

## Measurement of Cell-Mediated Immune Response in Woodchucks

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### 1. Introduction

Infection with hepatitis B virus (HBV) is a major health problem, with 350 million people chronically infected worldwide. Chronic HBV infection is characterized by severe hepatic disease sequelae, including chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma (*1*). The immunologic mechanisms that predispose to the development of chronic HBV infection are not completely understood. However, the cell-mediated immunity (CMI) to HBV is believed to play a crucial role in protection against viral infection, and development of efficient antiviral CMI is probably needed to avoid viral persistence and progression to chronic hepatitis (*1*).

The woodchuck hepatitis virus (WHV) and its natural host, the Eastern woodchuck (*Marmota monax*), have been used extensively as an animal model for HBV infection, disease, and prevention (e.g., *2*). This model is being applied further to study the immunopathogenesis, antiviral therapy, and immunotherapy of chronic WHV infection (*3–8*). Such studies have demonstrated the need for more detailed investigation of the immune response of woodchucks, and to develop new molecular and cellular immunologic assays for measuring this response (**Table 1**). For example, in vitro proliferation assays for measuring the responses of woodchuck peripheral blood mononuclear cells (PBMCs) to viral antigens are a prerequisite for studying the CMI of woodchucks during WHV infection and therapy (*3,4–7*). The use of a universal anti-CD3 antibody reagent indicated that woodchuck PBMCs that did not adhere to nylon wool and that proliferated after stimulation with WHV recombinant core antigen (rWHcAg) were CD3+ lymphocytes (*4*). However, antibodies to woodchuck CD4 and CD8 positive lymphocytes are needed to enable more detailed lymphocyte phenotyping in PBMC cultures, and for applications of separation and depletion of such lymphocytes. Functionally active woodchuck cytokines and anti-cytokine antibodies are also needed to develop enzyme-linked immunosorbent assay (ELISA) methods for determining the responding cell type in PBMC cultures, for example, for differenti-

**Table 1**  
**Molecular and Cellular Immunologic Assays for Studying the Host Immune Response Against WHV in the Woodchuck Model**

Assay/reagent	Method	Reference
Established for woodchuck model		
PBMC proliferation, Th epitopes, Th lines	Radiopurine, adenine	<i>3,4-7,9,10,11</i>
PBMC IL-2 production	IL-2 dependent murine NK cell bioassay	<i>3,12</i>
Macrophage, B cell, T cell staining	Immunostaining with anti-Lyz, anti-woodchuck IgG, anti-CD3 (Dako)	<i>4,8,13</i>
T cell cDNAs (CD3, 4, 8)	Endpoint/real-time RT-PCR	<i>9,13-16</i>
Cytokine cDNAs (IL-1 $\beta$ , 2, 4, 10, IFN- $\gamma$ , TNF- $\alpha$ )	Endpoint/real-time RT-PCR	<i>8,9,13-19</i>
Cytokines/Antibodies (IL-6, IFN- $\gamma$ , TNF- $\alpha$ )	Immunostaining, in vitro studies	<i>17,18</i>
MHC	PCR-based allotyping, antibodies	<i>19,20</i>
Needed for woodchuck model		
CTL assay/CTL epitopes/ CTL lines and clones	Autologous fibroblasts or PBMC blasts	
Anti-T cell antibodies (CD3, 4, 8)	Selection, depletion	
Anti-cytokine antibodies (IL-2, 4, 10, 12, IFN- $\gamma$ , TNF- $\alpha$ )	Th1/Th2 ELISA	
Hepatocyte cDNAs	Libraries, host response	

Anti-Lyz, antibody against lysozyme; CD, cluster of differentiation; CTL, cytolytic T cells; ELISA, enzyme-linked immunosorbent assay; IFN- $\gamma$ , interferon- $\gamma$ ; IgG, immunoglobuline G; IL, interleukin; NK, natural killer cells; MHC, major histocompatibility complex; PCR, polymerase chain reaction; RT, reverse transcription; Th, T-helper cells; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

ation of type 1 and 2 responses. At present, the activation of woodchuck T helper 1 (Th1) cells can be measured by the production of interleukin-2 (IL-2) using an IL-2-dependent murine cell line (3). Th1 and Th2 cell responses can be differentiated, moreover, by recently developed real-time polymerase chain reaction (PCR) assay for mRNAs of woodchuck leukocyte CD markers and type 1/type 2 cytokines in PBMC cultures and tissues (9).

