Detection of Cellular Immunity to Rabies Antigens in Human Vaccinees

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A nonradioactive multi-parameter flow cytometry assay was developed to identify antigen-specific lymphocytes in human subjects previously vaccinated against rabies virus and was subsequently compared to the standard tritiated thymidine method. A cell tracking dye, carboxyfluorescein succinimidyl ester, was used in combination with surface label for CD4 and CD8 cells in order to determine the response of lymphocytes to killed rabies virus in an antigen recall assay. The rabies virus-specific lymphocyte response was compared to the humoral immune response in each of ten vaccinated and five non-vaccinated subjects. Lymphocyte responses to rabies virus were observed in all ten vaccinated subjects; some noted as early as 3 days after stimulation while others were not until 7 days after stimulation. There was good agreement between the proliferation index of the CFSE assay and the simulation index of the [^{3}H]thymidine assay (*kappa* statistic = 0.73). An inverse relationship was detected between the level of rabies virus neutralizing antibody (RVNA) and the lymphocyte response to inactivated rabies virus in the vaccinated subjects. The association between cytokines production and level of humoral and cellular response was investigated in four representative subjects. Two vaccinated subjects with high proliferation indices and low RVNA titers produced Th1 type cytokines to rabies virus stimulation, whereas two vaccinated individuals with low proliferation indices and high RVNA titers responses did not produce these cytokines.

KEY WORDS: Carboxyfluoroscein succinimidyl ester; *in vitro* proliferation; flow cytometry; rabies; vaccine response; cytokines.

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INTRODUCTION

In spite of the accessibility of effective cell culture rabies vaccines, the ever-increasing use of post exposure prophylaxis, and the availability of proven methods to effectively control stray animals, rabies continues to be a major public health problem ranking 11th among the infectious diseases causing the most global human deaths (1). Rabies has the highest case fatality rate of any infectious disease known to man and is considered to be virtually 100% fatal once symptoms are evident. Fortunately, the rabies virus neutralizing antibody (RVNA) produced in response to vaccination, unquestionably provides protection from this horrific disease (2, 3). In reality, rabies vaccination not only induces RVNA but also results in the production of long-lived plasmacytes and memory B and T cells. However, because of the critical importance of RVNA in protecting patients exposed to rabies virus through animal bite wounds inflicted by infected animals or other means, and because laboratory methods to measure the humoral immune response are easier to perform than methods that measure the cellular immune response, RVNA assays are considered to be the 'gold standard' for verifying that an immune response after rabies vaccination has occurred. A dichotomy of RVNA responses has been reported in rabies vaccinated subjects indicating that some individuals respond to vaccination earlier and produce high titers (early/high responders) while others respond to vaccination later and produce lower titers (late/low responders) (4, 5). Though a correlation between survival and the level of RVNA has been reported previously, there are also published reports of vaccinated animals that had no detectable RVNA present and yet survived rabies challenge thus indicating other immune mechanisms protect against rabies infection (6, 7).

Rabies is one of the 'Th2-healing diseases' in which activation of B cells, with the help of CD4$^+$ T cells, is

important for immune protection [8]. When activated, primarily by the N protein of the rabies virus, CD4$^+$ T cells produce specific cytokines (i.e. IL-4) that drive antibody production, referred to as a Th2 response. In contrast, rabies-specific CD8$^+$ T cells cause neuronal damage when a Th1 response (IFN-γ and TNF-α) is the predominate immune response against rabies infection [9, 10]. Previous studies have reported that CD4$^+$ cells are the primary T lymphocyte subset that responds to rabies vaccination in humans; however, the cytokine profiles associated with rabies antigen-specific CD4$^+$ memory cells have not been well defined [11, 12]. The 'gold standard' method of analysis used to measure lymphocyte proliferation is the tritiated thymidine ([^{3}H]thymidine) assay. The disadvantages of this assay are the required use of radioactive material to detect proliferating cells and the fact that it only measures the bulk proliferation of lymphocytes in culture at a specific time point. Recently, new methods that utilize cell tracking dyes in conjunction with flow cytometry have been used to quantitate generations of proliferating cells in culture while simultaneously using fluorescent-labeled antibodies to cluster differentiation (CD) antigens to identify specific subsets of lymphocytes [13, 14]. The objective of this study was to develop an assay using the cell tracking dye, carboxyfluoroscein succinimidyl ester (CFSE), for the cumulative measurement and subset characterization of lymphocyte responses to rabies viral antigens in subjects vaccinated against rabies, to compare this novel assay to the traditional [^{3}H]thymidine assay, and to determine if there was a correlation between the humoral response of subjects and their cellular immune response after vaccination.

