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Obtaining anti-type 1 melatonin receptor antibodies by immunization with melatonin receptor-expressing cells

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ABSTRACT

Antibodies (Abs) specific to cell-surface receptors are attractive tools for studying the physiological role of such receptors or for controlling their activity. We sought to obtain such antibodies against the type 1 receptor for melatonin (MT_1). For this, we injected mice with CHO cells transfected with a plasmid encoding human MT_1 (CHO-MT_1-h), in the presence or absence of an adjuvant mixture containing Alum and CpG1018. As we previously observed that the immune response to a protein antigen is increased when it is coupled to a fusion protein, called ZZTat101, we also investigated if the association of ZZTat101 with CHO-MT_1-h cells provides an immunogenic advantage. We measured similar levels of anti-CHO and anti-MT_1-h Ab responses in animals injected with either CHO-MT_1-h cells or ZZTat101/CHO-MT_1-h cells, with or without adjuvant, indicating that neither the adjuvant mixture nor ZZTat101 increased the anti-cell immune response. Then, we investigated whether the antisera also recognized murine MT_1 (MT_1-m). Using cloned CHO cells transfected with a plasmid encoding MT_1-m, we found that antisera raised against CHO-MT_1-h cells also bound the mouse receptor. Altogether our studies indicate that immunizing approaches based on MT_1-h-expressing CHO cells allow the production of polyclonal antibodies against MT1 receptors of different origins. This paves the way to preparation of MT_1-specific monoclonal antibodies.

1. Introduction

Melatonin receptors (MTRs) are membrane proteins belonging to the G protein-coupled receptor (GPCR) family (Ebisawa et al., 1994; Reppert et al., 1994; Roca et al., 1996). Many studies of MTRs have sought to characterize their physiological role or to regulate their activity (Cos and Fernandez, 2000; Abbas et al., 2010; Pévet, 2003; Boutin et al., 2005; Pandi-Perumal et al., 2008; Devavry et al., 2012), often by seeking new ligands that can help to specify receptor localization in various tissues or which have agonist or antagonist characteristics.

Numerous MTR ligands have been identified by conventional screening of small chemical molecules or by high-throughput screening of very large compound libraries (Audinot et al., 2003; Audinot et al., 2008; Yan et al., 2008). Antibodies (Abs) might represent attractive alternatives to such chemical molecules because they have a longer half-life and are well suited to biodistribution studies (Hutchings et al., 2010). In addition, they may be directed against different epitopes of the target, which can give them agonist or antagonist characteristics (Allard et al., 2013; Kozer et al., 2011). As the extracellular loops of GPCRs may contain epitopes accessible to recognition by B-lymphocytes, specific anti-GPCR Abs can be raised using peptides with sequences corresponding to the extracellular loops. However, linear peptides rarely accurately mimic the conformation that the same sequence has within the whole protein (Cuniasse et al., 1995) and often raise Abs with low affinity for the native protein (Léonetti et al., 1990, 1995), which might explain the low number of functional Abs raised against GPCRs (Hutchings et al., 2010). Another strategy is to use the full-length protein derived by solubilization of the plasma membrane or by using recombinant approaches (Freson et al., 2008; Hutchings et al., 2010). However, this approach has yielded variable results, probably because of the difficulty of maintaining the three-dimensional structure of the protein outside its membrane environment. To overcome these drawbacks, one approach is based on immunization with cells transfected with a plasmid encoding the receptor sequence (Goetzl et al., 2004; Ohno et al., 2008; Allard et al., 2013). In this approach the receptor expressed at the cell membrane has a native conformation, which is particularly suitable for the preparation of Abs with high affinity. We chose to use this approach to raise Abs specific to type 1 melatonin receptor (MT_1).