The CMI of woodchucks can be studied by in vitro proliferation of PBMCs using polyclonal activators (e.g., concanavalin A [Con A], lipopolysaccharide [LPS], phytohemagglutinin [PHA], human recombinant IL-2) and WHV antigens (e.g., rWHcAg, e antigen [WHeAg], surface antigen [WHsAg], x antigen [WHxAg], and antigen-derived peptides) for stimulation (Fig. 1). Woodchuck PBMCs do not incorporate sufficient tri-

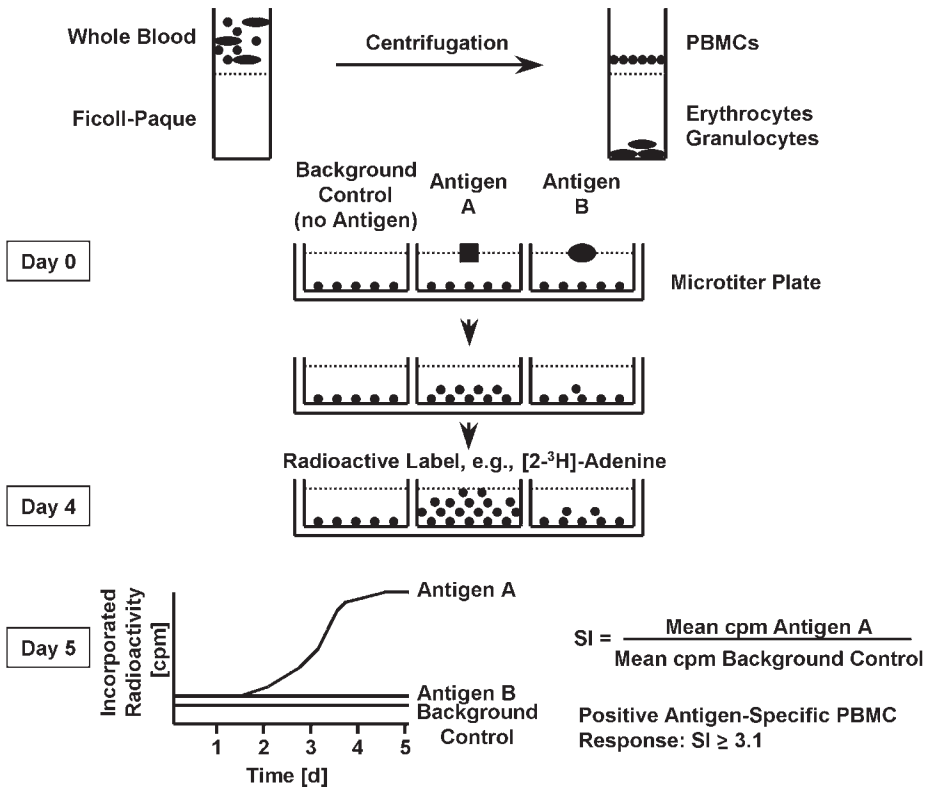


Fig. 1. Overview of the isolation of woodchuck PBMCs from whole blood and their use in the *in vitro* proliferation assay with radioactive labels (e.g., [<sup>3</sup>H]adenine) for the detection of CMI against WHV in woodchucks.

tiated thymidine in proliferation responses to polyclonal stimulators (**Table 2**) and WHV antigens (**Table 3**), which apparently relates to the inefficient transcription of the cytosolic thymidine kinase 1 gene (**3,10,11**). However, woodchuck PBMCs incorporate sufficient tritiated adenine, serine, adenosine, and deoxyadenosine (**3,4–6,10,11**), which enables the development of a meaningful proliferation assay (**Tables 2 and 3**).

