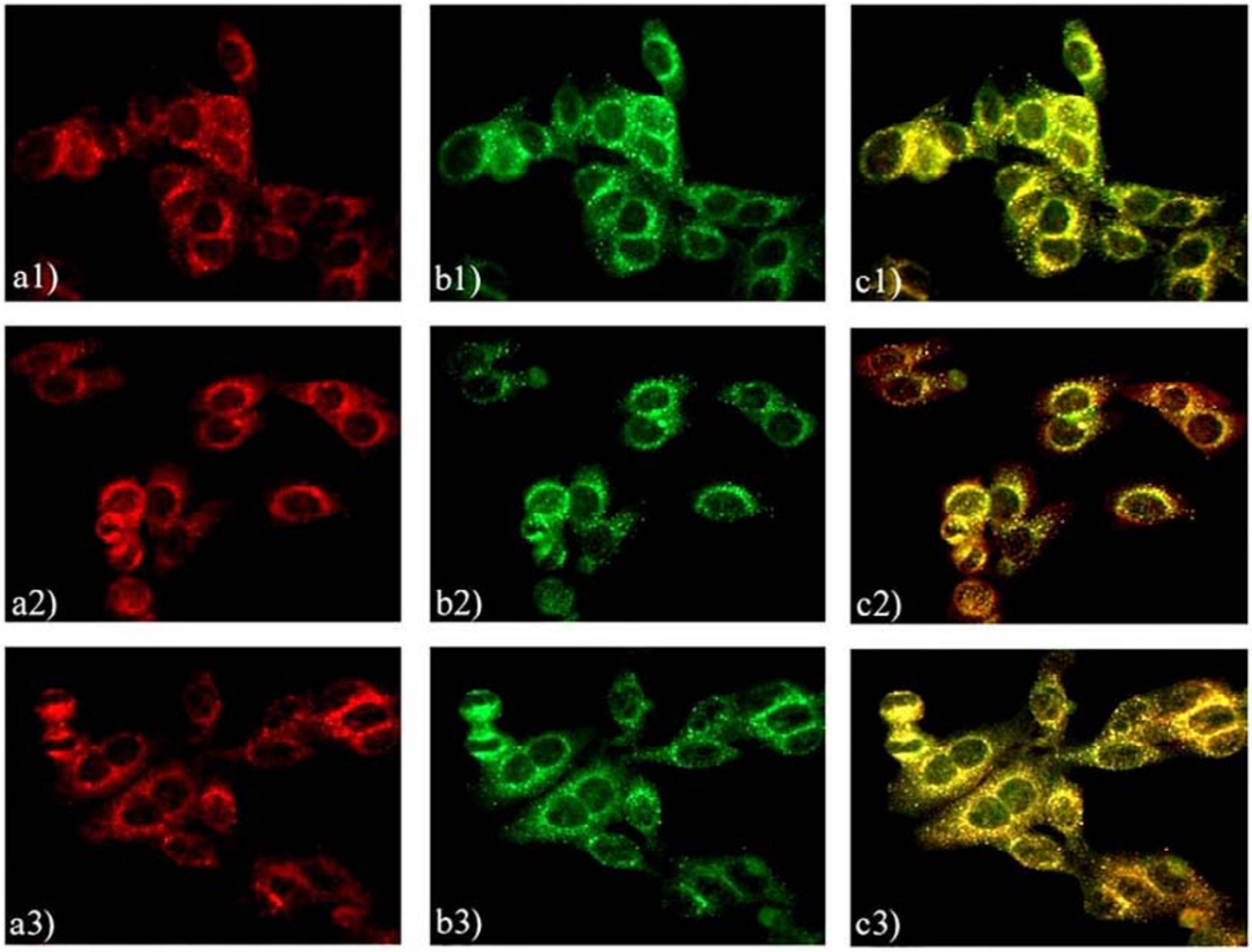

Figure 1
Indirect immunofluorescence of anti-EEA1 sera induced in SWR/J (top row), C57BL/6J (middle row) and BALB/CJ (bottom row) mice by immunization with purified recombinant EEA1. The immunized mice produced a cytoplasmic vesicular staining pattern (panels a: red) that co-localized with the human prototype anti-EEA1 serum (panels b: green) on HEp-2 cells. The red (a) and green (b) merged panels are shown in panels c. Original magnification 400 ×.

conducted by immunizing thirty-six female mice with EEA1 recombinant protein, followed by the evaluation of the neurological and behavioral skills two months after immunization. The observations in this study indicated that mice bearing anti-EEA1 antibodies developed impaired neurological and behavioral skills.