METHODS

Human Subjects

Fifteen healthy volunteers participated in this study. Written informed consent was obtained from all subjects after explanation of the study and prior to study initiation. The study protocol was approved by the Committee for Research Involving Human Subjects at Kansas State University (Institutional Review Board protocol). Five volunteers formed a sex- and age-matched control group to evaluate the cellular reactivity to rabies virus proteins in subjects that had not received rabies vaccine. Ten of the subjects in the study had been previously vaccinated with either human diploid cell rabies vaccine (HDCV) or purified chick embryo cell culture rabies vaccine (PCECV) from 5 months to 19 years earlier and had received an average of five total rabies vaccinations (Table I). All except

Table I. Demographic and Immunization Data of Ten Rabies Subjects Previously Vaccinated with Either HDCV or PCECV and Five Sex-Matched Non-Vaccinated Subjects

Subject number	Age (years)	Sex (M/F)	Δ Years	Total # of vaccines
1	37	M	5	7
2	35	F	5	8
3	45	F	5	4
4	26	F	0.5	4
5	27	F	1	5
6	36	F	5	4
7	35	F	2	5
8	49	F	19	5
9	33	F	12	3
10	36	M	12	5
11C	42	F		
12C	30	M		
13C	48	F		
14C	49	F		
15C	47	N		

Notes. M, male; F, female; Δ, difference between year of study and year of last immunization; Total # is the sum of all vaccines given including pre-exposure series; control subjects are designated by C following the subject number.

one subject received at least one booster dose of vaccine between the first vaccination series and the blood collection. The vaccinated subjects all worked or had worked in a rabies research/diagnostic laboratory where there exists a potential risk of rabies exposure.

Rabies Vaccine

HDCV is prepared from the Pitman Moore rabies virus strain (PM) and PCECV is prepared from the Flury low egg passage rabies virus strain (Flury LEP). Both vaccines are produced from inactivated whole rabies virus. All cell culture rabies vaccines are required to have a potency ≥ 2.5 IU/intramuscular dose as per the National Institutes of Health (NIH) test. Vaccine potency variation of >2.5 IU/dose has not been demonstrated to be a factor in the level of the humoral response after intramuscular administration of cell culture rabies vaccine [5].

Antibody Determinations

RVNA levels expressed as either titers or IU/mL were determined by the rapid fluorescent focus inhibition test (RFFIT) as previously described [15]. Briefly, five-fold dilutions of heat-inactivated serum were incubated with a 50 TCID$_{50}$ dose of live rabies virus, Challenge Virus Strain-11 (CVS-11) (Centers for Disease Control and Prevention, Atlanta, GA) in 8-well chamber slides for 90 min at 37°C, followed by the addition of baby hamster kidney cells (BHK-21) (ATCC, Manassas, VA) and further incubation at 37°C in a 5% CO$_2$ for 20–24 h. After

fixation in cold 80% acetone and staining with fluorescent conjugated anti-rabies antibody (Chemicon, Temecula, CA), the cells in 20 fields per well were examined for the presence of fluorescence under $160 \times$ magnification. The 50% endpoint titer was calculated based on the proportion of fields present with detectable infected cells using the Reed and Muench method (16). Assays were performed on stored sera and reported in international units per mL (IU/mL) by comparing the 50% endpoint titers with the 50% endpoint titer of the U.S. standard for rabies antibodies (CBER/FDA, Bethesda, MD) diluted to 2.0 IU/mL and tested in parallel. An adequate immune response to rabies vaccination is defined by the Advisory Committee on Immunization Practices (ACIP) as complete neutralization at the 1:5 dilution and by the World Health Organization (WHO) as ≥ 0.5 IU/mL (17, 18).

