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# Memory T-Cell Response to Rotavirus Detected with a Gamma Interferon Enzyme-Linked Immunospot Assay

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**Measurements of serum-neutralizing antibody and anti-rotavirus immunoglobulin A (IgA) are the current standard for assessing immune responses following rotavirus vaccination. However, there is ongoing debate as to whether antibody titers correlate with protection against rotavirus gastroenteritis. Children recovering from rotavirus gastroenteritis have increased gamma interferon release from cultured peripheral blood mononuclear cells (PBMCs), suggesting that cell-mediated immunity (CMI) may play a role in viral clearance and protection from subsequent gastroenteritis. We have developed a gamma interferon enzyme-linked immunospot (ELISPOT) assay for evaluation of CMI responses to rotavirus using frozen PBMCs obtained from healthy adults. Responses to three different rotavirus antigen types were analyzed—a peptide pool based on the human VP6 sequence; reassortant human:bovine vaccine strains; and cell culture-adapted (CCA) human G1, G2, G3, G4, and bovine (WC3) G6 strains. The reassortant strains consist of a bovine WC3 genome background expressing the human rotavirus surface proteins VP7 (G1, G2, G3, or G4) or VP4 (P1). Responses to titrations of the peptide pool as well as CCA and reassortant strains were assessed. Gamma interferon ELISPOT responses were similar for CCA and reassortant strains, whether live or UV inactivated, and when tested either individually or pooled. For most subjects, responses to the VP6 peptide pool positively correlated with responses to CCA and reassortant strains. Cell depletion studies indicate the memory responses detected with these frozen adult PBMCs were primarily due to the $CD4^+$ T-cell population. This gamma interferon ELISPOT assay provides a new tool to apply in clinical studies for the characterization of natural or vaccine-induced CMI to rotavirus.**

Rotavirus is the most common cause of severe diarrhea among children worldwide. By age five years, 70% of children in the United States will have developed rotavirus disease (15, 34). The typical illness is characterized by fever and vomiting, followed by diarrhea. Rotavirus is a mucosal pathogen that replicates in the mature villous epithelial cells at the mucosal surface of the small intestine. Similar to other mucosal pathogens, rotavirus infection is generally limited to the small intestinal mucosa, resulting in incomplete immunity (21). As a result, protection from reinfection is incomplete; generally resulting in less severe disease symptoms than primary infection (24). Another contributing factor to reinfection is the fact that there are multiple serotypes and strains for rotavirus and some of the protection gained from infection is type specific.

The current standard for assessing immune responses following rotavirus vaccination in humans is the measurement of antirotavirus immunoglobulin A (IgA) and serum-neutralizing antibody to rotavirus antigen VP7 or VP4. There is ongoing debate as to whether serum antibody levels correlate with protection (4, 14, 19, 21, 39). Better correlation between the presence of serum antibodies and protection has been observed in natural rotavirus infection studies than in the limited number of candidate vaccine studies evaluating neutralizing responses to date (14). Furthermore, conflicting results as to whether serum levels of IgG may represent correlates of protection have been presented in several studies of natural rotavirus infection in children (9, 12, 14, 29, 36). Serum-neutralizing antibodies, as correlates of protection, are also under debate. Several studies have shown that preexisting homotypic neutralizing antibodies in children correlate with protection from rotavirus infection and disease (5, 6, 14, 29, 30), whereas Ward et al. (40) found some correlation only with heterotypic neutralizing antibodies.

Experiments in animal models have demonstrated an important role for cell-mediated immunity (CMI) in protection from rotavirus shedding (38). Using suckling mice, Offit et al. have shown that rotavirus-specific cytotoxic T lymphocytes are present in the intestinal mucosa following rotavirus inoculation (25) and that protection from rotavirus disease can be mediated through adoptive transfer of immune $CD8^+$ T cells (26). In SCID mice, a role for CMI in clearance of chronic rotavirus infection was shown by the adoptive transfer of both immune splenic and intestinal $CD8^+$ T cells (11) and splenic $CD4^+$ T cells (16). By in vivo depletion of the $CD4^+$ T cells in BALB/c mice, VanCott et al. (35) demonstrated $CD4^+$ T cells were necessary to resolve primary infection. McNeal et al. (18) recently published data indicating that CD4 T cells are the only lymphocytes needed to protect mice against shedding after intranasal immunization with VP6.

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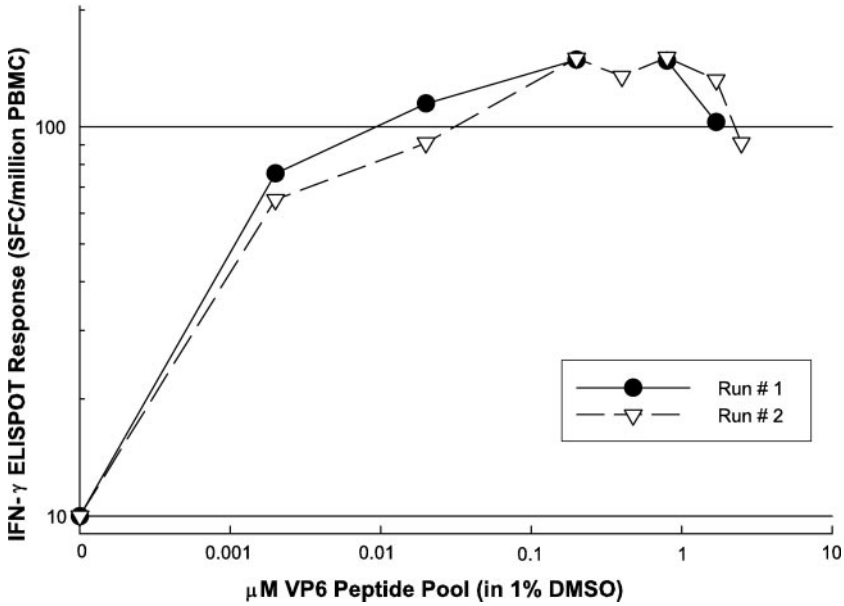


FIG. 1. Titration of rotavirus VP6 peptide pool with donor 5020 PBMCs.

The role of CMI in humans for protection from rotavirus infection or disease is not as well characterized. Using a lymphoproliferation assay, Offit et al. (27) observed a positive correlation between the presence of rotavirus-specific T cells and previous exposure to rotavirus in infants. In another study, they observed that a majority of the children monitored had detectable levels of circulating rotavirus-specific T cells after infection (28). Intracellular staining and an enzyme-linked immunospot (ELISPOT) assay with antigen-stimulated peripheral blood mononuclear cells (PBMCs), from both adults and children recently infected with rotavirus, exhibited increased levels of gamma interferon (IFN-γ)-secreting, rotavirus-specific $CD4^+$ and $CD8^+$ T cells (13, 31). Culture supernatants of rotavirus-stimulated human PBMCs from healthy adult donors have been shown to contain IFN-γ (41). Azim et al. (2) have shown an increase in IFN-γ release from cultured human PBMCs of children with rotavirus diarrhea compared with controls. These data suggest that IFN-γ could be used as a marker for T-cell responsiveness to infection. Recent in vitro findings by Bass (3) that IFN-γ inhibits rotavirus entry into the human intestinal cell lines Caco-2 and HT-29 suggest that IFN-γ plays a beneficial role in controlling rotavirus infection. The mechanism by which CMI responses may protect against rotavirus infection, as shown in animal models, may be through production of cytokines such as IFN-γ by activated T cells (3, 16, 35, 41). We show here the development of an IFN-γ ELISPOT assay for evaluation of CMI responses to rotavirus using PBMCs obtained from healthy human adults. With this assay, we have detected memory T-cell responses (both CD4 and CD8) to VP4, VP6, and VP7 rotavirus antigens.