Results

Generation of anti-EEA1 autoantibodies in mice

All mice from the experimental group that were immunized with the recombinant EEA1 protein but not those that received adjuvant alone, developed autoantibodies that displayed an endosomal cytoplasmic staining pattern that co-localized with index human serum (Figure 1). Some C57BL/6J mice anti-EEA1 sera displayed weak nuclear staining in addition to the vesicular cytoplasmic staining pattern.

The antibody response was followed and quantitated using the addressable laser bead immunoassay (Table 1). All pre-immune mouse sera had median fluorescence units (MFU) of <700. Eight weeks after the initial immunization, the MFU increased and was sustained at >4500 in SWR/J, >4300 in C%&BL/6J, and >7500 in BALB/CJ mice.

Table 1: Measurement of antibodies to early endosome antigen 1 (EEA1) by an addressable laser bead immunoassay[a]

Mouse Strain	Pre-immune MFU (range)	Eight Weeks After Immunization MFU (range)
SWR/J	235 (197–254)	4545 (989–9677)
C57BL/6J	209 (226–373)	4303 (675–5396)
BALB/CJ	329 (221–666)	7854 (3842–10660)

[a] results of assay shown as the mean of median fluorescence units (MFU)

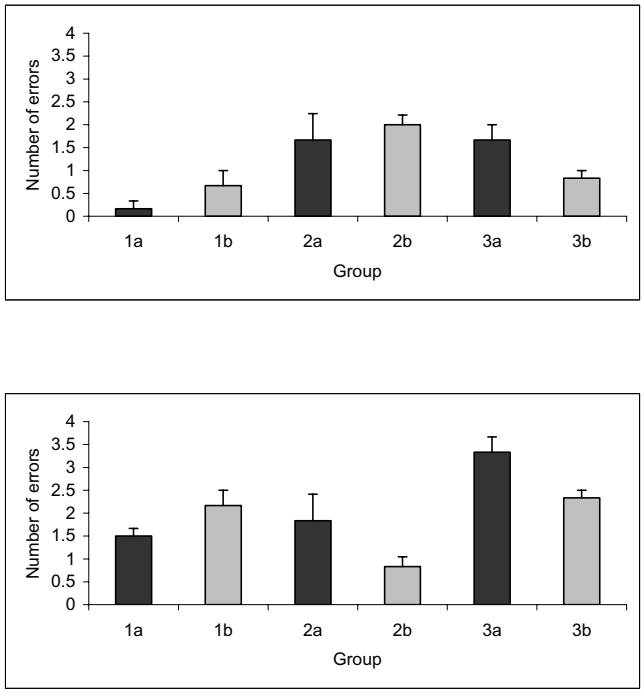

Figure 2
Forelimb (top panel) and hind limb errors (bottom panel) in SWR/J (1), C57BL/6J (2) and BALB/CJ (3) mice while traversing a grid. Immunized mice bearing anti-EEA1 antibodies (1a, 2a, 3a respectively) were compared to the same strain of non-immune control mice (1b, 2b, 3b). This test revealed that the BALB/CJ mice bearing anti-EEA1 antibodies made significantly more forelimb errors than the control non-immune group.

Grid walking and forelimb strength

Eight weeks after immunization, the mice were subjected to the five neurological and behavioural tests. The average number of forelimb and hind limb errors that were recorded while each mouse traversed the grid (Figure 2). The analyses showed that there were no statistically significant differences between immune and control mice on forelimb or hind limb errors for SWR/J (t = -1.3, p = .21, t = 1.14, p = .28), or C57BL/6J (t = .54, p = .60, t = -1.6, p = .14) mice. However, there was a significant difference between immune and non-immune BALB/CJ mice on forelimb errors (t = 2.24, p = .049), but not on hind limb errors (t= p = .18).

