Veterinary Immunology and Immunopathology 149 (2012) 1–5

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Flow cytometric determination of allergen-specific T lymphocyte proliferation from whole blood in experimentally asthmatic cats

Carol R. Reinero*, Hong Liu, Chee-Hoon Chang

Department of Veterinary Medicine and Surgery and the Comparative Internal Medicine Laboratory, College of Veterinary Medicine, University of Missouri, 900 East Campus Drive, Columbia, MO 65211, USA

ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form 30 April 2012
Accepted 10 May 2012

Keywords:
Clinical immunology
Antigen recall
Allergy
Mitogen

ABSTRACT

The ability to quantify feline lymphocyte proliferation, especially to specific antigen or allergen, would be valuable in experimental models and naturally developing disease where activated lymphocytes drive immune responses. Traditional proliferation assays may pose radioactivity hazards, lack the ability to distinguish viable from non-viable cells, and cannot discriminate individual populations of proliferating lymphocytes (e.g., the CD4+ T cell class). We hypothesized that in an experimental model of feline allergic asthma a four-color flow cytometric assay capable of simultaneously detecting division, viability and cell surface markers (pan T cell marker CD5 or CD4) would allow characterization of lymphocytes stimulated ex vivo using the sensitizing allergen, Bermuda grass (BGA).

Peripheral blood mononuclear cells were harvested from eight experimentally asthmatic cats to validate and optimize use of a cell proliferation dye or bromodeoxyuridine (BrdU) with BGA-specific stimulation in a lymphocyte proliferation flow cytometric assay. Only the latter reagent was suitable in the cat. After a 3 day incubation, antibodies with different fluorochromes were used to identify BrdU, viable cells, CD5 and CD4 for subsequent flow cytometric analysis. In asthmatic cats, the group mean ± SEM of proliferating CD5+ lymphocytes was 2.3 ± 0.5%. The group mean ± SEM of proliferating CD4+ lymphocytes was 1.2 ± 0.3%. Flow cytometry is a sensitive method for detecting simultaneous proliferation and viability of very minor populations of allergen-specific lymphocytes in experimentally asthmatic cats.

1. Introduction

Determination of lymphocyte proliferation to mitogens, specific antigen or specific allergen is applicable in both research and clinical settings for the study of congenital and acquired immune defects and response to therapeutics as it reflects the ability of lymphocytes to respond to external signals. Established techniques for lymphocyte proliferation in the cat use incorporation of a radioactive nucleoside ([^{3}H]thymidine) into cellular DNA (Than et al., 1982) or, to avoid radioactivity, commercially available ELISA kits can be used which incorporate bromodeoxyuridine (BrdU) with a chromogenic reaction (Reinero et al., 2006). Radioactive assays, while having the advantage of high sensitivity, are a biological hazard requiring special licensure and secured facilities. The latter technique, while convenient and suitable for use with mitogens which broadly activate the majority of lymphocytes in a non-specific manner, are relatively insensitive in comparison for identifying antigen/allergen-specific cells which constitute the minority of the T cell population. Additionally, neither of these radioactive nor non-radioactive assays can simultaneously discriminate proliferative responses of individual lymphocyte populations—e.g. T cell vs B cell, or CD4+ vs CD8+ T cells. An assay which is highly sensitive, non-radioactive, and capable of discriminating individual lymphocyte

* Corresponding author. Tel.: +1 573 882 7821; fax: +1 573 884 5444.
E-mail address: reineroc@missouri.edu (C.R. Reinero).

0165-2427/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetimm.2012.05.014

populations in the cat would be highly valuable for feline immunology research. Flow cytometric assays using stains which bind to cellular DNA and either divide providing half of the stain to daughter cells (e.g., carboxyfluorescein diacetate succinimidyl ester (CSFE) or cell proliferation dye efluor® (CPD)) or incorporate a pulsed marker (BrdU, a pyrimidine analogue) into dividing cells have been successfully used in other species. Additionally, flow cytometry can be used to identify viable cells using other markers, providing additional immunologic information.

Allergic asthma is a common bronchopulmonary disorder in cats; this species can serve as an excellent pre-clinical model to evaluate immunopathogenic mechanisms and novel therapeutics for both cats and humans. In susceptible individuals with appropriate genetic and environmental contributions, inhalation of aeroallergens triggers T helper 2 (Th2) lymphocytes to orchestrate airway inflammation, airway hyperresponsiveness and airway remodeling. Understanding more about how allergen-specific lymphocyte proliferation can be modulated to attenuate the allergic inflammatory cascade would be beneficial. We hypothesized that in an experimental model of feline allergic asthma a polychromatic flow cytometric assay capable of simultaneously detecting division, viability and relevant cell surface markers (pan T cell marker CD5 or CD4) would allow characterization of lymphocytes stimulated ex vivo using the sensitizing allergen, Bermuda grass.