## MATERIALS AND METHODS

**Healthy adult donors.** Blood was collected from random volunteers recruited by either Biological Specialty Corporation (Colmar, PA) or Merck & Co., Inc (West Point, PA). Ages ranged from 21 to 69 years. All donors resided in Southeastern Pennsylvania. History of contact with infants is unknown.

**Isolation and freezing of PBMCs from whole blood.** Whole blood was collected from healthy adult donors in either heparin- or EDTA-containing Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). The blood was diluted with Hanks' balanced salt solution without calcium and magnesium (HBSS; GIBCO Invitrogen Corp., Carlsbad, CA), and the PBMCs were separated on a density gradient using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) or in Accuspin System-Histopaque-1077 tubes (Sigma-Aldrich). The tubes were centrifuged ($1{,}000 \times g$ for 10 min for the Accuspin System tubes or $400 \times g$ for 30 min for the Histopaque-1077 alone), and the buffy layer containing the PBMCs was removed. The cells were washed, and the red blood cells lysed with ACK lysing buffer (GIBCO Invitrogen Corp.). The PBMCs were then washed twice with HBSS and counted using a Z1 Dual Particle counter (Beckman Coulter Inc., Fullerton, CA). Any particle greater than 45 fl was counted. The cells were washed again and resuspended to a concentration of $1 \times 10^7$ cells/ml in freezing medium consisting of 90% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The cells were distributed into cryogenic vials and placed into a Nalgene 1°C Cryo freezing container (Fisher Scientific, Pittsburgh, PA). The freezing container was stored at −70°C for up to 3 days, and frozen cell samples were transferred to liquid nitrogen (vapor phase, <−130°C) for long-term storage.

**Thawing of frozen PBMCs.** Complete medium consisting of RPMI medium 1640 (GIBCO Invitrogen Corp.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 10 mM HEPES buffer (GIBCO Invitrogen Corp.), 1 mM L-glutamine (GIBCO Invitrogen Corp.), 100 U penicillin G sodium and 100 μg streptomycin sulfate per ml (GIBCO Invitrogen Corp.), and $5 \times 10^{-5}$ M β-mercaptoethanol (Sigma-Aldrich) was prepared and stored at 4°C. The medium was then warmed to room temperature prior to use. Benzonase-supplemented complete medium was made by adding Benzonase (EMD Chemicals Inc., Gibbstown, NJ) to complete medium at a final concentration of ≥50 U/ml. Frozen PBMC aliquots were thawed at 37°C, and Benzonase-supplemented complete medium was slowly added. Cells were washed and resuspended in Benzonase-supplemented complete medium. Cells were washed again, resuspended in complete medium without Benzonase, and counted with a Z1 Dual Particle counter (Beckman Coulter Inc.). Cells were washed and then resuspended in complete medium without Benzonase at a concentration of $1 \times 10^7$ cells/ml. These cells were then used immediately in either the IFN-γ ELISPOT assay or for cell depletion assays.

**CD4 and CD8 cell depletion.** CD4 or CD8 T-cell populations were depleted from thawed PBMCs using the Dynabead magnetic bead system (Dynal Biotech ASA, Oslo, Norway), according to the manufacturer's recommended procedures. The remaining cells were resuspended in complete medium at $1 \times 10^7$ cells/ml for antigen stimulation in the ELISPOT assay. Depletion was confirmed by fluorescence-activated cell sorter (FACS) analysis after staining cells with phy-

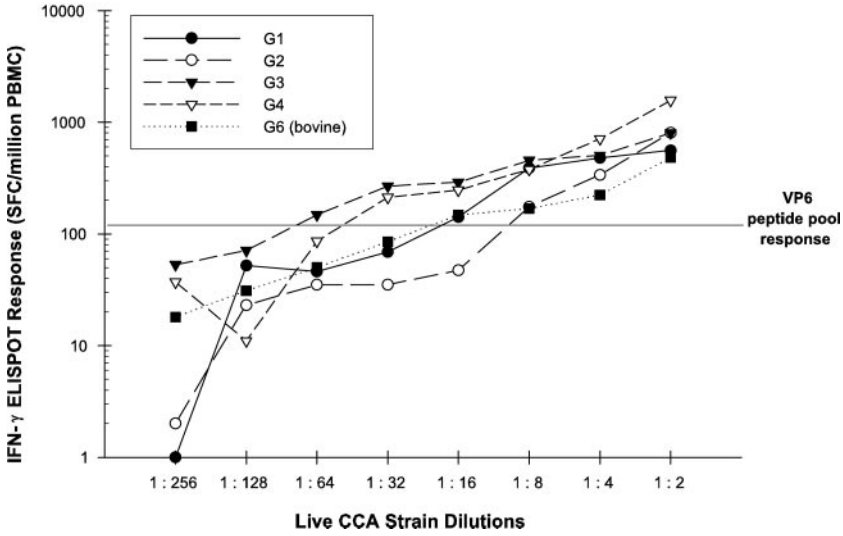


FIG. 2. Titration of five live cell-culture adapted rotavirus strains with donor 5020 PBMCs. Dilutions of cell-culture adapted strains were made from the initial stock concentrations. Response to the VP6 peptide pool (0.8 μM) is indicated by the reference line.

coerythrin-conjugated anti-human CD4 antibody (BD PharMingen, San Diego, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-human CD8 antibody (BD PharMingen).

**Antigens.** The rotavirus VP6 peptide pool consists of 37 individual 20-mer peptides, with each peptide overlapping the next by 10 amino acids. The 37 peptides span amino acids 1 to 376 of the human VP6 gene of Indian neonatal rotavirus strain 116E (10). This human VP6 group A sequence, from GenBank accession number AAB46985, was chosen for being as close as possible to a consensus sequence for multiple strains yet still representing a single specific rotavirus strain sequence. The peptides were synthesized by Mimotopes Pty., Ltd. (Clayton Victoria, Australia). Each peptide was reconstituted to ~10 mM in DMSO (Sigma-Aldrich) and stored at −70°C. All 37 peptides were pooled at a concentration of 270 μM for each peptide. The final VP6 peptide pool dilution in the assay (0.8 μM for each peptide, in complete medium with 0.5% DMSO) was selected from titration experiments. A 0.5% DMSO control was incorporated in the assay to serve as background control for the VP6 peptide pool.

Both the cell culture-adapted (CCA) strains (human G1, G2, G3, G4, and bovine G6 rotavirus strains) and reassortant human-bovine vaccine strains were analyzed in the IFN-γ ELISPOT assay. The strains tested were representative of prevalent human serotypes, including the virus serotypes in the vaccine presently under development by Merck & Co., Inc.

Human and bovine rotavirus strains were grown in MA-104 cells, clarified by centrifugation (8,400 × *g* at 4°C), and pelleted at 101,000 × *g* at 4°C, and pellets were resuspended in phosphate-buffered saline (PBS). Infectivity titers were determined by plaque assay in MA-104 cells and stained with neutral red. Four CCA human strains (G1 W179 at $4 \times 10^7$ PFU/ml, G2 SC2 at $5 \times 10^7$ PFU/ml, G3 W178 at $1.4 \times 10^8$ PFU/ml, and G4 BrB at $2.1 \times 10^8$ PFU/ml) and one CCA bovine strain (G6 WC3 at $6 \times 10^8$ PFU/ml) were grown and concentrated. Both live and UV-inactivated individual strains were tested as indicated. The human viral pool contained UV-inactivated CCA human G1, G2, G3, and G4 strains. Individual UV-inactivated CCA human strain concentrations in the pooled stock were tested at $1 \times 10^5$ PFU equivalents/well in the ELISPOT assay. Complete medium or UV-inactivated rotavirus vaccine uninoculated control (spent culture medium from uninfected Vero cell cultures) was incorporated in the assay to serve as a background control for the CCA strains.

