Video Article

Preparation of Mouse Pituitary Immunogen for the Induction of Experimental Autoimmune Hypophysitis

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Abstract

Autoimmune hypophysitis is a chronic inflammation of the pituitary gland caused or accompanied by autoimmunity[1]. It has traditionally been considered a rare disease but reporting has increased markedly in recent years. Hypophysitis, in fact, develops not uncommonly as a "side effect" in cancer patients treated with antibodies that block inhibitory receptors expressed on T lymphocytes, such as CTLA-4[2] and PD-1 receptors. Autoimmune hypophysitis can be induced experimentally by injecting mice with pituitary proteins mixed with an adjuvant[3]. In this video article we demonstrate how to extract proteins from mouse pituitary glands and how to prepare them in a form suitable for inducing autoimmune hypophysitis in SJL mice.

Video Link

The video component of this article can be found at https://www.jove.com/video/2181/

Protocol

Experimental Protocol

The first step in this experimental protocol is to collect a large number of mouse pituitary glands. The second step is to homogenize the glands to prepare a protein extract. The third step is to emulsify these proteins with an oily mixture.

Step 1: Collection and storage of mouse pituitary glands

The mouse pituitary gland sits at the base of the skull in a depression of the sphenoid bone called the sella turcica, surrounded laterally by the trigeminal nerves and anteriorly by the optic chiasm. The gland is composed of a larger anterior lobe and smaller posterior and intermediate lobes, and weighs on average 1.9 mg. Pituitary glands are collected from mice of mixed strains, sexes, and ages, destined to be euthanized by the animal care facility.

To isolate the pituitary gland, the mouse is euthanized, the skin above the skull dissected, and the skull bone cut open with scissors. The brain is lifted to expose the pituitary gland, which is then isolated from the surrounding dura mater, scooped out of the sphenoid bone and stored in a plastic tube on dry ice.

In general we use 300 mouse pituitary glands for one protein preparation, a number that can be collected by one person in 9 hours.

Once the collection is completed, the tube containing the pituitary glands is stored at -80 °C until ready for the next step.

Step 2. Extraction of mouse pituitary proteins

1. Add the homogenization buffer to a 15-ml Falcon tube (catalog no 352059) kept in an ice bath, using 250 µL of buffer for every 100 pituitary glands collected. Use a buffer of ionic strength similar to the one that is physiologically present inside the cytoplasm of the cell (0.2 M) and of physiologic pH (7.4). We use phosphate buffered saline (0.161 M, pH 7.4), supplemented with a protease inhibitor cocktail (Sigma-Aldrich, catalog no P8340-1ML).
2. Add the pituitary glands to the buffer just before homogenization, and then place the generator (5-mm diameter) of the Polytron homogenizer at the bottom of the Falcon tube. Homogenize at maximum rpm for 30 seconds, gently moving the Falcon tube up and down while keeping it in the ice bath. Then, rest the homogenate on ice for 1 min. Repeat the homogenization and cooling steps two more times. This gentle homogenization should break the cells and release cytosolic proteins without disrupting the nuclei.

3. To remove nuclei, unbroken tissue and insoluble materials (such as connective tissue and denatured protein complexes), centrifuge the homogenate at low speed (1000g) for 10 min at 4 °C.
4. Transfer the supernatant (now called Post-Nuclear Supernatant, PNS) to a new Falcon tube, taking care to leave the pellet undisturbed, and store the PNS on ice.
5. Resuspend the pellet in the original volume of homogenization buffer (250 µL of buffer for every 100 pituitary glands collected), homogenize as above, centrifuge as above, and then combine the second PNS with the first one.
6. Aliquot the two pooled PNS samples into microcentrifuge tubes and store at -80 °C until ready for the next step. This protein preparation can be stored at -80 °C but gradually looses its potency, so we recommend to use it within 6 months from the preparation. Use a small aliquot to determine the protein yield and concentration by the BCA assay (Pierce catalog no. 23225), and the protein quality by gel electrophoresis. In general, we obtain about 60 mg of protein from 300 mouse pituitaries in 1.5 mL of homogenization buffer, at a concentration around 40 mg/ mL.

Step 3. Preparation of the emulsion for immunization

1. Thaw rapidly the frozen protein aliquots needed for the experiment and adjust the protein concentration to 20 mg/ mL. This protein extract will be emulsified 1:1 with Complete Freund's Adjuvant (CFA, Sigma F-5881) containing 5 mg/ mL of heat killed Mycobacterium tuberculosis (Becton Dickinson, 231141). Each mouse will receive 1 mg of pituitary extract in 100 µL of emulsion.
2. The example given here is for immunizing 10 mice, where each mouse will be injected with 100 µL of the emulsion containing 1 mg of pituitary proteins. Allow two extra mice for material loss during the preparation, so use 12 mg of pituitary proteins. Aspire 600 µL of the 20 mg/ mL pituitary protein extract describe above using a 2.5 mL Hamilton gastight syringe. Resuspend the CFA by shaking, and aspire 600 µL of CFA into another 2.5 mL Hamilton syringe. Attach and secure a 22-gauge micro-emulsifying needle to the syringe filled with CFA. Slowly push the plunger of the CFA syringe such that the needle is filled with CFA, then attach and secure the other end of the needle to the syringe filled with pituitary extract.
3. Slowly advance the plunger of the CFA syringe to push the oil component into the acqueous pituitary extract, such that only a small amount is mixed. Then push back the plunger of the pituitary extract syringe into the CFA syringe. Repeat this action gradually increasing the amount of material mixed. Continue until the entire content of the two syringes are mixed. You will obtain a whitish and evenly distributed solution that represents the water-in-oil emulsion.
4. Once the emulsion is formed, repeat the back-and-forth motion at least 500 times to ensure the emulsion is thoroughly mixed. After the final mixing cycle, the 1:1 water-in-oil emulsion is ready to use and contains a pituitary protein concentration of 10 mg/mL. The emulsion will be injected the same day subcutaneously into anesthetized SJL mice, as detailed in the companion JoVE article.

Discussion

Despite the fact that the first patient with autoimmune hypophysitis was reported in 1962[4], the pathogenic pituitary autoantigens remain to be identified[5]. Animal models can be very useful to identify these autoantigens, aiding the discovery of biomarkers that are translatable to patient care. This article has demonstrated a straightforward procedure for preparing pituitary proteins in a form that is adequate for inducing autoimmune hypophysitis in experimental animals.

Disclosures

No conflicts of interest declared.

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