When forelimb strength was analyzed separately for each strain, a significant difference was observed between the immune and non-immune groups of SWR/J (F(1,10) = 6.347, p = .03) mice (Figure 3). No significant differences were observed between the study groups of C57BL/6J or BALB/CJ mice (F(1,10) = 3.685, p = .08, F(1,10) = .702, p = .42). In summary, the experimental group of strain SWR/J was significantly weaker than the control group. The difference in strength scores for the C57BL/6J mice bearing anti-EEA1 antibodies compared with the non-immune control group approached statistical significance. This suggested a trend toward greater weakness in the immunized experimental group in comparison to the non-immune control group.

Open field, reaching and rotatrod tests

The performance of the mice in the open field, reaching and rotarod tests did not reveal any statistically significant differences between the non-immune control and immunized experimental groups (Figures 4,5,6,7). The total distance traveled during 20 min of observation in the open field for all three mouse strains is shown in Figure 4. A repeated measures analysis of variance (ANOVA) showed that there was no statistically significant difference between SWR/J control and experimental groups (F(1,40) = .051, p = .83) (Figure 4, top panel). Both groups did travel progressively less over bins (F(4,40) = 82.12, p < .0001) and changed similarly over the bins (F(4,40) = 1.163, p = .34). The C57BL/6J non-immune control group tended to be more active overall than the immunized group (Figure 4, middle panel), however, a repeated measure ANOVA showed that this difference was not statistically significant (F(1,40) = 1.05, p = .33). Both groups did travel progressively less over the bins (F(4,40) = 11.39, p < .0001) and there was a trend for a significant interaction between bin and distance traveled but this difference was not statistically significant (F(4,40) = 2.17, p = 0.09).

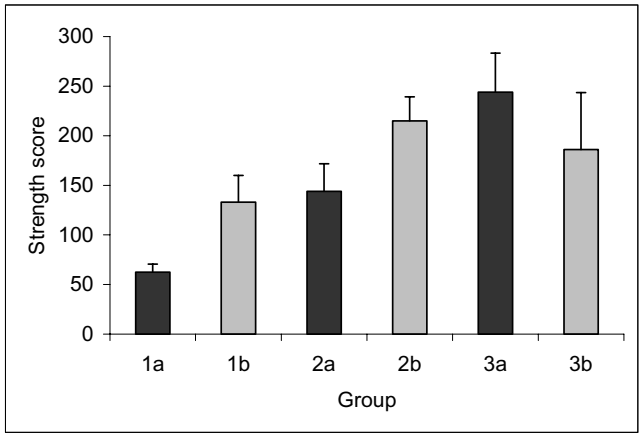

Figure 3
The average forelimb strength was tested in the immunized group (a) and non-immune control group (b) of SWR/J (1), C57BL/6J (2) and BALB/CJ (3) mice. The immunized SWR/J mice were significantly weaker than the control non-immune group, whereas the difference in strength scores for the C57BL/6J mice bearing anti-EEA1 antibodies did not achieve statistical significance when compared to the non-immune control group.