The rotavirus reassortant human:bovine vaccine (7, 8) consists of a bovine WC3 genome background expressing the human rotavirus surface proteins VP7 (G1, G2, G3) or VP4 (P1). The human rotavirus VP7 (G4) strain was naturally reassorted in cell culture using the same methodology as the other strains cited above. The identity of reassortant vaccine strains were confirmed by electropherotyping and sequencing. The concentrations of the stocks for the five individual reassortant vaccine strains used in this study were as follows: G1 at $2.3 \times 10^7$ PFU/ml, G2 at $3.4 \times 10^7$ PFU/ml, G3 approximated to be $2 \times 10^7$ PFU/ml, G4 at $2.3 \times 10^7$ PFU/ml, and P1 at $11.4 \times 10^7$ PFU/ml. The reassortant vaccine strains were cultured in Vero cells and clarified by centrifugation. Infectivity titers were determined by plaque assay in MA-104 cells and stained with neutral red. All reassortant strains were UV inactivated prior to testing individually or as a pooled stock. The reassortant viral pool contained UV-inactivated G1, G2, G3, G4, and P1 reassortant strains. The final concentrations for the individual reassortant strains in the reassortant viral pool were tested at the highest concentration possible when pooling the individual strains (i.e., a 1:10 final dilution of each individual strain stock in ELISPOT assay well).

UV-inactivated rotavirus vaccine uninoculated control was incorporated in the assay as a background control for the reassortant vaccine strains. This vaccine uninoculated control was spent culture medium from uninfected Vero cell cultures grown under the same conditions as the virus-infected cultures. Final dilution in the assay was selected to align with the dilution used for the reassortant strain preparations (i.e., a 1:10 final dilution in the ELISPOT assay well).

The CCA strains and reassortant vaccine strains were UV inactivated using a Stratalinker 2400 (Stratagene, La Jolla, CA) within a biosafety cabinet. For each strain, 200 μl of concentrated stock was added to each well of a sterile 24-well plate then placed inside the Stratalinker with the lid removed. Strains were treated with 800 μJ × 100 three times, rotating the plate between treatments. Individual wells of the same strain were then pooled and aliquots frozen at −70°C. Infectivity titers were performed on each of the individual strains to confirm inactivation of the virus by the UV light treatment. The UV-treated strains did not have detectable infectivity (<50 PFU/ml).

Phytohemagglutinin (PHA-M) (Sigma-Aldrich) at 5 μg/ml was assayed as a positive control mitogen.

**IFN-γ ELISPOT assay for rotavirus responses.** Development of the IFN-γ ELISPOT assay for response to rotavirus antigens was based upon the procedure previously described by Smith et al. (32) for varicella-zoster virus. Briefly, 100 μl of an anti-human recombinant IFN-γ monoclonal antibody (catalog no. M-700A; Endogen Rockford, IL), diluted to 1 μg/ml in sterile PBS, was added to each well of a 96-well MultiScreen-IP membrane plate (Millipore, Bedford, MA) and stored overnight at 4°C. The wells were washed three times with sterile PBS, then blocked by adding 200 μl of complete medium and incubated at 37°C with 5% $CO_2$ for 1 to 4 h. The wells of the plate were washed once with complete medium.

Fifty μl of complete medium containing the appropriate antigen was added to each well. Next, 50 μl of thawed PBMC cell suspension, at $1 \times 10^7$ cells/ml, was added to each of two replicate wells for each antigen tested, with $5 \times 10^5$ cells added to each well. Plates were incubated for 16 to 20 h at 37°C, 5% $CO_2$, and 95% humidity. Plates were washed six times with PBS containing 0.005% Tween-20 (ELISPOT wash buffer). A biotinylated anti-human recombinant IFN-γ antibody (catalog no. M-701-B; Endogen) was diluted to 1 μg/ml in PBS containing 5% heat-inactivated fetal bovine serum and 0.005% Tween 20 (2nd antibody buffer), and 50 μl was added to the wells. After overnight incubation at 4°C, the plates were washed six times with ELISPOT wash buffer. Streptavidin-alkaline phosphatase (Pierce, Rockford, IL) was diluted 1:3,000 in 2nd antibody buffer, and 100 μl was added to each well of the assay plate. After incubation at room temperature for 1.5 to 2.5 h, the plates were washed three times with ELISPOT wash buffer followed by three washes of PBS. 1-Step NBT/BCIP

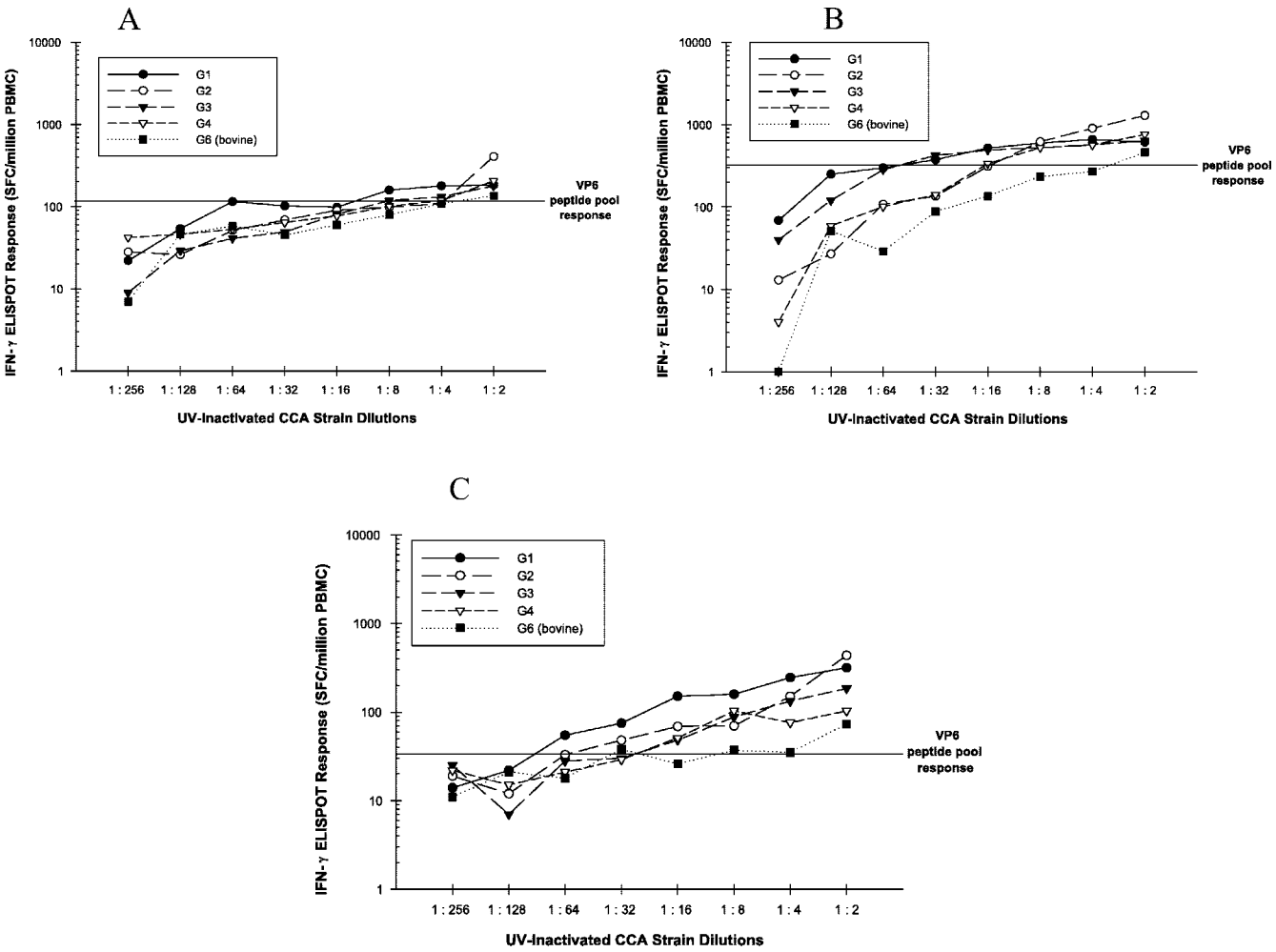


FIG. 3. Titration of five UV-inactivated cell culture-adapted rotavirus strains with PBMCs from donors 5020 (A), 42 (B), and 3742 (C). Concentrations for all cell culture-adapted strains were normalized to $4 \times 10^7$ PFU/ml equivalents, and subsequent dilutions were made. Response to the VP6 peptide pool (0.8 μM) is indicated by the reference line.