To induce an anti-MT_1 humoral response, we used CHO cells transfected with a plasmid encoding human MT_1, called CHO-MT_1-h. In addition, we assessed whether the Ab response is enhanced when

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cells are first coupled to a fusion protein containing the transcriptional transactivator of HIV-1, called Tat101, and a double Ig-binding domain derived from protein A of *Staphylococcus aureus*, called ZZ. We used this recombinant protein, called ZZTat101, since the Ag-specific Ab-response is increased when the immunizing Ag is previously coupled to Tat (Gadzinski et al., 2012) or ZZTat101 (Ait Mebarek et al., 2013, and Knittel et al. submitted). Lastly, we examined whether the Abs raised against MT_1-h can recognize mouse MT_1 (MT_1-m), the extracellular loop sequences of which differ by 25.3% from those found in humans.

2. Materials and methods

2.1. Cells and antigens

CHO and HEK cells were kindly provided by Servier Inc. (France). The human and mouse MT1 receptors were fused in their C-terminal region to a M2 Tag (DYKDDDDK sequence). The CHO-K1 cell line stably expressing the human MT1 receptor (Audinot et al., 2003) was grown in HAM-F12 supplemented with 10% FCS, 1% GlutaMAX™, and 400 μg/mL Geneticin®. Wild-type (WT) CHO cells were grown in HAM-F12 supplemented with 10% FCS and 1% GlutaMAX™. CHO-MT_1-m cells were grown in Kaighn's F12 supplemented with 10% FCS, 1% GlutaMAX™, 600 μg/mL Geneticin®, and 10 μg/mL puromycin. WT CHO cells used for MT_1-m transfection were grown in Kaighn's F12 supplemented with 10% FCS, 1% GlutaMAX™, and 600 μg/mL hygromycin. HEK-MT_1-m cells were grown in DMEM supplemented with 10% FCS, 1% sodium pyruvate, 400 μg/mL Geneticin®, 1 μg/mL puromycin, and 1% GlutaMAX™. WT HEK cells were grown in DMEM supplemented with 10% FCS, 100 μg/mL hygromycin, and 1% GlutaMAX™.

The HEK293 cell line expressing MT1 (Hassaine et al., 2013) was grown in DMEM/F-12 medium supplemented with 10% FBS, 4 mM GlutaMAX™, 10 mg/mL blasticidin, and 200 mg/mL hygromycin.

The ZZTat101 fusion protein was prepared using recombinant technology as previously described (Ait Mebarek et al., 2013). Briefly, Tat101 (101 residues, MW = 11,507 Da) was designed using a synthetic sequence encoding Tat101. This sequence was ligated in a *KpnI*/*BamHI*-opened pCP vector (Drevet et al., 1997) coding for ZZ (MW = 16,752 Da). The recombinant protein (MW = 26,409) was produced in *Escherichia coli* BL21(DE3)plysS after IPTG induction. Bacteria were mechanically lysed in the presence of AEBSF protease inhibitor (Pefabloc, Sigma). ZZTat101 was purified by 20-min incubation at 4 °C in NaCl (2 M) and protamine sulphate (2 mg/mL) followed by centrifugation to remove nucleic acids. Then, the samples were dialyzed in PBS-Tween 20 (0.1%) and loaded on an affinity column grafted with Abs (IgG Sepharose 6Fast flow #17–0969-02 Amersham) to purify the fusion protein using the Ig-binding ability of its ZZ-moiety. The bound protein was eluted from the column using 5 mM acetic acid pH 3.4 and kept lyophilized until use.

2.2. Animals and immunizations

C57BL/6J mice were from Charles River France. The care and use of animals strictly followed European and national regulations for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (facility licence #C75-05-18). Care and use of animals also complied with internationally established principles of replacement, reduction and refinement in accordance with the Guide for the Care and Use of Laboratory Animals. Mice were immunized intraperitoneally four times at 14-day intervals with 100 μL of a mixture containing $1.5 \cdot 10^6$ to $4.5 \cdot 10^6$ of either CHO-MT_1-h or ZZTat101/CHO-MT_1-h cells (50 μL) previously mixed with 50 μL of either PBS or aluminium hydroxide (alum, Serva) and CpG (40 μg/mouse) (CpG1018, Trilink) adjuvants. Mice were bled before immunization and two weeks after the second (S1), third (S2) and fourth (S3) immunizations.