Antigen for In vitro Assays

Rabies virus CVS-11 was propagated in BHK-21 cells. The virus suspension was filtered through a 0.45 μm filter (Nunc, Rochester, NY) and determined to have a viral titer of $10^{5.2}$. The virus suspension was inactivated by adding β propiolactone (Sigma Chemical Company, St. Louis, MO) at a dilution of 1/4000 and incubating at 37°C for 2 h (shaking every 10–15 min). Complete inactivation of the filtrate was confirmed by serial passage of the treated suspension in BHK cells and microscopic examination daily for the presence of virus infected cells using fluorescent conjugated anti-rabies antibodies as a marker.

[3H]thymidine Cell Proliferation Assay

Peripheral blood mononuclear cells (PBMCs) were obtained from 10 to 20 mL whole blood by Ficoll-paque density gradient (1.077) centrifugation. The mononuclear cells were resuspended in RPMI1640 media (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine and 110 U of penicillin per mL, 100 μg of streptomycin (Gibco) per mL, and 10% heat-inactivated fetal bovine serum (FBS) (Gibco) at a concentration of 1×10^6 cells per mL. The assays were conducted in triplicate by plating out 10^5 cells in three separate wells of a 96-well flat bottom culture plate (Costar, Corning, NY). To determine optimization, cells were initially stimulated in culture with phytohemagglutinin-P (PHA) (Sigma Chemical Company) at 10 μg/mL, and inactivated rabies virus (RV) at 1/10, 1/50 and 1/100 dilutions for intervals of 3, 4, 6, or 8 days. For the remaining experiments, cells were stimulated with PHA at 10 μg/mL and inactivated RV at 1/50 dilution for 3 and 7 days. Unstimulated PBMC cultures served as background controls for the study. The cells were incubated at 37°C and 5% CO_2. Eigh-

teen hours prior to each respective time point of harvest, the cells were pulsed with 0.2 uCi/well [3H]thymidine (Amersham Biosciences, Uppsala, Sweden). The level of incorporation of [3H]thymidine into DNA of proliferating lymphocytes was measured by the radioactive counts per minute (cpm) in a liquid scintillation counter (Packard Instrument Company, Meridan, CT). The mean cpms were determined from each triplicate data set and expressed as a stimulation index (SI) (ratio mean cpm for mitogen- or antigen-stimulated cells/mean cpm of unstimulated cells). Evidence of antigen-induced proliferation was defined as a SI greater than two standard deviations of the mean SI of the negative control subjects.

CFSE Cell Proliferation Assay

The PBMCs were suspended in PBS at a concentration of 2×10^7 cells/mL and mixed with an equal volume of a working solution of 10 mM CFSE (Molecular Probes, Carlsbad, CA) diluted to 1/500 (final concentration 10 μM) in initial experiments and to 1/1000 (final concentration 5 μM) in subsequent experiments. After incubation in the dark at 37°C for 10 min, 5 mL of cold FBS was added to stop the uptake of the CFSE dye. The PBMCs were washed $1 \times$ in PBS and resuspended in RPMI1640 media supplemented with 2 mM L-glutamine and 110 U of penicillin per mL, 100 μg of streptomycin per mL, and 10% heat-inactivated (FBS) at a concentration of 1×10^6 cells per mL. CFSE-unlabeled cells were used as a negative control.