(Nitro Blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolylphosphate ρ-toluidine salt) (Pierce) was added to each well, and spots were developed for 3 to 15 min at room temperature. The wells were rinsed with water to stop the reaction, and the plates were allowed to dry overnight. The resulting spots were examined using an ImmunoSpot Image Analyzer system (Resolution Technology Inc./Cellular Technology Limited, Cleveland, OH) for automated plate scanning, well imaging, and spot enumeration.

ELISPOT responses were expressed as net spot-forming cells (SFC) per million PBMCs. Final antigen responses were calculated by subtracting out background response. DMSO was used as a background control for the VP6 peptide pool. Media or the rotavirus vaccine uninoculated control was used for the CCA strains. The rotavirus vaccine uninoculated control was used as a background control for the rotavirus reassortant vaccine strains.

**Concordance analysis details.** Responses to the VP6 peptide pool and responses to the reassortant viral pool were independently compared to responses to the human viral pool. A concordance intercept and slope were estimated separately for each comparison. The estimated slope was utilized to establish the percent change in response (22).

## RESULTS

**Antigen titrations.** The VP6 peptide pool was titrated so that each peptide in the pool was titrated from 2.5 μM to $2 \times 10^{-3}$ μM. All peptide pool levels were tested with a final concentration of 1% DMSO in the preliminary titration studies. Titrations were tested in multiple runs against PBMCs from donor 5020 (Fig. 1). A concentration of 0.8 μM was selected to be used in all subsequent assays. The final DMSO concentration was decreased to 0.5% in subsequent assays to reduce any toxic effects the DMSO could have on the PBMCs during incubation. In the following CCA and reassortant strain titration studies (Fig. 2, 3A to C, and 4A to C) the responses to the VP6 peptide pool, tested concurrently in each donor run, are indicated by the reference line in each plot.

Live CCA strains were titrated by twofold dilutions from their initial stock concentrations and tested against PBMCs from donor 5020 (Fig. 2). The measured IFN-γ ELISPOT response to each of the CCA strains increased as the PFU concentration was increased. None of the five strains was observed to reach a plateau in response level, even at the most concentrated PFU tested. To determine whether the rising response to the live CCA strains was related to their ability to replicate, each strain was subsequently UV light treated to inactivate infectivity. The UV-inactivated CCA strains were

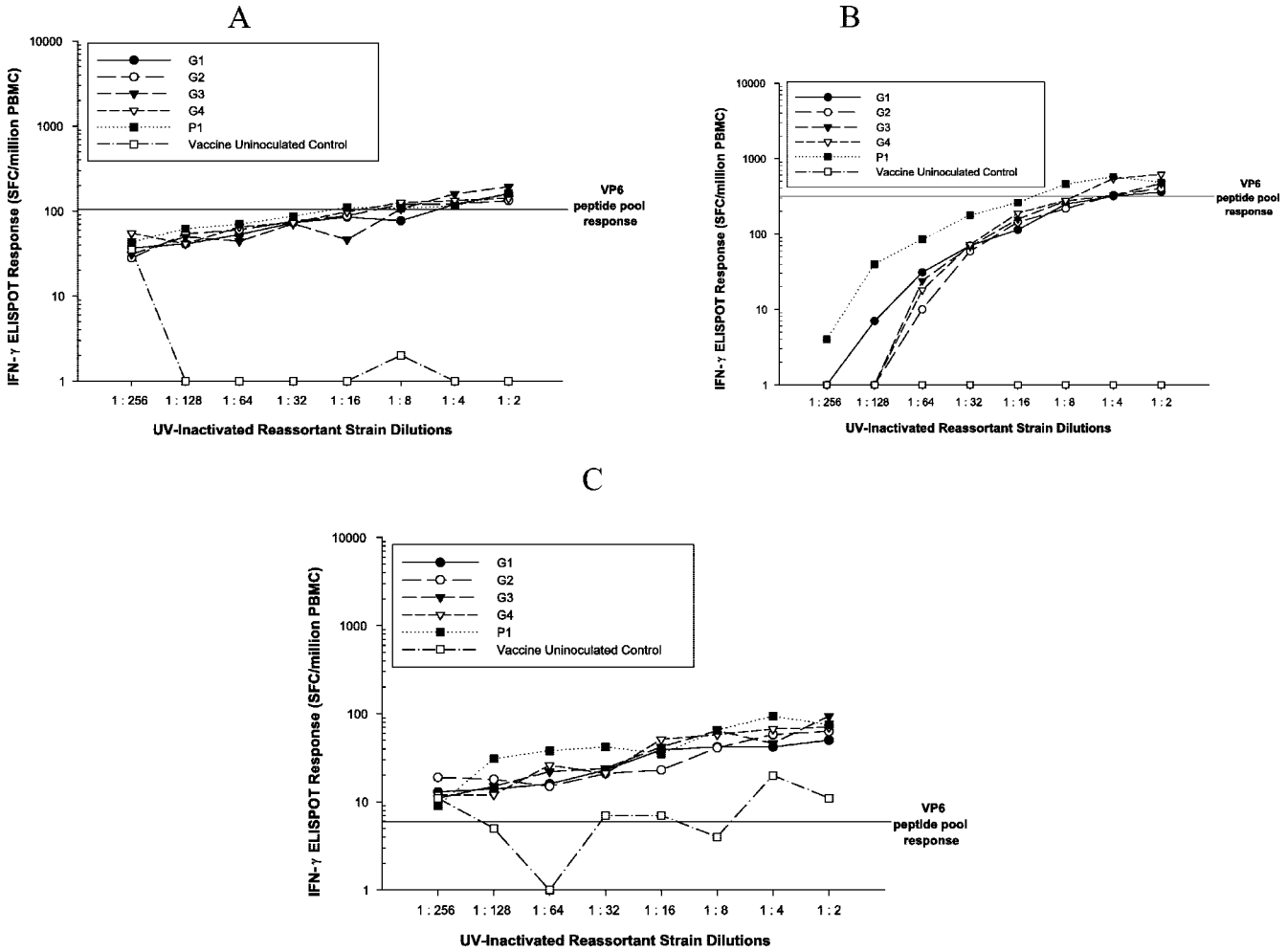


FIG. 4. Titration of five UV-inactivated rotavirus reassortant vaccine strains with PBMCs from donors 5020 (A), 42 (B), and 3742 (C). Dilutions of reassortant strains were made from initial stock concentration. Response to the VP6 peptide pool (0.8 μM) is indicated by the reference line.

normalized to $4 \times 10^7$ PFU/ml equivalents and then titrated in twofold dilutions (Fig. 3A) with PBMCs from the same donor bleed used in Fig. 2. The IFN-γ ELISPOT response with donor 5020 PBMCs, expressed as net SFC per million PBMCs, was reduced by approximately twofold compared with the responses measured to the non-UV-inactivated (live) CCA strains. No response plateaus were observed with any of the UV-inactivated strains, even at the highest PFU equivalent levels tested. This is consistent with the pattern observed using the non-UV-inactivated (live) strains. Two additional donors were tested, donor 42 and donor 3742 (Fig. 3B and C). The response profiles observed were similar to those observed with donor 5020; no response plateaus were observed with any of the strains for either donor.