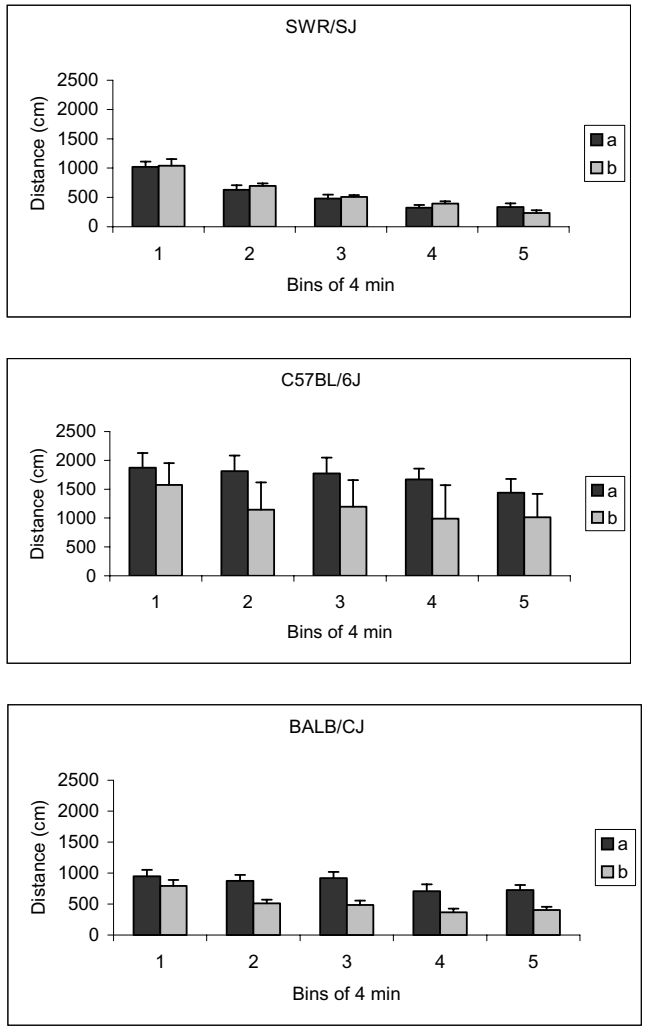

Figure 4
The distance travelled in the open field test was measured in the immunized mice (a) and the non-immune control group (b). Statistically significant differences were not observed in these groups.

A repeated measures ANOVA showed that BALB/CJ control group traveled less distance than the experimental group ($F(1,40) = 11.38$, $p = .007$) (Figure 4, bottom panel). Both groups did travel progressively less over the 5 bins ($F(4,40) = 10.38$, $p < .0001$) and the groups changed similarly over the bins ($F(4,40) = .203$, $p = .23$).

The total number of rears (supported and unsupported) and the number of unsupported rears for each mice strain is summarized in Figure 5. A one-way ANOVA shows that SWR/J control and experimental groups do not differ in the number of rears ($F(1,10) = .257$, $p = .62$), nor in the number of unsupported rears ($F(1,10) = 1.905$, $p = .198$). Similar observations were recorded for the two BALB/CJ study groups: rears ($F(1,10) = .907$, $p = .36$); unsupported rears ($F(1,10) = 1.348$, $p = .27$). By comparison, the differences of rears ($F(1,10) = 4.514$, $p = .0596$) and unsupported rears ($F(1,10) = 4.233$, $p = .0667$) between C57BL/6J non-immune control and immune groups did approach but did not achieve statistical significance.

Over the five days of conditioning, the mice learned to retrieve food pellets from the tray by reaching with their forepaw through the vertical bars. On the sixth day, the animals were individually placed in the reaching box and reaching was videotaped for 5 consecutive minutes, beginning with the first reaching attempt. Figure 6 shows the total number of attempted reaches and the percentage of hits (number of successful reaches over the total number of attempted reaches). One way ANOVA analysis of the data for each strain showed that there were no significant differences between the immune and non-immune groups of each mouse strain ($F(1,10) = 1.079$, $p = .32$, $F(1,10) = 2.026$, $p = .188$, $F(1,10) = .331$, $p = .58$ for SWR/J, C57BL/6J, and BALB/CJ strains respectively).

After all mice were trained to walk on a rotating drum (6 sessions over 2 days), the rotational speed of the rotarod at time when the mouse fell from the drum was recorded (Figure 7). Repeated measure ANOVA shows that there were no statistically significant differences between the