Proliferation cultures were set up in duplicate with 7.5×10^5/well in 24-well flat bottom culture plates. PHA (10 μg/mL) and inactivated RV (1/50) were added in duplicate to designated wells containing the CFSE-labeled cells. Control wells containing CFSE-labeled cells in medium alone and CFSE-unlabeled cells in medium alone were tested in parallel. Plates were incubated in at 37°C incubator and 5% CO_2 for 7 days. Fresh medium was added at day 4 to day 5 as needed. On day 7, the cells were harvested. The PBMCs were washed $2 \times$ in PBS and suspended at a concentration of 10^6/mL.

Two-Color Parameter Flow Cytometric Analysis of Lymphocyte Subsets Proliferation and Expression of Activation Markers

CFSE-labeled cells were surface labeled for CD antigens according to established protocols (Becton Dickinson, San Jose, CA) using Becton Dickinson monoclonal antibodies R-PE-anti-CD4 (RPA-T4, IgG1) and R-PE anti-CD8 (RPA-T8, IgG1). An R-PE conjugated isotype control antibody (MOPC-21) was used in separate

tubes to determine background fluorescence and to adjust for compensation.

Data were collected on 10,000 to 20,000 cells and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), equipped with an argon and red diode laser and CellQuest software (Becton Dickinson, San Jose, CA). Proliferating lymphocyte subsets (CD4 and CD8) were distinguished by multi-parameter flow cytometry. Specifically, CFSE stained cells were detected using the FL-1 detector for 530 nm emission and CD4 and CD8 positive lymphocytes were detected using the FL-2 detector for 585 nm emission. Analysis was performed using two-parameter dot plots.

Measuring Lymphocyte Subset Proliferation with CFSE

Analysis of proliferating generations was performed using Proliferation Wizard program of ModFit LT 3.1 software (Verity Software House, Inc., Topsham, ME). Cells were gated according to the forward and side scatter of the lymphocyte population. In addition, gates were placed on R-PE-labeled CD4 and CD8 lymphocytes in a sample. The intensity of the nonproliferating (parental) cells was determined by analysis of the sample cultured in medium without antigenic or mitogen stimulation. Percentages were given for the proportion of cells under each peak or generation from which a proliferation index (PI), nonproliferative fraction, precursor frequency, and upper level generation proliferation index (UGPI) were determined by the software program. The UGPI is a measure of the relative number of cells that have gone through more than a three divisions. The UGPI is more useful for long-term incubations because the width of the parental peak of the histogram can spread due to leakage of the dye and resting cells may start to slowly proliferate from "bystander" effect of soluble factors in the medium. Similar to the SI used for the [3H]thymidine assay, the final proliferation index (PI) was determined by calculating the ratio of the average PI for mitogen- or antigen-stimulated cells divided by the average PI of unstimulated cells. Proliferation indices that were greater than two standard deviations of the mean PI of the negative control subjects were considered as evidence of antigen-induced proliferation. Calculation of the UGPI was conducted in the same manner. A gate was placed on the blast population based on an increase in the forward scatter (cell size) properties of these cells compared to the resting population. The percentage of blasts was determined and referred to as the percentage blast transformation, %BT. A "weighted PI (WtPI)" or "weighted UGPI index (WtUGPI)" was obtained by multiplying the UGPI by the %BT.

Cytometric Bead Array Assay for Cytokine Measurement

Using PMBCs from four rabies vaccinated subjects, culture supernatants from cells stimulated with inactivated rabies virus and from unstimulated cells were collected at day 7 and kept frozen at −20°C until used for cytokine measurements. Quantities of Th1 (TNF-α and IFN-γ) and Th2 cytokines including (IL-2), (IL-4), (IL-5), and (IL-10) were measured using the standard protocol of the Human Th1/Th2 Cytokine Cytometric Bead Array Kit (BD Biosciences, San Diego, CA). The lower detection limits of this method were 2.6 pg/mL for IL-2 and IL-4, 2.4 pg/mL for IL-5, 2.8 pg/mL for IL-10 and TNF, and 7.1 pg/mL for IFN-γ.