The rotavirus reassortant vaccine strains were UV inactivated and titrated in twofold dilutions from their initial stock concentrations in an ELISPOT assay with PBMCs from donors 5020, 42, and 3742 (Fig. 4A to C). The ELISPOT responses increased along with increase in the PFU equivalent of reassortant vaccine stock added. No response plateaus were observed for any of the reassortant strains with any of the three donors, even at the highest PFU equivalent concentrations tested. The observed difference in responses to the UV-inactivated reassortant strain titrations compared with the UV-inactivated CCA human strains were donor dependent. For donor 5020, the responses with both viral strain types were similar for all five serotypes. Donor 42 had a twofold reduction in IFN-γ ELISPOT response to the reassortant strains compared with the CCA strains. While donor 3742, whose overall response to the viral strains is low, showed a higher variability in fold response changes between the viral strain types ranging from similar responses for G3 and G4, a twofold reduction for reassortant strain G2, and a threefold reduction for reassortant strain G1 when compared to the responses to the respective CCA strains.

Endotoxin testing was performed on the individual UV-inactivated CCA strains, using the Limulus Amebocyte Lysate Endosafe KTA kit from Charles River Endosafe, and indicated the presence of a low level of endotoxin in the G2 and G4 strains but not the G1, G3, or G6 strains (data not shown). At $1 \times 10^5$ PFU/well, the concentration of endotoxin activity was 2.0 endotoxin units/ml for the G2 strain and 0.2 endotoxin units/ml for the G4 strain. In an effort to determine if the endotoxin activity was responsible for the IFN-γ ELISPOT

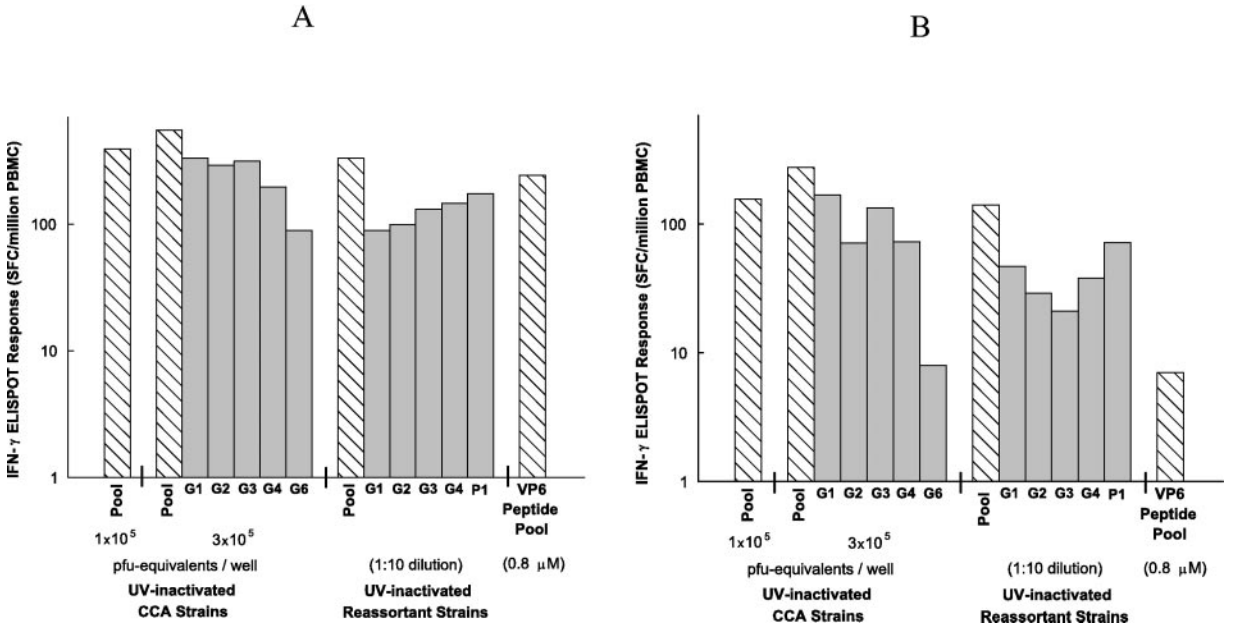


FIG. 5. IFN-γ ELISPOT response comparison of individual versus pooled UV-inactivated viral strains with PBMCs from donors 42 (A) and 38 (B). The human viral pool consisted of human G1, G2, G3, and G4 strains. The reassortant viral pool consisted of G1, G2, G3, G4, and P1 reassortant strains.

responses observed with the G2 and G4 strains, polymyxin B (10 μg/ml) was added to titrated concentrations of all the CCA strains and tested in the IFN-γ ELISPOT assay using PBMCs from three different donors (data not shown). For two of the donors, the addition of polymyxin B did not change the kinetics of the observed response plots for any of the five strains. For the third donor, the addition of polymyxin B did not change the kinetics of the observed response plots for the G3 and G6 strains. However, with the G1, G2, and G4 strains, there was a reduction in IFN-γ ELISPOT response, but only observed at concentrations greater than $1.25 \times 10^5$ PFU/well. This appears to be a donor-specific observation.

**Individual versus pooled responses.** A concentration of $3 \times 10^5$ PFU equivalents/well was initially selected for each of the CCA strains. Each CCA strain was tested at $3 \times 10^5$ PFU equivalents/well alongside a pool of the G1, G2, G3, and G4 CCA strains, with each strain in the pool at $3 \times 10^5$ PFU equivalents/well. Due to the small volume of concentrated CCA strains available for this study, a second human viral pool was also tested with the G1, G2, G3, and G4 strains at a concentration of $1 \times 10^5$ PFU equivalents/well.

TABLE 1. Comparison of IFN-γ ELISPOT responses to two different concentrations of UV-treated human viral pool

| Donor | Mean SFC/$10^6$ PBMCs at human pool concn of [a]: | |
|---|---|---|
| | $1 \times 10^5$ | $3 \times 10^5$ |
| 08 | 58 | 170 |
| 09 | 32 | 39 |
| 11 | 84 | 108 |
| 31 | 34 | 84 |
| 33 | 176 | 369 |
| 34 | 292 | 446 |
| 38 | 152 | 271 |
| 40 | 648 | 819 |
| 41 | 130 | 250 |
| 42 | 396 | 556 |
| 4597 | 88 | 115 |
| 5020 | 98 | 240 |

[a] Human pool concentrations are given in PFU equivalents of each strain/well.

The reassortant vaccine strain stock concentrations were lower than the CCA strain stocks, restricting the highest concentration for assay testing. The concentration chosen was the highest PFU concentration possible for each of the five strains when pooled together. Therefore, each strain was tested in the assay well at a final dilution of 1:10 from the initial stock concentration, both for individual strain testing and when combined within the reassortant viral pool.