2. Materials and methods

2.1. Animals

Eight purpose bred male one year old cats were obtained from a commercial vendor (Liberty Research, Waverly, New York) for use in this study. The study was conducted with the approval of the University of Missouri Animal Care and Use Committee and cats were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Allergen sensitization and challenge

Eight cats were sensitized and challenged with Bermuda grass allergen (BGA) to induce an asthmatic phenotype. Allergen sensitization was performed as previously described (Reinero et al., 2011). Briefly, cats were initially evaluated by intradermal skin test (IDST) on day 0 to ensure that they were naive to the allergens of interest; they also had collection of bronchoalveolar lavage fluid (BALF) to confirm absence of preexisting eosinophilic airway inflammation. Cats then received subcutaneous injections of BGA (12 μg of BGA in 10 mg of alum) and 100 ng *Bordetella pertussis* toxin (Sigma–Aldrich, St. Louis, MO). On day 14, the cats were administered an intranasal solution containing 75 μg of BGA in 0.2 ml PBS. Subcutaneous injections of BGA in alum were repeated on day 21. On day 28, IDST confirmed reactivity to BGA and cats subsequently received local delivery of BGA to the airways using a series of 7 aerosol challenges with BGA over the next 2 weeks. Twenty four hours after the last allergen challenge, BALF was collected to confirm induction of asthma (mean ± SEM BALF % eosinophils, 64 ± 8%). Weekly aerosol challenges were performed thereafter to maintain a chronic asthmatic phenotype.

2.3. Collection of peripheral blood mononuclear cells

Whole blood was collected by jugular venipuncture into sodium heparinized or sodium EDTA tubes and processed within one hour of collection. Assay optimization included harvesting PBMCs by density gradient centrifugation using either Percoll® (P4937 Sigma–Aldrich, St. Louis, MO, 43% and 62.5% layers) or histopaque® 1077 (density 1.077, Sigma–Aldrich, St. Louis, MO). Results were similar for both anticoagulants and types of density gradient media (data not shown); sodium EDTA and histopaque were used in the final assay protocol.

2.4. Markers to track cell proliferation

A series of experiments were performed to optimize a commercially available cell proliferation dye (CPD efluor® 670, eBioscience, San Diego, CA) that binds cellular proteins containing primary amines and distributes the dye equally to daughter cells upon division. Cell concentrations of 1×10^5, 5×10^5, and 1×10^6 were used, and the CPD efluor® 670 was added to washed PBMCs at concentrations of 2.5 μM, 5.0 μM and 7.5 μM. Cells were incubated for 10 min at 37 °C in the dark and labeling was stopped by addition of 5 volumes of cold complete RPMI (500 ml cRPMI; RPMI 1640 with 10% FBS, 5 ml 1 M Hepes, 0.35 μl diluted beta-mercaptoethanol, 5 ml penicillin-streptomycin-glutamine). Cells were washed 3 times with cRPMI and transferred in triplicate to 96 well plates (#3595, Costar, Corning, Inc., Corning, NY). Pilot studies used concanavalin A (con A) at 5 μg/ml as a mitogen which induces T cell proliferation in a much larger percentage of T cells than BGA. Plates were incubated at 37 °C in humidified 5% CO_2/95% air (Hera Cell 150, Kendro Laboratory Products, Langenselbold, Germany) for 3 days prior to labeling cells for flow cytometric analysis (see below).

The second marker of cell proliferation evaluated was 5-bromo-2′-deoxyurindine (BrdU B5002, Sigma–Aldrich, St. Louis, MO) followed by labeling with an anti-BrdU FITC antibody (clone PRB-1, eBioscience, San Diego, CA). Of note, this clone has since been discontinued, but the alternative clone BU20A (eBioscience, San Diego, CA) works similarly. After harvesting and washing PBMCs, cells were resuspended in cRPMI and incubated at a concentration of 2×10^5 cells/well in triplicate in a 96 well plate (#3595, Costar, Corning, Inc., Corning, NY). The cell concentration was optimized to be 2×10^5 cells/well (range, 1×10^5–1×10^6 cells/well; data not shown) in a final volume of 200 μl/well. Initially con A (5 μg/ml) was used as a mitogen with a 3 day incubation; subsequently BGA (25 or 50 μg/ml) was used as an allergen-specific proliferative stimulus with a 7 day incubation (compared with a 5 day incubation, data not shown) inclusive of a media change at the beginning of day 4. For each well with conA or BGA, there was an appropriate control well with cells in cRPMI alone to allow analysis of stimulated compared with unstimulated cells in the following flow cytometric assay. For cells incubated in conA, BrdU was added at a final