Statistical Analysis

Statistical comparisons between responders and non-responders were conducted by either the *t*-test for parametric data or Mann–Whitney rank sum test for nonparametric data (Sigma Stat, Richmond, CA). Normality of the data was determined using Kolmogorov–Smirnov (K–S) statistics (Sigma Stat, Version 3.1, Systat Software, Inc., Point Richmond, CA). Differences in cellular proliferation between low, intermediate, and high antibody responders were determined using one-way analysis of variance (ANOVA) (Sigma Stat, Version 3.1, Systat Software, Inc., Point Richmond, CA). Agreement between the proliferative responses obtained using the [3H]thymidine assay and the CFSE assay was determined by the *kappa* test (Analyse-it Software, Ltd., Leeds, England). Spearman rank (*rs*) order test (Sigma Stat, Version 3.1, Systat Software, Inc., Point Richmond, CA) was used to determine both the correlation between [3H]thymidine stimulation indices obtained using unstained and CFSE stained lymphocytes and whether or not a significant association existed between demographic data and RVNA levels. Significant difference between cytokine levels produced in response to rabies vs. media was determined by the *t*-test (Sigma Stat, Version 3.1, Systat Software, Inc., Point Richmond, CA). Statistical significance was established at *p* values <0.05.

RESULTS

Optimization for Cellular Proliferation Assays [3H] and CFSE

The T lymphocyte mitogen, PHA, optimally stimulated proliferation at 3 days of culture. The mean cpm (and SEM) for cells cultured in medium alone or with PHA at 3 days was 371 (37), and 67008 (1539), respectively.

Using the inactivated RV as a recall antigen, the mean SIs of the PBMCs obtained from three rabies vaccinated subjects evaluated in this study indicated that the maximum lymphocyte proliferation occurred on day 8 (Fig. 1). There were no significant differences in cell proliferation for the three different dilutions of rabies virus used for stimulation due to the variability in the mean SI between the three subjects and the large standard deviations (Fig. 1). There was, however, an increase in cell death noted on day 8 (data not shown). Thus, the 1/50 dilution of inactivated rabies virus and cell harvesting on day 7 were selected as the optimum criteria for future assays.

Initial experiments of the CFSE assay using the same three subjects demonstrated similar proliferation patterns as observed in the [³H]thymidine assay with the 1/50 dilution of inactivated rabies virus and PHA-stimulated lymphocytes. The PHA-stimulated lymphocytes demon-

strated a progressive reduction in fluorescent intensity after 3 days, whereas inactivated rabies-stimulated lymphocytes demonstrated proliferation at day 7. The analyses of the CFSE assay results permitted visualization of cell generations and hence determination of when cells started to divide thus eliminating the need to harvest cells on different days because the cumulative proliferation of all cells was measured regardless of the day the proliferation response began. Therefore, day 7 was selected as the day of harvest because it allowed detection of both the immediate proliferation of PHA-stimulated PBMCs and the later proliferation of inactivated RV-stimulated PBMCs.

Inactivated Rabies Virus Cell Proliferation Measured with the [³H]thymidine Assay