The VP6 peptide pool at 0.8 μM, individual UV-inactivated CCA strains at $3 \times 10^5$ PFU equivalents/well, UV-inactivated human viral pool at $3 \times 10^5$ PFU equivalents/well and $1 \times 10^5$ PFU equivalents/well, and the UV-inactivated individual and pooled reassortant vaccine strains at 1:10 final dilution were all tested against a panel of 12 donors. See Fig. 5A for data representative of these 12 donors. IFN-γ ELISPOT responses obtained using the human CCA viral pools at $3 \times 10^5$ PFU equivalents/well were approximately 1.5-fold higher than responses obtained with the $1 \times 10^5$ PFU equivalent/well concentration (Table 1). However, responses to CCA strains for individual donors were still quantifiable above background and strain response profiles remained consistent, while reducing the amount of concentrated CCA stocks required to support testing. Therefore, $1 \times 10^5$ PFU equivalents/well was selected for all subsequent analysis and assays. For all donors tested, there was a modest increase observed in IFN-γ ELISPOT response to the pooled strains when compared with the responses to the individual strains for both the CCA and reas-

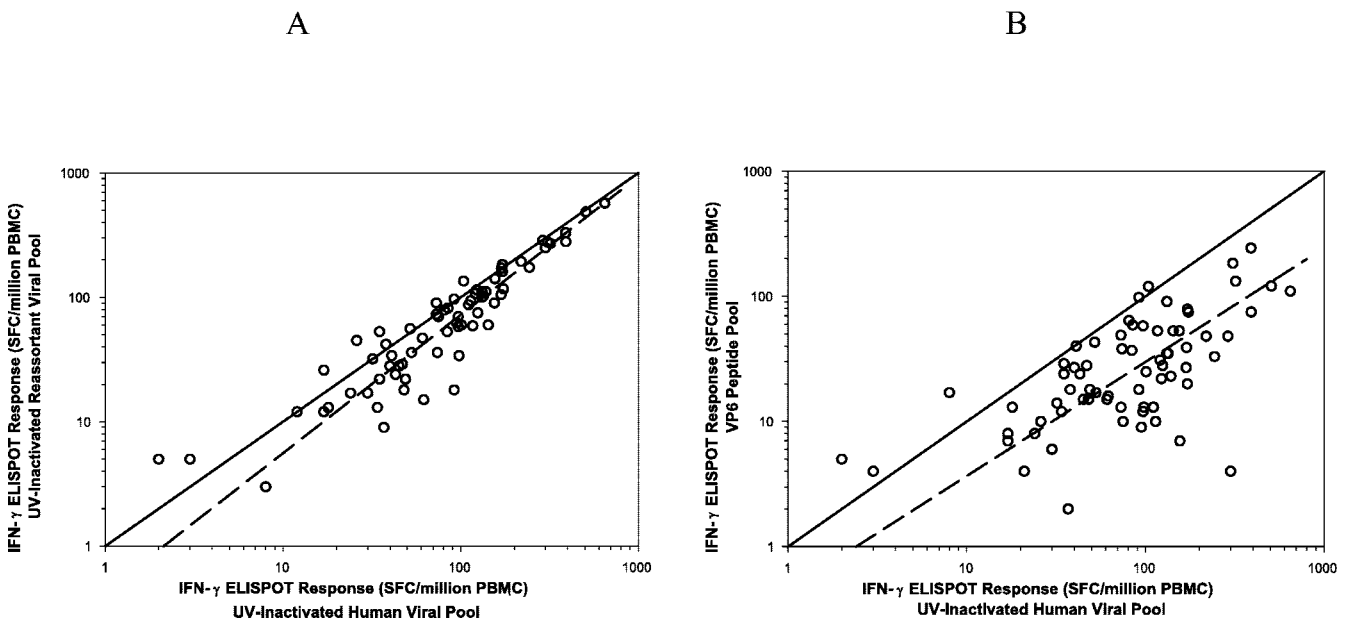


FIG. 6. Concordance line plots for rotavirus antigen pools. The VP6 peptide pool was tested at 0.8 μM; each strain in the human viral pool was tested at $1 \times 10^5$ PFU equivalents/well, and each strain in the reassortant viral pool was tested at a 1:10 dilution from concentrated stock. The predicted concordance line is solid, and the observed concordance line is hatched.

sortant strains. Ten of the 12 donors tested had a ≤1.6-fold difference in IFN-γ ELISPOT response to the human and reassortant viral pools, with the largest of these fold differences observed with the lowest responders (data not shown). For most of the donors tested, the ELISPOT response to VP6 peptide pool was similar to the responses detected with both the CCA and reassortant strains. However, donor 38 (Fig. 5B) had a very low response to the VP6 peptide pool but a high response to both the CCA and reassortant vaccine strains. This may represent a donor with minimal response to conserved epitopes between the three antigen stocks (i.e., the VP6 antigen) but stronger response to epitopes in the more variable domains of the antigen stocks (e.g., VP7 and VP4 antigens).

Testing pooled viral strains, as opposed to individual strains, improves practicality and throughput for donor screening due to the greatly reduced number of PBMCs (and reagents) required per subject test. An additional 61 donors were tested against the VP6 peptide pool and the human CCA and reassortant viral pools. The concordance in response between the reassortant viral pool and human viral pool and between the peptide pool and the human viral pool was statistically assessed (Fig. 6A and B). The concordance slope comparing the reassortant viral pool to the human viral pool was 1.13 (90% confidence interval [CI] = 0.95, 1.35), meaning that, on average, a 10-fold difference in response in the human viral pool corresponds to a 13.5-fold difference (90% CI = 8.9-fold, 22.2-fold) in the reassortant viral pool. The concordance slope comparing the peptide pool to the human viral pool was 0.94 (90% CI = 0.70, 1.26), meaning that, on average, a 10-fold difference in response in the human viral pool corresponds to an 8.7-fold difference (90% CI = 5.1-fold, 18.2-fold) in the peptide pool. Responses detected with the reassortant and VP6 peptide pools were, on average, 28.8% (90% CI = −41.1%, −13.9%) less and 68.4% (90% CI = −75.2%, −59.8%) less than the response detected with the human viral pool, respectively. When we defined a positive response as ≥20 net SFC/$10^6$ PBMCs, 89% of the 73 donors tested responded to the human viral pool, 78% responded to the reassortant viral pool, and 55% responded to the VP6 peptide pool. This analysis indicates that the difference in IFN-γ ELISPOT responses between the human and reassortant viral pools could be significant, but the response rate of 78% for the reassortant pool is high enough to be considered as an alternative to testing with the human viral pool for indicating that a particular donor has an IFN-γ ELISPOT response to rotavirus.

**T-cell phenotype.** T-cell depletion studies were performed to investigate the phenotype of the T cells contributing to the IFN-γ ELISPOT responses to the various antigens. Initially, PBMCs from three donors were thawed with fractions being depleted of $CD8^+$ T cells, $CD4^+$ T cells, or left undepleted. After depletion, the remaining cells were counted again and $5 \times 10^5$ of the remaining cells were added to each well. Therefore, the cell population where the $CD8^+$ T cells were depleted is now enriched for the $CD4^+$ T cells and vice versa. IFN-γ ELISPOT responses were assessed to the VP6 peptide pool and the individual CCA strains and reassortant strains (Fig. 7A to C). Donors 33 and 38 had minimal IFN-γ ELISPOT responses detected to any of the three antigen stocks following $CD4^+$ T-cell depletion (<2% $CD4^+$ T cells by FACS [data not shown]), but little to no reduction in detected response was observed when the $CD8^+$ T-cell population was depleted (<2% $CD8^+$ T cells by FACS [data not shown]). Donor 42 also exhibited little to no reduction in response when the $CD8^+$ T-cell population was depleted. However, a strong response to the VP6 peptide pool was still observed with donor 42 following $CD4^+$ T-cell depletion.

