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Analyzing Cellular Immunity to AAV in a Canine Model Using ELISpot Assay

Zejing Wang[1], **Rainer Storb**[1,2], **Stephen J Tapscott**[3,4], and **Stanley Riddell**[2,5]

[1]Program in Transplantation Biology, Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA 98109

[2]Department of Medicine, University of Washington, Seattle, WA 98195

[3]Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109

[4]Department of Neurology, University of Washington, Seattle, WA 98195

[5]Program in Immunology, Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA 98109

Abstract

Adeno-associated viral vector (AAV) mediated gene transfer represents a promising gene replacement strategy for treating various genetic diseases. One obstacle in using viral-derived vectors for *in vivo* gene delivery is the development of host immune responses to the vector. Recent studies have demonstrated cellular immune responses specific to capsid proteins of various AAV serotypes in animal models and in human trials for different diseases. We developed a canine specific ELISpot assay to detect such immunity in dogs received AAV treatment. Here, we describe in detail the use of a constructed panel of overlapping peptides spanning the entire VP1 sequence of AAV capsid protein to detect specific T cell responses in peripheral blood in dogs following intra-muscular injection of AAV. This high-throughput method allows the identification of T cell epitopes without the need for large cell numbers and the need for MHC matched cell lines.

Keywords

adeno-associated virus; AAV; peptide library; dog; ELISpot assay

1. Introduction

Vectors based on the adeno-associated virus (AAV) have shown promise for gene therapy of a number of acquired and inherited diseases in pre-clinical studies and clinical trials (1–6). However, one major hurdle in using viral vectors for *in vivo* gene delivery is the development of host cellular immune responses to the vector which may lead to elimination of transgene expression (7–12).

Corresponding Author: Zejing Wang, MD, PhD, Fairview Av N, D1-100, Seattle, WA 98109-1024; zwang@fhcrc.org; Phone: 206-667-5558; Fax: 206-667-6124.

Cellular immune responses are mediated through the recognition of peptide epitopes presented by MHC molecules on antigen presenting cells by T-cell receptors, and can be assessed by several approaches. The chromium release assay has been a standard to measure specific cytotoxic T lymphocyte (CTL) activity (13). However, this assay requires knowledge of the peptide epitopes that are recognized and major histocompatibility molecule (MHC)-matched target cells. The chromium release assay has low sensitivity and is not practical for assessing consecutive blood samples or for high-throughput screening to identify new epitopes. The use of a flow cytometry-based staining assay with MHC tetramers is more sensitive for detecting specific T cells than a chromium release assay, but depends on prior knowledge of both the MHC restricting allele and the immunogenic epitopes (14, 15). The other disadvantage is that MHC tetramer staining measures only the frequency of T cells with a specific T-cell receptor and not the function of the T cell. Intracellular cytokine staining (ICS) is another developed flow cytometry-based assay that can detect cytokine expression from responding T cells stimulated with peptides or peptide pools (16, 17). It is a sensitive assay without the need of knowing the precise MHC restricting allele, and when combined with multi-parameter staining with antibodies to diverse cell surface markers can simultaneously detect the phenotypes of the responding T cells. However, the technique does not lend itself to high-throughput testing.

Interferon gamma (IFNγ) ELISpot assay is a cytokine enzyme-linked immunospot assay that has many advantages over other methods (18–22). It can be used on blood samples without the need to add additional MHC-matched target cells. IFNγ ELISpot can be designed in a high-throughput format to screen all potential epitopes in a given viral antigen using an overlapping peptide library, and thereby, identify novel immunogenic epitopes. It is both sensitive and quantitative, and does not require large number of cells. Furthermore, the ELISpot assay can be performed on frozen cells which would allow testing of multiple samples from different subjects or conditions or consecutive samples from the same subject within the same assay (23, 24). It has been applied in analyzing peptide-specific immune responses in patients with various diseases including vaccine trials for monitoring cell immune responses (25–29).

Here, we describe the use of INFγ ELISpot assay with a constructed panel of overlapping peptides spanning the entire VP1 sequence of AAV6 capsid protein to detect specific T cell responses in peripheral blood in dogs given AAV6 vectors.