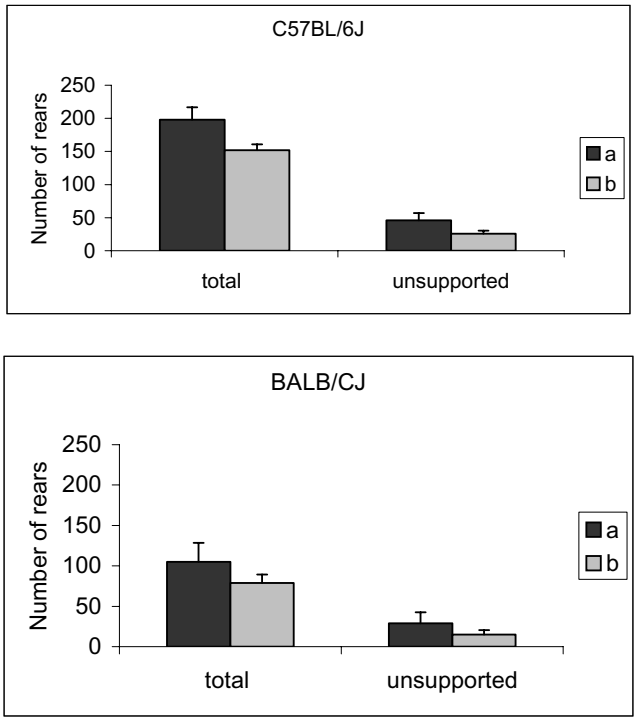

Figure 5
The number of rears during 20 min in the open field was recorded in the immunized group (a) and non-immune control (b) mice. The differences of rears ($F(1,10) = 4.514$, $p = .0596$) and unsupported rears ($F(1,10) = 4.233$, $p = .0667$) between C57BL/6J non-immune control and immunized groups approached, but did not achieve, statistical significance. Statistically significant differences were not observed in the other genetic strains of mice.

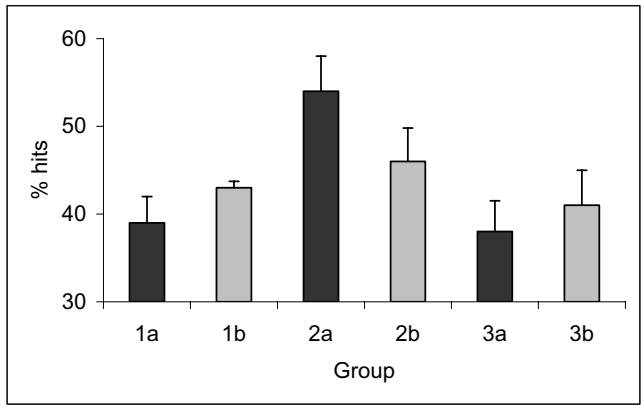

Figure 6
The percent of hits achieved in reaching for sweet pellets was recorded in SWR/J, C57BL/6J and BALB/CJ immunized mice (1a, 2a and 3a respectively) and were compared to the same strain of non-immune control mice (1b, 2b, 3b). Statistically significant differences were not observed in these groups of mice.

study groups of any of the mouse strains ($F(1,10) = 2.85$, $p = .12$, $F(1,10) = 0.07$, $p = .80$, $F(1,10) = .85$, $p = .38$). SWR/J mice did show a significant improvement on subsequent trials ($F(1,5) = 5.5$, $p = .0004$), whereas C57BL/6J and BALB/CJ mice did not show significant improvement ($F(1,5) = 1.5$, $p = 0.21$, $F(1,5) = 0.81$, $p = .55$).

In this study we used mice immunized with EEA1 as an approach to determine if anti-EEA1 autoantibodies were associated with functional neurological deficits *in vivo*. The experimental approach to neurological and functional behavior abnormalities that we used in these studies are detailed and validated by others http://www.neurod.com/behavior_w.htm[13]. We observed that mice immunized with an EEA1 recombinant protein displayed a statistically significant impaired performance on two out of five of these tests. Significantly reduced forelimb strength was recorded in SWR/J mice that had sustained anti-EEA1 antibodies when compared to the control non-immune mice. The immunized BALB/CJ mice demonstrated significantly more forelimb errors on the grid walk test than did the control non-immune mice. In addition, C57BL/6J mice had lower forelimb strength scores that approached statistical significance, indicating that the experimental group tended to be weaker than the control group. Of interest, it was observed that the non-immune control group showed a tendency to be less active and to show less rearing than the experimental group. This suggested that mice bearing anti-EEA1 may have expressed other neurological features, such as hyperactivity, that were not formally or objectively evaluated in this study.

It is important to note that the data collected for the behavioral studies reported here were expressed as mean values for each group of mice and this approach may obscure profound abnormalities in a single animal. For example, if only 50% of animals immunized with anti-EEA1 antibodies developed the neurological features of disease symptoms, the mean performance score on each test would be less obvious than if individual values would be compared.