This chapter describes a method for the isolation of woodchuck PBMCs from whole blood and their use in proliferation assays. The *in vitro* PBMC proliferation assay is optimized for woodchuck PBMCs and enables the high-throughput analyses of CMI from large numbers of woodchucks to many stimulators in a given experiment. This assay circumvents the problem of insufficient incorporation of tritiated thymidine mainly by the use of tritiated adenine (or other radioactive purine labels). The availability of this assay facilitates the characterization of host immune response kinetics in woodchucks with applications to the continued modeling of chronic HBV infection and therapy in humans.

**Table 2**  
**Incorporation of Tritiated Thymidine, Serine, Adenine, Adenosine, and Deoxyadenosine by Woodchuck PBMC Cultures After 4 D of Stimulation with Polyclonal Activators in the In Vitro PBMC Proliferation Assay**

Stimulator	Outcome	SI (± SD)					[8- <sup>3</sup> H] Deoxyadenosine
		[ <sup>3</sup> H]Thymidine	L-[3- <sup>3</sup> H]Serine	[2- <sup>3</sup> H]Adenine	[2- <sup>3</sup> H]Adenosine		
ConA	Control	3.2 ± 0.2	47.3 ± 3.3	42.4 ± 3.2	37.8 ± 9.4		34.2
	Acute	3.2 ± 0.3	47.6 ± 4.5	42.6 ± 4.5	20.6		35.6
	Chronic	3.2 ± 0.2	48.0 ± 3.8	43.7 ± 3.6	nd		nd
PHA	Control	1.3 ± 0.1	6.9 ± 0.7	4.9 ± 0.6	nd		nd
	Acute	1.4 ± 0.1	7.1 ± 1.0	5.1 ± 0.8	nd		nd
	Chronic	1.4 ± 0.1	6.9 ± 0.9	5.0 ± 0.6	nd		nd

5 × 10<sup>4</sup> woodchuck PBMCs were cultured with ConA (8.0 µg/mL; 20 µg/mL for [2-<sup>3</sup>H]adenosine and [8-<sup>3</sup>H]deoxyadenosine) or PHA (2.4 µg/mL) in microtiter plates for 4 (3 d for [2-<sup>3</sup>H]adenosine and [8-<sup>3</sup>H]deoxyadenosine) including a 16- to 20-h pulse with 37 kBq of the respective <sup>3</sup>H labels. Stimulation indices (SI) represent the mean SI value of triplicate cultures from all woodchucks in each outcome group of infection. Four adult woodchucks per outcome group were tested (two control and one acute WHV-infected woodchucks for [2-<sup>3</sup>H]adenosine, one control and one acute WHV-infected woodchuck for [8-<sup>3</sup>H]deoxyadenosine). Control: WHV-uninfected woodchucks. Acute: Woodchucks with acute self-limited WHV infection (4–8 wk after experimental infection). Chronic: Woodchucks with chronic WHV infection. nd, not done. The mean cpm values of unstimulated PBMCs in medium alone (background control) of woodchucks in each outcome group were: [<sup>3</sup>H]thymidine, control: 803 ± 39, acute: 812 ± 34, chronic: 795 ± 38; L-[3-<sup>3</sup>H]serine, control: 1326 ± 62, acute: 1409 ± 95, chronic: 1357 ± 101; [2-<sup>3</sup>H]adenine, control: 2857 ± 228, acute: 2956 ± 345, chronic: 3027 ± 287; [2-<sup>3</sup>H]adenosine, control: 2107 ± 1061, acute: 2722; [8-<sup>3</sup>H]deoxyadenosine, control: 2304, acute: 2223.

**Table 3**

**Incorporation of Tritiated Thymidine, Serine, Adenine, and Adenosine by Woodchuck PBMC Cultures After 5 D of Stimulation with WHV Antigens in the In Vitro PBMC Proliferation Assay**