2. Materials

2.1. Intra-muscular vector injection

1. AAV vectors carrying CMV-canine factor IX (cFIX) (provided by Drs. Jeffrey Chamberlain, University of Washington, Seattle, WA, and Dusty Miller, Fred Hutchinson Cancer Research Center, Seattle, WA). The vectors were purified either by affinity purification through a HiTrap heparin column (Amersham, Piscataway, NJ) or by double CsCl gradient centrifugation followed by dialysis into Hank's buffered salt solution (Invitrogen, Carlsbad, CA).
2. Hank's buffered salt solution (HBSS) (Invitrogen, Carlsbad, CA)

3. 31 gauge syringes (Becton-Dickinson, Franklin Lakes, NJ).
4. Anesthesia reagents: acepromazine (Fort Dodge Animal Health, Fort Dodge, Iowa, USA); atropine (Baxter Health Corporation, Deerfield, IL, USA); butorphanol (Fort Dodge Animal Health); glycopyrrolate (American Regent, Inc, Shirly, NY, USA); propofol (Abbott Laboratories, North Chicago, IL, USA); lidocaine hydrochloride, injectable-2% at the site (VEDCO, Inc, St. Joseph, MO, USA).
5. Surgical instruments and sutures: sterile forceps and scissors (World Precision Instruments, Inc. Sarasota, FL, USA), 4-0 Maxon (US Surgical, Tyco Healthcare LP, Norwalk, CT); 4-0 Prolene non-absorbable suture, blue microfilament (Ethicon, Inc. Somerville, NJ, USA), scalpel #10 blade (Medline Industries, Inc. Mundelein, IL, USA), skin stapler (US Surgical, Tyco Healthcare LP, Norwalk, CT, USA).

2.2. Peripheral blood mononuclear cells collection

1. Heparin sulfate (APP Pharmaceuticals, LLC Schaumburg, IL, USA).
2. 1 × Dulbecco's phosphate-buffered saline (PBS, Gibco-Invitrogen, Carlsbad, CA)
3. Ficoll-Hypaque (D =1.074, FHCRC, Seattle) Tongue depressor (Fisher Scientific).
4. Waymouth medium (Gibco-Invitrogen, Carlsbad, CA)
5. Nonessential amino acids (Mediatech Inc., Herndon, VA)
6. 50ml corning tubes (Corning Inc. Corning, NY)
7. Centrifuge - Sorvall Lengend Heraeus, 750064446 B (Thermo Fisher Scientific, Waltham, MA, USA).
8. Sterile plastic transfer pipette (Fisher Scientific, Pittsburgh, PA)

2.3. ELISpot assay for detecting T cells to AAV capsid protein

1. Peptide panel encompassing the entire AAV VP1 capsid protein sequence synthesized
2. Dimethyl Sulphoxide (DMSO, Sigma, St. Louis, MO)
3. Canine IFN- development module (Store at 4°C, R&D, Minneapolis, MN)
4. ELISpot blue color module (Store at 4°C, R&D, Minneapolis, MN)
5. ISCOVE medium (Gibco-Invitrogen, Carlsbad, CA)
6. Waymouth medium (Gibco-Invitrogen, Carlsbad, CA)
7. Nonessential amino acids (Mediatech Inc., Herndon, VA)
8. Sodium pyruvate (Mediatech Inc., Herndon, VA)
9. Penicillin streptomycin (Gibco-Invitrogen, Carlsbad, CA)
10. L- glutamine (Gibco-Invitrogen, Carlsbad, CA)
11. Heat inactivated dog serum (HIND, Gemini Bio-Products, West Sacramento, CA)

12. Phytohemagglutinin, PHA (Store at −20°C, Sigma, St. Louis, MO)
13. Sucrose (Sigma, St. Louis, MO)
14. 96-well PVDF microplates (Millipore, Bedford, MA)
15. Tween 20 (Sigma, St. Louis, MO)
16. Automatic reader (CTL, Cleveland, OH)

3. Methods

3.1. Intra-muscular AAV vector injection

All research experiments performed on dogs follow the *Guide for Laboratory Animal Facilities and Care* prepared by the National Academy of Sciences, National Research Council and after approval by the Institutional Animal Care and Use Committee. All dogs are immunized for leptospirosis, distemper, hepatitis, papillomavirus and parvovirus, de-wormed. Three muscles in hind limbs are chosen for intra-muscular AAV injection for easy access: biceps femoris, semitendinosus and semimembranosus.