2.3. Assessment of MT_1 expression by Western blot

One million cells were lysed using Triton (0.15%) and the total amount of protein was measured using a micro-BCA assay. Then, 10 μg of protein was added to a polyacrylamide gel (12%). After electrophoretic migration, proteins were transferred to a nitrocellulose membrane. The membrane was saturated with a PBS/0.1% Tween/0.5% milk mixture and incubated with a mouse anti-FLAG Ab. Ab binding was revealed using a goat anti-mouse IgG Ab coupled to peroxidase (1/500 dilution) and Luminata Forte (Millipore) substrate.

2.4. Cloning of CHO cells transfected with a plasmid encoding murine MT_1-m

CHO-MT1-m transfected cells were cloned by limiting dilution (0.3 cells/well) in 96-well microtiter plates using culture medium (Kaighn's F12, 10% FCS, 1% GlutaMAX™, 600 μg/mL Geneticin®, 10 μg/mL puromycin). Approximately 21 days later, clusters of growing cells were found and transferred to 6-well plates. Cells were collected at 90% confluence to assess MT_1-m expression by Western blot.

2.5. Association of ZZTat101 with CHO-MT_1-h cells

CHO-MT1-h cells (10^5 cells) were incubated with ZZTat101 or ZZ (5 μg/10^5 cells) for 30 min at 4 °C in 200 μL of a PBS mixture containing BSA (0.1%) and horse normal serum (5%). Then, cells were washed and incubated with non-immune rabbit IgGs (1 μg). After one hour, $F(ab')_2$ goat anti-rabbit IgG coupled to FITC was added. One hour later, cells were washed and analysed by flow cytometry using a Guava apparatus (Millipore, France).

2.6. Assessment of Ab response using cell-binding assays

Preimmune and immune sera were diluted (100 μL at 1/100) and incubated in the presence of 10^5/100 μL cells (WT CHO, HEK, HEK-MT_1, or CHO-MT_1-m) for 30 min at 4 °C in PBS-0.5% BSA. Cells were washed twice and incubated for 30 min with a fluorescein-labelled donkey anti-mouse $F(ab')_2$ (Jackson Immunoresearch, UK). Cells were washed and binding to cells was assessed by flow cytometry using a Guava cytometer. Data were analysed using Guava Express Pro software. For each group of immune sera, Ab binding to MT1 was determined by dividing their mean binding to MT1-cells by their mean binding to wild-type cells. For each group of preimmune and immune sera ability to bind cells was determined by dividing the mean binding of immune sera by the mean binding of preimmune sera.

3. Results

3.1. Coupling of ZZTat101 to CHO-MT_1-h transfected cells

As we have previously shown that Tat increases the immune response against soluble protein antigens (Ags) (Gadzinski et al., 2012) and that ZZTat101 has the same behaviour in vitro (Ait Mebarek et al., 2013) and in vivo (Knittel et al. submitted), we assessed whether the immune response raised against CHO-MT_1-h cells is increased when they are associated beforehand with the fusion protein. For this, we first produced ZZTat101 using a recombinant approach. Then, we took advantage of the ability of Tat to bind heparan sulphate proteoglycan (HSPG) and of the large amounts of HSPG on the surface of CHO cells (Léonetti et al., 2010) to form complexes between ZZTat101 and CHO-MT_1-h cells. We assessed formation of these complexes by cytometry. For this, we used the capacity of the ZZ moiety to bind the Fc portion of IgGs (Léonetti et al., 1998) and, in particular, rabbit IgGs. We revealed CHO/ZZTat/rabbit-IgG interaction using a $F(ab')_2$ anti-rabbit IgG coupled to FITC. As shown in Fig. 1, fluorescence increased when cells were pre-incubated with ZZTat101, which reflects binding of the fusion