T-cell depletion studies were repeated with donor 42 and an additional five donors, with ELISPOT testing performed using

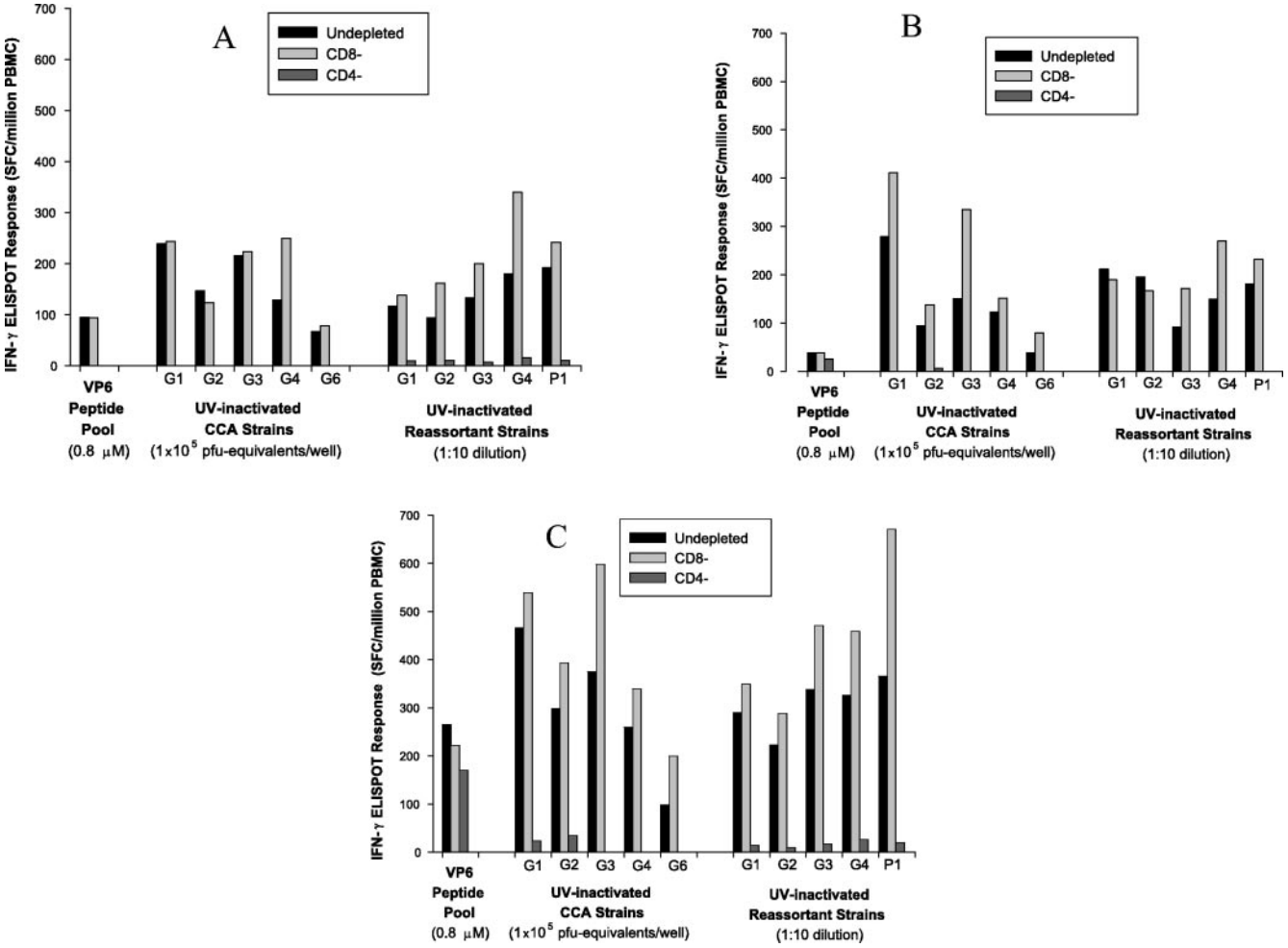


FIG. 7. T-cell phenotype of response to peptide pool versus individual viral strains with PBMCs from donors 33 (A), 38 (B), and 42 (C).

the human and reassortant viral pools instead of the individual strains (Fig. 8A to C). Consistent with the previous results, the response with donor 42 PBMCs (Fig. 8A) to the VP6 peptide pool was not greatly diminished following $CD4^+$ T-cell depletion. Two additional donors had a substantial antigen response following $CD4^+$ T-cell depletion (<1% $CD4^+$ T cells by FACS [data not shown]): donor 41 (Fig. 8B) to the peptide pool and donor 34 (data not shown) to the reassortant viral pool. All six donors had some increase in IFN-γ ELISPOT response to the VP6 peptide pool and viral pools following $CD8^+$ T-cell depletion (<1% $CD8^+$ T cells by FACS [data not shown]). This would suggest that the IFN-γ ELISPOT response to the rotavirus antigens tested for these three donors (41, 42, and 34) is a mix of $CD4^+$ and $CD8^+$ T-cell components. The three donors 5107 (Fig. 8C) and 40 and 5020 (data not shown) had no significant response to any of the rotavirus antigens following $CD4^+$ T-cell depletion, exhibiting a completely $CD4^+$ T-cell response profile to all rotavirus antigens tested.

## DISCUSSION

Cellular immunity has been shown to be important for rotavirus disease prevention in animal models, suggesting a role during the human immune response to rotavirus infection (11, 16, 25, 26, 35, 38). However, exploration of CMI response as a possible correlate of protection in humans remains to be determined. There is a need for development of improved assays for performing these types of assessments to facilitate further studies. To this end we have developed an IFN-γ ELISPOT assay that can both detect and quantitate the human cellular immune response to rotavirus antigens. We demonstrated that approximately 90% of the healthy adult donors tested in this new assay exhibited a memory response to rotavirus antigens. In many cases, the ELISPOT response to a human VP6 peptide pool was predictive of the individual's overall response to both CCA and reassortant vaccine strains. It is not surprising that the response to VP6 represents a major component of the overall cellular immune response, as VP6 is the most abundant viral protein (51% of the virion) (15) and is the major structural component of the viral particle. Also, the amino acid sequence of the VP6 protein is highly conserved (87 to 99%) among group A rotaviruses in mammals (33). T-cell depletion data suggested that the response to the VP6 peptide pool, while on average predictive of overall response to virus, represents only a subset of the total cellular immune response to rotavirus. There is a positive correlation between