1. Shave injection sites;
2. Dogs are under general anesthesia then placed in a lateral decubitus position; the site of incision is infiltrated with 2% lidocaine hydrochloride S.C. ;
3. A 4–6 cm incision is made in the skin along the longitudinal axis of the hind limb to expose selected muscles;
4. Non-absorbable 4-0 sutures are placed in the belly of the muscle as marker for each injection site;
5. Slowly inject 1×10^{11} to 1×10^{12} vector genome of rAAV vectors in 250 μl of HBSS per site into the muscle belly right underneath each suture using 31 gauge syringes;
6. Close skin, and monitor animals daily for recovery.

3.2. Peripheral blood mononuclear cells collection

To examine cellular immune responses to AAV capsid proteins, blood samples are collected for isolation of PBMC before vector injection (pre) and at 4 and 12 weeks after AAV injection or at a desired time of your study.

1. Collect 30 ml of blood containing 10% heparin as anticoagulant;
2. Dilute blood in 30 ml pre-warmed PBS (37°C);
3. Transfer 15 ml of Ficoll into each 50ml corning tubes, tilt the tube and layer 30ml of diluted blood very slowly on top of the Ficoll by gently pipetting the blood onto the side of the tube;
4. Centrifuge at 1300 rpm for 40 minutes at room temperature (RT), with the lowest speed acceleration rate.

5. Remove the lymphocyte layer that collects above the Ficoll with a sterile plastic pipette, transfer to a 15 ml tube, and then fill the tube with Waymouth medium supplemented with 2% nonessential amino acid (Way-N) to 15 ml and centrifuge at room temperature for 10 minutes at 1000 rpm.
6. Remove supernatant, re-suspend the cell pellet and combine cells from all tubes and transfer to a new 15-ml tube. Fill the tube with Way-N and centrifuge at RT for 10 minutes at 800 rpm.
7. Remove supernatant, re-suspend cells at 2×10^6 cells/ml in 50% Waymouth 50% ISCOVE medium supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin streptomycin, 5% L- glutamine and 10% HIDS (Way-ISC). Or alternatively, freeze down in 10%DMSO90%HIDS at a concentration of 10–15 $\times 10^6$cells/ml in liquid nitrogen.

3.3. ELISpot assay for detecting T cells to AAV capsid protein

To examine cellular immune responses to AAV capsid proteins, blood samples are collected at different time points described above for isolation of PBMC, and each sample will be subjected for stimulation with peptides. PBMC collected before vector treatment stimulated with peptides and medium only and PBMC collected after vector treatment stimulated with medium only will be used as negative controls. PHA stimulation will be used as positive control for each sample.

3.3.1. Generation of peptide pools—

1. Obtain a peptide library consisting of 15mer peptides each overlapping by 11 amino acids with adjacent peptides. HPLC-purified peptides (purity >90 %) will be ideal (Reutlingen, Germany; or Sigma, St. Louis, MO);
2. Dissolve each peptide in 100 % DMSO at a concentration of 20 mg/ml. Number the peptide stocks sequentially and store at −80°C;
3. Design a two-dimentional array for the individual peptides. For AAV6 VP1, there are 182 peptides in the panel, so a 14 × 13 array was designed (Table).
4. Generate a peptide pool by combining equal quantity of each peptide stock along an axis and a volume of DMSO sufficient to result in a final concentration of 2 mg/ml/peptide in each pool. For example, pool 1 contains all peptides in the first column, and pool 15 contains all the peptides in the top row. There are total 27 pools for AAV6 VP1 (Table). Store pools at −80°C until use.

3.3.2. ELISpot assay—

1. Add 50 μl of 70 % ethanol to each well;
2. Wash immediately 4 × 200 μl of sterile PBS;
3. Calculate the total volume of Capture Antibody needed and dilute to the working concentration using PBS;