Stimulator	Outcome	SI ( $\pm$ SD)			
		[ $^3$ H] Thymidine	L-[ $^3$ - $^3$ H] Serine	[2- $^3$ H] Adenine	[2- $^3$ H] Adenosine
Core	Control	1.1 $\pm$ 0.1	1.3 $\pm$ 0.1	1.6 $\pm$ 0.2	1.0
	Acute	1.4 $\pm$ 0.1	23.8 $\pm$ 8.1	11.9 $\pm$ 1.7	4.1 $\pm$ 0.7
	Chronic	1.1 $\pm$ 0.1	1.6 $\pm$ 0.6	1.3 $\pm$ 0.5	nd
Surface	Control	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1	0.9 $\pm$ 0.1	1.2
	Acute	1.3 $\pm$ 0.1	11.0 $\pm$ 4.8	5.7 $\pm$ 2.2	3.5 $\pm$ 2.8
	Chronic	1.0 $\pm$ 0.1	1.2 $\pm$ 0.3	1.1 $\pm$ 0.3	nd

$5 \times 10^4$  woodchuck PBMCs were cultured with recombinant WHV core antigen (core, 1.0  $\mu$ g/mL; 0.75  $\mu$ g/mL for [2- $^3$ H]adenosine) or WHV surface antigen (surface, 2.0  $\mu$ g/mL; 2.5  $\mu$ g/mL for [2- $^3$ H]adenosine) in microtiter plates for 5 d (7 d for [2- $^3$ H]adenosine) including a 16- to 20-h pulse with 37 kBq of the respective  $^3$ H labels. Stimulation indices (SI) represent the mean SI value of triplicate cultures from all woodchucks in each outcome group of infection. Seven adult woodchucks per outcome group were tested (three woodchucks per outcome group for [ $^3$ H]thymidine; one control and three acute WHV-infected woodchucks for [2- $^3$ H]adenosine). Control: WHV-uninfected woodchucks. Acute: Woodchucks with acute self-limited WHV infection (4–8 wk after experimental infection). Chronic: Woodchucks with chronic WHV infection. nd, not done. The mean cpm values of unstimulated PBMCs in medium alone (background control) of woodchucks in each outcome group were: [ $^3$ H]thymidine, control: 763  $\pm$  64, acute: 838  $\pm$  22, chronic: 832  $\pm$  108; L-[ $^3$ - $^3$ H]serine, control: 1110  $\pm$  364, acute: 1279  $\pm$  201, chronic: 1162  $\pm$  543; [2- $^3$ H]adenine, control: 2273  $\pm$  952, acute: 2381  $\pm$  458, chronic: 2365  $\pm$  869; [2- $^3$ H]adenosine, control: 2507, acute: 2621  $\pm$  953.

## 2. Materials

### 2.1. Stimulators

1. Polyclonal activators: ConA (Sigma, St. Louis, MO, cat. no. C-2010); PHA (Sigma, cat. no. L-8902).
2. WHV antigens: rWHcAg and WHsAg are prepared as recently described (3–7).

### 2.2. Solutions

1. Isolation of PBMCs from blood: Ficoll-Paque (Amersham Pharmacia Biotech, Arlington Heights, IL, cat. no. 17-1440-03); 0.9%, NaCl (Baxter, Deerfield, IL, cat. no. 2F7124); glacial acetic acid (Fisher Scientific). Store all solutions at room temperature.
2. Medium for PBMC cultures: Complete medium contains AIM-V medium (*see Note 1*), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and  $5 \times 10^{-5}$  mM  $\beta$ -mercaptoethanol (6,10,11). AIM-V medium (Gibco BRL, Rockville, MD, cat. no. 12055-09); FBS (Sigma, cat. no. F-2442);  $\beta$ -mercaptoethanol (Sigma, cat. no. M-6250). Complete medium is light sensitive. Store at 2–8°C (refrigerator temperature).
3. Radioactive labels: [2- $^3$ H]adenine, specific activity = 703 GBq/mmol (Amersham Pharmacia Biotech, cat. no. TRK311); methyl[ $^3$ H]thymidine, specific activity = 925 GBq/mmol (Amersham Pharmacia Biotech cat. no. TRK120); L-[3- $^3$ H]serine, specific activity = 999 GBq/mol (Amersham Pharmacia Biotech, cat. no. TRK308); [2- $^3$ H]adenosine, specific activity =

740–925 GBq/mmol (Amersham Pharmacia Biotech cat. no. TRK423); [8-<sup>3</sup>H]deoxyadenosine, specific activity = 185–925 GBq/mmol (ICN Pharmaceuticals, Costa Mesa, CA, cat. no. 24053). All labels are radiation hazards. Store at 2–8°C (refrigerator temperature).