The tests of forelimb strength and grid walk were used to evaluate deficits in descending motor control and global muscular tone. Although it is possible that antibodies to EEA1 were responsible for these neurological deficits, it is appreciated that the co-morbid variables that combine to express a disease state might not be the simple result of a

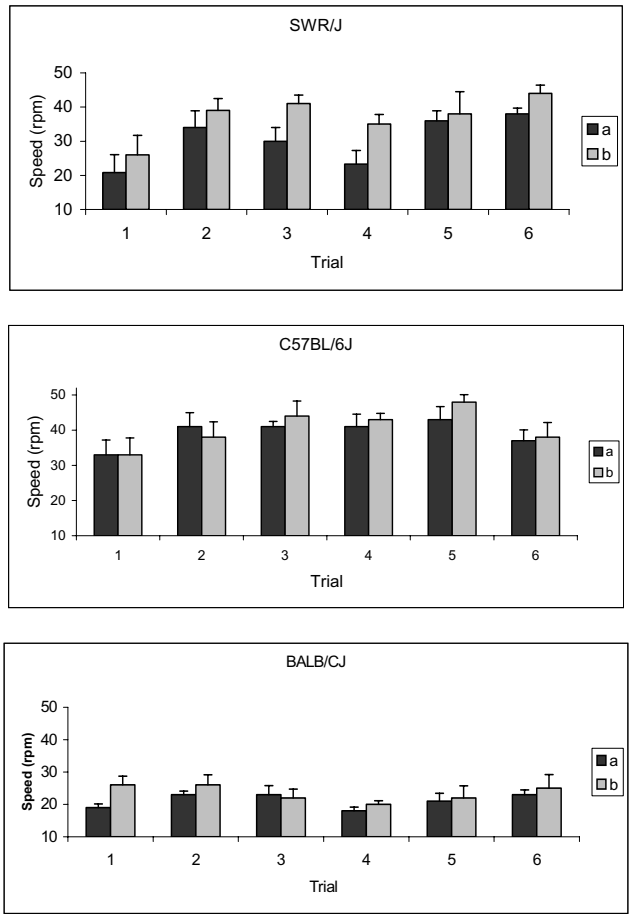

Figure 7
The rotational speed of the rotating drum at which mice SWR/J, C57BL/6J and BALB/CJ mice fell off was recorded for the immunized experimental (a) and non-immune control (b) groups. Although SWR/J mice did show a significant improvement on subsequent trials, statistically significant differences were not observed in these groups of mice.

immunization with an autoantigen. Genetic, environmental and hormonal influences are known to operate in concert to result in pathogenesis and expression of disease features [2,14,15]. For example, experimental autoimmune encephalitis, which shows pathological similarities to human multiple sclerosis, can be induced in SJL mice by immunization with myelin basic protein (MBP) [16], whereas BALB/C mice are resistant to development of the disease, despite generation of highly MBP-specific T cell clones that recognize MBP peptides presented in the complex with MHC class II molecules [17,18]. Of interest, the different genetic strains of mice used in these studies demonstrated different quantitative responses to the EEA1 immunogen. Notably, BALB/C mice had significantly higher levels of anti-EEA1 than the other strains and they exhibited the most remarkable abnormalities in testing performance.

It is difficult to induce readily detectable pathological changes in animals comparable to those observed in human disease. For example, C57BL/6 mice are used as an animal model of MG, however multiple immunizations with acetylcholine receptor in complete Freund's adjuvant were required to induce signs of muscular weakness which can be detected in only 20%-60% of the animals [19]. This was in contrast to a study that showed pathogenic autoimmunity to affinity-purified mouse acetylcholine receptor that was induced without adjuvant in BALB/c mice [20]. In our mice the abnormalities were observed after an initial immunization of EEA1 in Freund's adjuvant followed by a single booster immunization. It is possible that more profound deficits could be induced by repeated immunization and the induction of higher levels of anti-EEA1 responses. In addition, there is the possibility that the immune response of mice to the human recombinant antigen may produce antibodies which differ in specificity and perhaps pathological effect compared to the human autoantibodies. To address this issue, epitope mapping of the antibodies produced in mice and comparison to epitopes bound by naturally occurring autoantibodies in humans [7] may be informative.

EEA1 is expressed in a number of tissues [21] and is polarized in hippocampal neurons, epithelial cells and fibroblasts [10]. One of the challenges in understanding the role of autoantibodies in the pathogenesis of many diseases is to consider mechanisms other than traditional antibody-mediated mechanisms (i.e. anti-acetylcholine antibodies in myasthenia gravis) or immune complex formation (i.e. anti-DNA antibodies in lupus glomerulonephritis). An interesting departure from these traditional pathogenetic mechanisms is the observation that anti-mitochondrial antibodies (AMA), the serological hallmark of primary biliary cirrhosis [22], do not directly cause hepatocyte or bile duct damage but when antigen-presenting dendritic cells that were pulsed with antigen bound to the cognate autoantibody there was an increase of cytokines that may then mediate inflammation and cytotoxicity [23]. This is an important model and may have relevance to anti-EEA1 because, just like EEA1, the targets of AMA (i.e. pyruvate dehydrogenase complex) are ubiquitous in many cells and tissues, but the pathogenic expression of disease is almost exclusively restricted to the bile ducts.