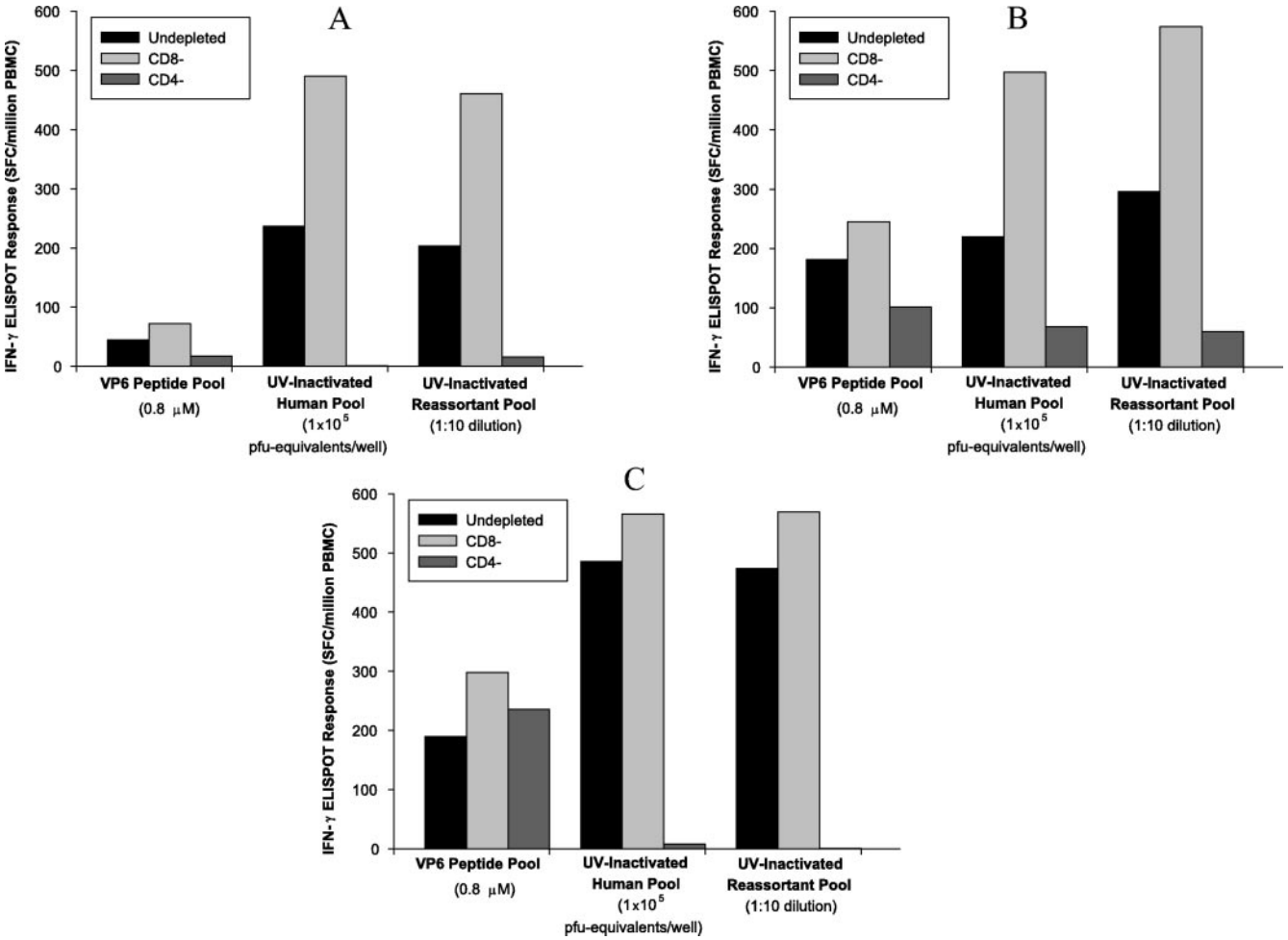


FIG. 8. T-cell phenotype of response to peptide pool versus pooled viral strains with PBMCs from donors 42 (A), 41 (B), and 5107 (C).

the ELISPOT response to this human VP6 peptide pool and to the reassortant vaccine strains (data not shown), which have a bovine (WC3) strain VP6 background. We speculate that the observed ELISPOT response differences between the VP6 peptide pool and both the CCA and reassortant viral strains most likely represent serotype-specific responses due to the VP7, VP4, and perhaps even virion NSP proteins or other virus-associated proteins.

When comparing ELISPOT responses to the human and reassortant viral pools versus individual strains, the differences were minimal. There was no additive effect observed when the ELISPOT response to the individual viral strains was compared to the response with the viral pools, for either CCA or reassortant strains. The majority of detected ELISPOT response may be to epitopes conserved between the strains, including the highly conserved VP6 viral protein. There are practical advantages to using viral strain pools instead of the individual strain stocks as the cellular response to multiple serotypes can be investigated simultaneously with fewer PBMCs required for assay testing. This is especially important for clinical testing applications where the volume of blood that can reasonably be obtained is limited, especially in pediatric patient populations.

The IFN-γ ELISPOT assay we developed has added advantages over another IFN-γ ELISPOT assay recently published by Rojas et al. (31). The first advantage is in the choice of antigen. While we use pools of common serotypes, whether they are CCA or vaccine reassortants, Rojas et al. used rhesus rotavirus (RRV), representing a single serotype. The VP7 protein from RRV is similar to the human serotype 3, but the VP4 (serotype 5B) for RRV is not related to any human strains (20). This antigen testing approach for human CMI analysis increases the chance of missing responses in subjects within populations exposed to multiple serotypes, either following natural infection or immunization. This risk is demonstrated by the following analyses. We tested 12 donors for ELISPOT responses to the individual CCA and reassortant vaccine strains and observed some distinct differences in response patterns to the individual strains. One subject did not have a detectable ELISPOT response to the CCA G3 strain; however, this subject did have detectable responses to G1 and G2 strains, as well as the human viral pool (>20 SFC/$10^6$ PBMCs above background; data not shown). Three donors did not have detectable ELISPOT responses to the reassortant vaccine G3 strain. Only one of these three donors would have been negative for detectable CMI response based on response to the

reassortant viral pool (data not shown). By using a pool of common serotypes, there is increased opportunity for detecting responses to epitopes not conserved across serotypes.

The second advantage is in the method we use for our depletion assay which ensures that the antigen-presenting cells are present throughout the assay for the $CD4^+$ IFN-γ T-cell response assessment. While we depleted the total cell population of $CD8^+$ T cells and then stimulated the remaining cells for 16 to 20 h in the ELISPOT assay, Rojas et al. stimulated the total cell population for only 6 h, performed a positive selection for $CD4^+$ T cells and placed only those activated $CD4^+$ T cells in the ELISPOT assay with IFN-γ-specific monoclonal antibodies. Using their IFN-γ ELISPOT assay, Rojas et al. (31) detected RRV-specific T-cell responses in children with diarrhea that appeared to correlate with a history of exposure to rotavirus. They reported that $CD8^+$ T cells were predominantly responsible for the response detected in their IFN-γ ELISPOT assay with PBMCs from recently infected children. They further reported comparable $CD4^+$ and $CD8^+$ T-cell response components in all healthy adults tested. As Rojas et al. mentioned, the presence of antigen-presenting cells is necessary for a functional $CD4^+$ IFN-γ T-cell response but not a $CD8^+$ T-cell response. We performed $CD4^+$ and $CD8^+$ T-cell depletions on healthy adult donor PBMCs and compared ELISPOT responses to the undepleted PBMCs in our ELISPOT assay. The predominant IFN-γ ELISPOT response in healthy adults was detected in the $CD8^+$ T-cell-depleted populations, with all eight donors having comparable or increased IFN-γ ELISPOT responses following $CD8^+$ T-cell depletion compared with their respective undepleted cell population. When the $CD4^+$ T cells were depleted, five of the eight donors had no detectable IFN-γ ELISPOT response to rotavirus antigens. The other three donors had a mix of $CD4^+$ and $CD8^+$ T-cell responses to the antigens tested. Somewhat different from what was reported by Rojas et al. with ELISPOT response to RRV, our data indicate that the T-cell phenotype of healthy adult memory response to rotavirus is donor specific and can be a mixture of levels of $CD4^+$ and $CD8^+$ T-cell-mediated responses. A predominantly $CD4^+$ response was observed in all of these healthy adult subjects, with some subjects exhibiting levels of detectable $CD8^+$ T-cell response, mainly to the VP6 peptide pool. A different response phenotype profile to these antigens may be observed in subjects recently infected with rotavirus or in vaccinated children.

Assays for assessment of cell-mediated immunity would be helpful to characterize the role of CMI both during clearance of infection and for protection against infection. ELISPOT assays for detection of IFN-γ production have been effectively utilized in other infectious diseases for monitoring changes in cell-mediated immune responses (1, 17, 23, 32, 37). The ELISPOT assay described in this article represents an advance with the ability to use either a peptide pool based on a conserved protein (VP6) or pooled viral strains to effectively measure cell-mediated immune responses. Application of this assay in studies of vaccines and/or individuals naturally exposed to rotavirus may help identify a better correlate of protection against rotavirus disease to facilitate evaluations of vaccine candidates and to gain a broader understanding of the host response to rotavirus infections.

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