4. Liquid scintillation fluid: Betaplate Scint, Wallac, Gaithersburg, MD, cat. no. 1205–440. Store at room temperature.

### 2.3. Equipment

1. Isolation of PBMCs from blood: Sterile hood, for example, SterilGARD Hood (The Baker Company, Sanford, ME, cat. no. VBM-600); EDTA-containing vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, cat. no. 366454); conical tubes (Becton Dickinson, cat. no. 352070 for 50 mL, cat. no. 352196 for 15 mL); pipets (Becton Dickinson, cat. no. 357551 for 10 mL, cat. no. 357543 for 5 mL, cat. no. 357520 for 1 mL); Eppendorf tubes (1.5-mL), (Laboratory Product Sales, Rochester, NY, cat. no. L250502); hemocytometer (e.g., American Optical, Buffalo, NY).
2. In vitro proliferation assay: Microtiter plates (96-well round) (Becton Dickinson, cat. no. 353077); cell incubator (e.g., Forma Scientific, available through Fisher Scientific); automatic 96-well cell harvester, for example, MACH II 96 (Tomtec, Orange, CT); printed filter-mat A (Wallac, cat. no. 1205-401); sample bag (Wallac, cat. no. 1205-411; liquid scintillation counter (e.g., 1205 Betaplate, Wallac).

### 2.4. PBMCs for In Vitro Assays

Woodchuck whole blood of a volume of 5 mL or greater drawn into EDTA-containing vacutainer tubes (*see Note 2*) are recommended for density gradient centrifugation with Ficoll-Paque in 50-mL conical tubes. PBMCs have also been successfully isolated from smaller volumes of blood using 15-mL conical tubes instead of 50-mL tubes. For best results, PBMCs should be isolated immediately after whole blood is drawn and blood should be transported and kept at room temperature (e.g., if shipped overnight). The effects of delay in isolation or storage of blood at temperatures below room temperature on the viability or yield of isolated PBMCs have not been completely studied. Before starting with the isolation of PBMCs, tubes and solutions should be prepared as follows:

1. Label three conical tubes for each woodchuck with the proper animal ID number. Furthermore, label one tube for NaCl and another one for Ficoll.
2. **The following steps should be carried out under a sterile hood and with good sterile handling practice.** Dependent on the whole blood volume add the same volume of 0.9% NaCl into the NaCl-labeled tube to ensure a 1:2 dilution.
3. Add Ficoll-Paque in the same volume as the 1:2 dilution of blood and NaCl into the Ficoll-labeled tube.

## 3. Methods

### 3.1. Isolation of Woodchuck PBMCs From Whole Blood

**Precaution: Isolation of PBMCs must be performed under a sterile hood.**

1. After opening and flaming the opening of the EDTA-containing vacutainer tubes under a sterile hood, draw the whole blood into a pipet and add it **slowly** to the bottom of the NaCl-containing conical tubes. Then remove 2 mL of NaCl from the top using the same pipet and