Conclusions

Studies of mice immunized with recombinant EEA1 produced high titer antibodies and demonstrated behavioural deficits. Although the behavioral tests provided

valuable information about the potential pathological role of EEA1 antibodies, additional studies such as motor function, nerve conduction studies, T cell proliferative responses to EEA1 and immunohistological analysis would help prove that these antibodies autoreactive lymphocytes directly or indirectly participate in causing features of neurological disease. In addition, passive transfer of sera could also be conducted before more clear-cut conclusions can be drawn.

Methods

Mice strains

Since genetic factors, particularly the major histocompatability (MHC) locus, are known to play a significant role in expression of autoimmune phenomena, three strains of female mice (SWR/J, C57BL/6J and BALB/cJ, 12 of each strain) from different MHC haplotype ($H2^q$, $H2^b$ and $H2^d$) backgrounds were chosen for this study. All mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at three weeks of age and housed under standard housing and feeding regimens in infection controlled facilities in the Health Sciences Animal Resource Centre at the University of Calgary. All studies adhered to guidelines for animal studies developed by the Canadian Council on Animal Care.

Purification of EEA1 recombinant protein

EEA1-cDNA encoding a partial-length EEA1 protein ($EEA1_{82-1411}$) and characterized in our previous study [6] was subcloned into a glutatione-S-transferase (GST)-based vector (pGex6P1) and expressed and purified as a GST-fusion protein in a protease-deficient AB1899 *E. coli* strain using techniques essentially as previously published [7].

Immunization

Mice of each strain were divided into two groups (six in each group). One group (experimental group) received an intraperitoneal injection of 100 μg of the purified EEA1 recombinant protein in an equal volume of complete Freund's adjuvant. The other group (control) received an intraperitoneal injection of the GST cleavage buffer combined with an equal volume of complete Freund's adjuvant. Two weeks later, the mice were boosted with a subcutaneous injection of either 50 μg of the EEA1 protein in incomplete Freund's adjuvant, or an equal volume of GST cleavage buffer in incomplete Freund's adjuvant. Blood was obtained by retro-orbital collection in capillary tubes. The appearance and titer of EEA1 antibodies was monitored for two months by indirect immunofluorescence (IIF) of the collected sera on HEp-2 cells (HEp-2000; Immuno Concepts Inc., Sacramento, CA) using Alexa 488-conjugated goat anti-mouse IgG (H+L chain) antibody (Jackson ImmunoResearch Laboratories Inc). Co-localization of mouse antibodies with an index human anti-EEA1 antibody was performed using Cy3-conjugated goat anti-human IgG (H+L chain) (Molecular Probes, Eugene, OR) or the Alexa 488-conjugated goat anti-mouse IgG (H+L chain) antibody.

Laser bead immunoassay

Antibodies to EEA1 were detected and quantitated by using a set of addressable beads bearing laser reactive dyes (Luminex Corp., Austin, TX) that were covalently coupled to purified recombinant EEA1 protein as previously described [7]. To analyze reactivity with EEA1, mouse sera were diluted in QUANTA Plex™ sample diluent (INOVA, San Diego, CA) to a final dilution of 1/1000. To each well 40 μl of bead stock (1 part microspheres in blocking buffer to 40 parts QUANTA Plex sample diluent) and 10 μl of diluted mouse sera were added and incubated for 30 minutes on an orbital shaker. Then fifty μl of phycoerythrin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 1/50 was added to each well and incubated on the orbital shaker for an additional 30 minutes. The reactivity of the antigen-coated beads was determined on a Luminex 100™ dual laser flow cytometer (Luminex Corp.). The reactivity of antibodies in the assay was expressed as the median fluorescent intensity. A control monoclonal anti-EEA1 (CytoStore - Calgary, AB) and pre-immune mouse sera were included in the assay. The binding was compared to binding of an irrelevant monoclonal antibody (golgin97: CytoStore, Calgary, AB) as a negative control.