4. Add 100 μl diluted capture antibody to each well, cover and incubate overnight at 4°C;
5. Aspirate capture antibody and wash 4 times with wash buffer (0.05 % tween 20 in PBS, 350μl/well). After the final wash, remove any remaining liquid by inverting the plate and blotting it against a clean paper towel;
6. Block plates with 200 μl of blocking buffer (1 % BSA 5 %Sucrose in PBS) for 2 hours at RT;
7. During incubation, thaw peptide pool stocks and dilute each pool in culture medium (Way-ISC) to 4 μg/ml. The culture medium is used as negative control, and 5 μg/ml PHA is used as positive control;
8. Aspirate blocking buffer, and wash the plates once with 350 μl of culture medium;
9. Aspirate medium, and fill with 100 μl/well of each peptide pools or controls;
10. Add 100 μl of PBMC cell suspensions (2×10^6 cells/ml) to each well (final concentration of 2×10^5 cells/well, Note 1 and [2]) and incubate overnight at 37°C (Note 3);
11. Aspirate and wash plates 4 times with 350 μl wash buffer. After the final wash, remove any remaining liquid by inverting the plate and blotting it against a clean paper towel;
12. Calculate the total volume of Detection Antibody needed and dilute to the working concentration using Reagent Diluent (1% BSA in PBS);
13. Add 100 μl of the diluted Detection Antibody per well. Cover the plate with the lid and incubate overnight at 4° C;
14. Aspirate and wash plates 4 times with 350 μl wash buffer. After the final wash, remove any remaining liquid by inverting the plate and blotting it against a clean paper towel;

3.3.3. Color development—

1. Calculate total volume of Streptavidin-AP needed and dilute 1:60 in diluent reagent;
2. Add 100 μl to each well and incubate 2 hours at RT;
3. Wash the plates 4 x× 350 μl wash buffer, rinse again with deionized water, after the final wash, remove any remaining liquid by inverting the plate and blotting it against a clean paper towel;

[1]When using frozen cells, treat cells with 50 U/ml DNase for 3 minutes at RT after thaw to prevent cell aggregates. Then spin down at 1000g for 5 min, wash 2 times with 10 ml of the culture medium, spin at 800g for 7 minutes, then re-suspend cell pellet in appropriate volume that gives 2×10^6 /ml.

[2]Cell numbers per well should be determined empirically using 2×10^5 cells/well as a starting point. Too much background would require using reduced number of cells; or increasing cell numbers to obtain sufficient positive spots. Or alternatively, expand antigen specific T cells before subjecting to ELISpot assay (see Note 5 below).

[3]Incubation time can range from overnight to 3 days depending on the number or size of positive spots

4. Add 100 μl /well BCIP/NBT solution, cover, incubate 30 minutes in dark at RT;
5. Rinse 6 times with deionized water, invert plate and tap to remove excess water;
6. Allow plates to dry at RT for at least 3 hours;
7. Count spots using automated ELISpot reader (Note 4, [5]).

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[4]Once a positive pool is identified, each peptide within the pool should be subjected to a second round of ELISpot assay as stimulant for identifying individual antigenic peptides in a positive pool.

[5]To reduce background and increase the number of antigen specific T cells, in vitro T cell expansion can be performed as follow:

- Plate 1.5×10^6 cells/well in 500 μl medium in a 48-well plate;
- Dilute peptide pools in culture medium and add 500 μl to the cells for final concentration of 4 μg/ml. Incubate at 37°C for 48 hours.
- Take out 500 μl of the medium, and add IL-2 in 500 μl medium for final concentration of 2U/ml. Incubate at 37°C for 5 days;
- Spin down cells at 1000 rpm for 5minutes;
- Re-suspend in 350 μl of medium, add 100 μl/well for ELISpot assay.

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Table 1

AAV6 VP1 peptide panel.

Pools→ ↓	1	2	3	4	5	6	7	8	9	10	11	12	13	14
15	1	2	3	4	5	6	7	8	9	10	11	12	13	14
16	15	16	17	18	19	20	21	22	23	24	25	26	27	28
17	29	30	31	32	33	34	35	36	37	38	39	40	41	42
18	43	44	45	46	47	48	49	50	51	52	53	54	55	56
19	57	58	59	60	61	62	63	64	65	66	67	68	69	70
20	71	72	73	74	75	76	77	78	79	80	81	82	83	84
21	85	86	87	88	89	90	91	92	93	94	95	96	97	98
22	99	100	101	102	103	104	105	106	107	108	109	110	111	112
23	113	114	115	116	117	118	119	120	121	122	123	124	125	126
24	127	128	129	130	131	132	133	134	135	136	137	138	139	140
25	141	142	143	144	145	146	147	148	149	150	151	152	153	154
26	155	156	157	158	159	160	161	162	163	164	165	166	167	168
27	169	170	171	172	173	174	175	176	177	178	179	180	181	182

A total of 182 peptides was synthesized each of which is 15 amino acids long and overlapping by 11 amino acids with adjacent peptides. Twenty-seven peptide pools are generated containing all peptides in its corresponding columns or rows.