- rinse the EDTA-containing vacutainer tubes to obtain all the blood. Add the NaCl back to the conical tube and mix the whole blood and the NaCl together gently by pipetting up and down. Finish the samples for all animals before going to **step 2**. **Critical parameter: Flaming the opening of the EDTA-containing vacutainer tubes and any of the bottles of solutions before use reduces the possibility of contamination. Furthermore, it is important to transfer whole blood and NaCl under sterile conditions without touching the outside surface of the EDTA-containing vacutainer tubes or the conical tubes with the pipet.**
2. Transfer the NaCl whole blood mixture with a pipet and lay it **carefully and slowly** on the top of the Ficoll-Paque without mixing. Finish the samples for all animals before going to **step 3**. By the time all the samples are finished, some erythrocytes may already migrate through the Ficoll in the earliest samples. **Critical parameter: The overlay of Ficoll with whole blood can be done easily by dropping the blood slowly at the wall of the conical tube. Avoid forceful addition of blood, which will destroy the Ficoll layer. If working with an autopipet, use the largest pipet as possible (e.g., 10 mL). This will allow easier control of the rate of adding blood.**
  3. Centrifuge the conical tubes for 35–40 min at room temperature. For separation of lymphocytes from erythrocytes and granulocytes use approx 1700g for 15-mL conical tubes and approx 1300g for 50-mL conical tubes. **Critical parameter: It is important to run the centrifuge with the brake off, otherwise the gradient will be disrupted.**
  4. Centrifugation will result in three layers. The top layer contains NaCl–plasma, the layer in the middle Ficoll, and the layer at the bottom erythrocytes and granulocytes. The phase between NaCl–plasma and Ficoll contains the mononuclear cells, composed mainly of lymphocytes and some monocytes (hereafter referred to as PBMCs). Using a fresh pipet, transfer the PBMCs to the third conical tube. Fill the conical tube up to the top with NaCl. Finish the samples of all animals before going to **step 5**. **Critical parameter: Pick up the majority of PBMCs first. Make sure to leave enough NaCl on top layer to cover the line of PBMCs adhering to the wall of the conical tube. To increase the yield of isolated PBMCs scrape the wall of the tube with the pipet to also recover attached cells. Be careful to avoid transfer of any erythrocytes and granulocytes.**
  5. Wash PBMCs immediately by centrifuging the conical tubes at approx 300g for 10 min at room temperature to remove the Ficoll.
  6. The centrifugation will result in a visible (white) pellet of PBMCs at the bottom of the conical tube. Carefully remove all the supernatant to avoid any loss of PBMCs. After vortexing the PBMCs to mix, refill the conical tubes up to the top with NaCl and centrifuge at 300g for 10 min at room temperature. Repeat this step one more time.
  7. After the last wash carefully remove all the supernatant and vortex the PBMCs. Add 4 mL of complete medium and vortex again to mix.
  8. For counting of cells, transfer 100  $\mu\text{L}$  of PBMCs in complete medium from the conical tube into an Eppendorf tube by using a pipet. Dilute PBMCs 1:10 in 4% of glacial acetic acid using a fresh Eppendorf tube. After vortexing to mix, add approx 10  $\mu\text{L}$  of PBMCs to a hemacytometer. Calculate the total number of cells in the original 4 mL of complete medium. This can be done by counting the cells in the 64 squares of the four corner areas under the coverslip of the hemacytometer in a total volume of 0.1  $\text{mm}^3$ . Obtain the average cell number by dividing the total number of cells by 4. Multiply the average cell number by 10 to obtain the cell number per 1  $\text{mm}^3$ . Multiply this by 1000 to obtain the cell number per 1  $\text{cm}^3$ , which equals 1 mL of medium. Then account for the 10-fold dilution by multiplying the cell number by 10 to get the number of cells per 1 mL of medium. Multiply this number by 4 to achieve the total number of cells isolated. Add an appropriate volume of complete

medium to the conical tube to obtain a concentration of  $2.5 \times 10^6$  cells/mL. If the cell concentration in the 4 mL of complete medium is lower, centrifuge the PBMCs again at 300g for 10 min at room temperature and remove all the supernatant. Then add the appropriate volume of complete medium to get the desired cell concentration. **Critical parameter: 4% of glacial acetic acid is used to destroy any contaminating erythrocytes in the PBMC samples to be counted. If the PBMC sample contains a higher number of erythrocytes, the use of 8% of glacial acetic acid and incubation for 10 min at room temperature is recommended.**

### 3.2. In Vitro PBMC Proliferation Assay with Radioactive Labels

Stimulators are added to the wells of a 96-well microtiter plate in a volume of 20  $\mu$ L. This keeps the volume of stimulator preparation low (i.e., 1/10 of the final well volume), but easily dispersible by using a repeater pipet. Polyclonal activators and WHV antigens are used in triplicates at previously determined optimal concentrations (3,4,6): Con A (8.0  $\mu$ g/mL), PHA (2.4  $\mu$ g/mL), WHsAg (2.0  $\mu$ g/mL), and rWHcAg (1.0  $\mu$ g/mL). The desired concentrations of stimulators are achieved by diluting the stock solutions in an appropriate volume of 0.9% NaCl to obtain a 10-fold working solution. Controls may include irrelevant woodchuck serum protein as a control for WHsAg and irrelevant recombinant protein as a control for rWHcAg in the same concentration as above. For background control (unstimulated PBMCs in medium alone) add 20  $\mu$ L of 0.9% NaCl into eight wells of the microtiter plate. For control of WHV antigens add 20  $\mu$ L of irrelevant woodchuck protein into eight wells of the microtiter plate. Microtiter plates can be prepared in advance (and frozen) with all stimulators in the wells before transferring complete medium and freshly isolated PBMCs (see Note 3).