Design of the behavioral tests

The motor assessments were conducted at the facilities of NeuroDetective Inc., Lethbridge, Alberta http://www.neurod.com/behavior_w.htm[13]. The experimenters and behavioral analysts were blinded to mouse strain and immunized versus non-immunized control status. Thirty-six female mice (18 control and 18 experimental) were evaluated for functional motor assessment. The mice belong to 3 different strains with treated and controls within each strain (n = 6). The assessments included open field activity, skilled reaching, grid walking, strength, and rotarod walking. All behavioral experiments and study sessions were recorded on videotape. The tests did not measure behavioural motor deficits.

Open field testing was designed to evaluate hippocampal and basal ganglia damage, and hindlimb dysfunction. Each mouse was placed into an open field for 20 minutes (divided into 4 min bins for analysis). The distance traveled and the total number of supported (rearing while bracing against a wall with one or both forelimbs) and unsupported (rearing without bracing with the forelimbs) rears was counted. In the open-field test, mice had the opportunity toexplore a square-shaped arena for a fixed time. The dependent variablesrecorded were locomotor

activity and the balance between exploration and agoraphobia.

Reaching was a test that aimed to evaluate forelimb motor control and was sensitive to deficits in dopaminergic neurotransmission. The apparatus consisted of a 16 × 24 cm Plexiglas cage, one side of which consisted of vertical bars that were spaced with gaps between them that were just large enough for a mouse to reach its forelimb through to a food tray positioned just outside the bars (5 mm). The food tray contained sweetened pellets. Over the 5 days of conditioning the mice learned to retrieve food pellets from the tray by reaching their forepaw through the vertical bars. On the sixth day, individual animals were placed in the apparatus and reaching was videotaped for 5 consecutive minutes (beginning with the first reaching attempt). The total number of attempted reaches and the number of "hits" (defined as successful retrieval of a pellet) were analyzed and recorded.

Grid walking was sensitive to deficits in descending motor control. The grid walking apparatus consisted of two plastic panels 1 m long and 25 cm wide (5 mm thick) with holes drilled 1 cm apart along one long edge. The panels were placed 2.5 cm apart and connected via several metal bars (3 mm diameter) through the holes. The bars were randomly placed 1, 2, or 3 cm apart. The apparatus was suspended and oriented such that a narrow alley was formed 1 m long with walls 25 cm high. The grid bars formed the floor. The animals were individually placed at one end of the apparatus and filmed from the side as they walked across the bars to the opposite end. The number of forelimb and hind limb placement errors over the final 50 cm was analyzed. An error was counted whenever a limb missed a bar and extended through the horizontal plane of the grid floor.

Forelimb strength was assessed using a Grip Strength apparatus. Each mouse was held near a vertically oriented apparatus such that it grabbed with the forelimbs a handle that was fastened to a calibrated spring. Consistent and increasing pressure was applied by pulling the mouse downward until contact with the handle was broken. The distance the mouse pulled the handle was recorded as the strength score.

Rotarod was a test of sensorimotor coordination and was sensitive to damage in the basal ganglia and the cerebellum. All mice were trained to walk on a rotating drum (6 sessions over 2 days). During each test session the drum was rotated at 10 rpm for 30 s followed by 5 rpm increases every 10 s to a maximum of 45 rpm. The rotation speed at which the mouse fell from the rotating drum was recorded.

Statistics

A repeated measures analysis of variance (ANOVA) and unpaired *t*-tests were performed on grouped data for each mouse strain and for each of the behavioral tests.

Abbreviations

EDC, 1-ethyl-3-(3-dimethylaminopropyl) carboiimide hydrochloride; EEA1, early endosome antigen 1; GST, glutatione-S-transferase: IIF, indirect immunofluorescence; MBP, myelin basic protein; MG, myasthenia gravis; MHC, major histocompatability; PBS, phosphate buffered saline.

Author's contributions

SS carried out the immunizations, serum preparation and immunoassays. MF conceived of the study, and participated in its design and coordination participated in the design of the study and performed the statistical analysis. All authors were involved in writing the manuscript and have read and approved the final manuscript.

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