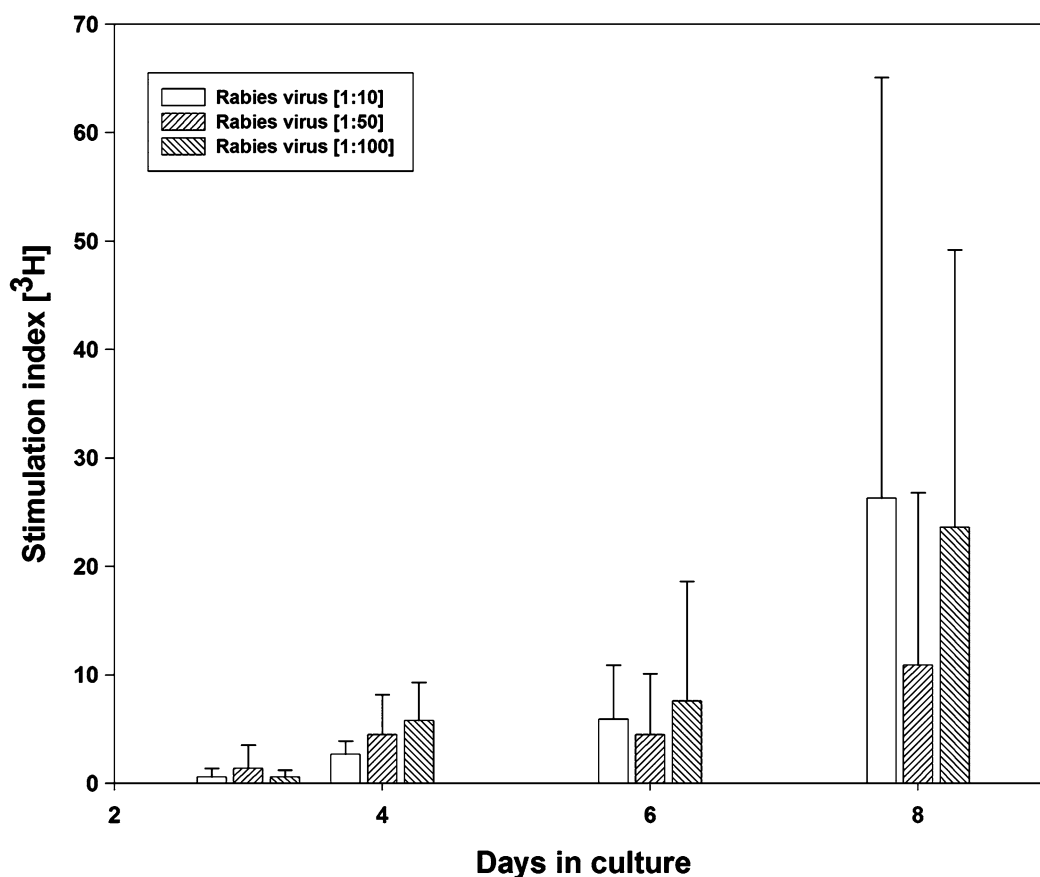Lymphocytes from all subjects proliferated to PHA stimulation of PBMCs (data not shown). Minimal or

Fig. 1. Optimization of antigen stimulation in [³H]thymidine assays. Peripheral blood mononuclear cells (PBMCs) from three rabies vaccinated human subjects were cultured (in triplicate) in the presence of 1/10, 1/50 and 1/100 dilutions of inactivated rabies virus (dose $10^{5.2}$) for 3, 4, 6 and 8 days. Stimulation indexes (SIs) were determined by dividing the mean cpm for the antigen-stimulated cultures by the mean cpm of the negative control cultures. Error bars indicate one standard deviation of the mean SI determined from three subjects.

no proliferation was detected to inactivated whole rabies virus preparation for the five control subjects with mean SI values ranging from 0.7 to 1.5 at day 3 and 0 to 0.8 at day 7. Of the ten rabies vaccinated subjects, six individuals, (subjects 1, 2, 3, 5, 6, and 8) had mean SI to inactivated whole rabies virus preparations approximately two times higher than the cutoff value for proliferation on day 3 (Table II). On day 7, five individuals (subjects 1, 5, 7, 8, 10) had mean SI above the cutoff value with three individuals ranging three to six times higher (Table II). Lymphocytes from two vaccinated subjects (4 and 9) had similar SI to inactivated rabies as the non-vaccinated subjects on both collection days. There was a significant difference in SI between vaccinated and non-vaccinated groups on both day 3 ($p = 0.023$) and day 7 ($p = 0.008$).