1. Add 160  $\mu$ L of complete medium to each well of the previously prepared microtiter plate by using a multichannel pipet.
2. After vortexing to mix the PBMCs in complete medium, add 20  $\mu$ L of PBMCs into each well of the microtiter plate by using a repeater pipet to reach the final concentration of  $5 \times 10^4$  cells per well (i.e., in a total well volume of 200  $\mu$ L).
3. Incubate cultured PBMCs for 5 d (see Note 4) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.
4. Add 37 kBq (= 1  $\mu$ Ci) of a certain radioactive label (e.g., [2-<sup>3</sup>H]adenine) to the PBMC cultures in each well of the microtiter plate on d 4 by using a repeater pipet. For transfer of 37 kBq of a radioactive label in a volume of 20  $\mu$ L, dilute the stock solution with an appropriate volume of complete medium.
5. Place radioactively labeled microtiter plates back into the incubator for another 16–20 h, before harvesting the PBMC cultures on d 5.
6. After 5 d of culture, radioactively labeled PBMCs are harvested onto filtermats by using an automatic 96-well cell harvester. After air-drying overnight place one filtermat into a sample bag. Add 10 mL of liquid scintillation fluid to the filtermat and seal the sample bag. Incorporated radioactivity is then measured by counting in a liquid scintillation counter. Results for triplicate cultures are expressed as the stimulation index (SI), which is determined by dividing the total counts per minute (cpm) obtained in the presence of stimulator by that in the absence of stimulator (e.g., background control = unstimulated PBMCs in medium alone, PBMCs cultured with irrelevant woodchuck protein; see Fig. 1). **Critical parameter: For calculation of the SI value average the cpm derived from the eight wells containing the background control or the controls for WHV antigens by removing the highest and lowest cpm value (see Note 5).**



#### 4. Notes

1. The use of AIM-V medium as main part of the complete medium for the culture of woodchuck PBMCs instead of RPMI 1640 (Gibco BRL) is recommended. AIM-V medium was demonstrated to significantly increase the uptake of radioactive label (i.e., [2-<sup>3</sup>H]adenine) by woodchuck PBMCs in the in vitro proliferation assay (10).
2. The use of EDTA as an anticoagulant for blood sampling instead of heparin is critical. EDTA was shown to increase the uptake of radioactive label (i.e., [2-<sup>3</sup>H]adenine) by woodchuck PBMCs in the in vitro proliferation assay (10).
3. For in vitro proliferation assays involving a large number of animals and many stimulators, plates can be prepared in advance for all test dates and stored at -20°C until use. Allow frozen plates to thaw before adding complete medium and freshly isolated PBMCs.
4. The culture period of 5 d is optimized for WHV antigen-specific stimulations of woodchuck PBMCs. If only polyclonal activators are used, the culture period should be decreased to 4 d to get best proliferation results (10,11).
5. SI values of  $\geq 3.1$  are considered positive for WHV antigen-specific PBMC responses of woodchucks. This positive cutoff is conservative in relationship to routine positive stimulations by WHV antigens; that is, the range of maximal antigen-specific SI values using [2-<sup>3</sup>H]adenine is 7.0–12 (e.g., 5,6). The 3.1 cutoff level, therefore, represents at least 25–45% of the observed response range for positive stimulations induced by antigens. At the 3.1 SI cutoff, the positive sample cpm for antigens always is greater than two standard deviations (SDs) above the mean cpm of background control (i.e., unstimulated PBMCs in medium alone) from the same woodchuck, and is characteristically more than two SDs above the average cpm for antigen-stimulated PBMCs from uninfected control woodchucks.

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