CFSE Does Not Interfere with Cell Proliferation

To determine whether or not CFSE labeling of lymphocytes interferes with proliferation to either mitogen or recall antigens, lymphocytes from three vaccinated and three non-vaccinated subjects were stained with CFSE and tested in the [^{3}H]thymidine assay in parallel with unstained lymphocytes and their respective SIs compared. There was no significant difference in the PHA or rabies virus SIs obtained from CFSE-labeled or -unlabeled lymphocytes at either day 3 or day 7 (Table III). Correlation between the SIs from CFSE-labeled and -unlabeled cells obtained with inactivated RV on day 7 was high ($rs = 0.94$,

Table II. Proliferation Response to Inactivated Rabies Virus

Vaccinated subject #	Day 3[a] SI mean (SEM)	Day 7[a] SI mean (SEM)
1	4.0 (2.0)	2.8 (1.2)
2	4.1 (0.4)	1.6 (0.1)
3	4.1 (0.9)	1.0 (0.4)
4	1.4 (0.1)	0.3 (0.1)
5	4.1 (0.1)	1.9 (1.0)
6	3.6 (1.9)	1.1 (0.0)
7	1.1 (0.2)	11.8 (2.2)
8	2.1 (0.3)	12.1 (5.9)
9	1.1 (0.1)	1.8 (0.6)
10	1.3 (0.2)	5.8 (1.9)
Mean	2.7[b]	4.0[b]

Notes. Lymphocyte stimulation index (SI) produced by recall antigen (inactivated rabies) determined for each vaccinated subject by the [^{3}H]thymidine assay is listed for day 3 and day 7 cultures. Cutoff value for day 3: mean SI of non-vaccinated subjects $(1.1) + 2$ standard deviations $= 1.7$. Cutoff value for day 7: mean SI of non-vaccinated subjects $(0.6) + 2$ standard deviations $= 1.8$.
[a] Each subject tested in triplicate, standard error of the mean for each subject in parentheses.
[b] The comparison of the mean SI of vaccinated vs. the mean SI of non-vaccinated subjects was significantly different ($p < 0.05$ Mann–Whitney rank sum test).

Table III. The Effect of CFSE Dye on Lymphocyte Proliferation

	CFSE cells mean SI (SEM), $n = 6$	Unstained cells mean SI (SEM), $n = 6$
Day 3 PHA stimulation	123 (28)	112 (23)
Day 3 Rabies stimulation	1.3 (0.1)	1.3 (0.2)
Day 7 PHA stimulation	35 (25)	42 (23)
Day 7 Rabies stimulation[a]	1.9 (0.7)	2.6 (1.2)

Notes. Stimulation index (SI) for three vaccinated and three non-vaccinated subjects on day 3 and day 7 with CFSE dyed and unstained cells determined in the [^{3}H]thymidine assay in cell cultures stimulated with either inactivated rabies virus or PHA. Each subject was tested in triplicate, the SI determined and means for each condition calculated; standard error of the mean for each condition in parentheses.
[a] Correlation between the SIs from CFSE-labeled cells and unlabeled cells obtained with inactivated RV; $rs = 0.94$, $p = 0.0167$.

$p = 0.0167$) indicating CFSE did not interfere with the lymphocyte responses.

Measurement of Lymphocyte Proliferation with CFSE

Proliferation of lymphocytes to either mitogen or recall antigen was determined by measuring the progressive decrease in intensity of the CFSE dye in each succeeding lymphocyte generation. In representative two-color dot plots, this decrease in CFSE intensity is depicted as a shift to the left along the *x*-axis for cells cultured with mitogen (PHA) (Fig. 2B and E) or recall antigen (rabies virus) (Fig. 2C and F), compared to cells cultured in medium alone (Fig. 2A and D). The relative numbers of CD4$^+$ and CD8$^+$ cells responding to stimulation in these two-color dot plots are evident in the upper left quadrants in Fig. 2B and C and E and F, respectively. The software used to analyze the dot plot data assumes that each daughter cell will have half of the fluorescence of the parent. It then converts the log fluorescence scale to a linear scale on a 256-resolution histogram where a 50% linear intensity decrease on log data is related to 19.19 linear channels. For example, in Fig. 3A which illustrates a typical proliferation pattern of PHA-stimulated cells after 7 days in culture, each generation is 19.19 linear channels apart reflecting a decrease in fluorescent intensity of the CFSE dye. The parent population (depicted in blue) has a mean fluorescent intensity of 196, whereas the second generation (depicted in orange) is 19.19 channels less at a mean fluorescent intensity of 176.81. With most of the parental cells proliferating in response to the mitogen, proliferation can be detected and quantitated. The extent of proliferation is indicated by the number of generations produced in the 7 days of culture and is also an indication of when the
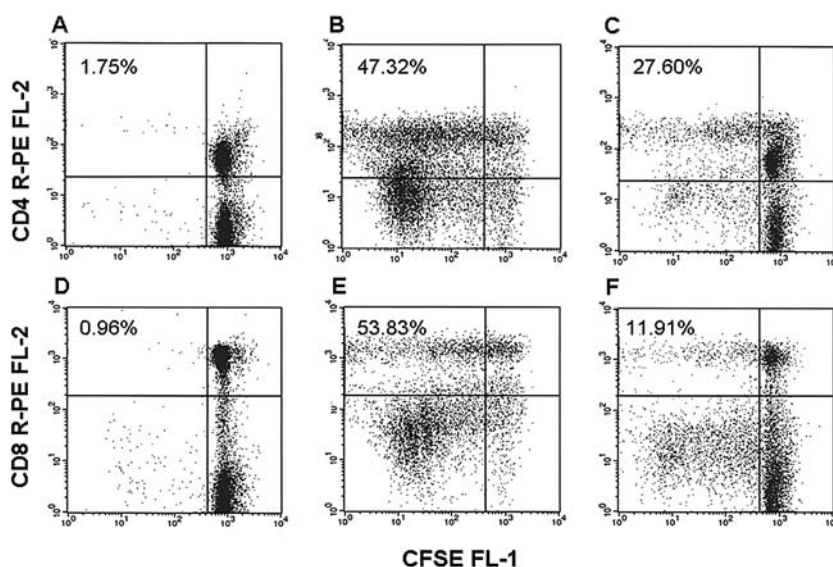
Fig. 2. Two-color dot plot analysis of nonproliferating CFSE stained $CD4^+$ and $CD8^+$ cells incubated with medium alone (A and D) and proliferating CFSE stained $CD4^+$ and $CD8^+$ cells stimulated with PHA (B and E) or rabies virus antigen (C and F). Nonproliferating $CD4^+$ and $CD8^+$ cells are found in the upper right quadrant with high CFSE fluorescent intensity. Nonproliferating $CD4^-$ and $CD8^-$ cells are found in the lower right quadrants. Percentages of proliferating $CD4^+$ and $CD8^+$ cells are found in the upper left quadrant with decreasing CFSE fluorescent intensity. Proliferating $CD4^-$ and $CD8^-$ cells are found in the lower left quadrant with decreasing CFSE fluorescent intensity.

cells started dividing. In addition, the percentage of cells calculated to be present in each generation contributes to the measure of the proliferative response. Because rabies-specific memory T cells are expected to number less than 1/200 to 1/50,000, gating first on cells which have undergone blast transformation enhances the detection of these cells as has been demonstrated previously (13). Figure 3B illustrates how after 7 days in culture, proliferation of RV-stimulated cells was difficult to appreciate in generations 5 through 7 of the analysis histogram. By gating on the blasts (Fig. 3C) the proliferation pattern became clearly evident (Fig. 3D). This gating method allowed for the discrimination between high numbers or low numbers of proliferating cells in subjects with similar proliferation patterns but differed by the height of the peaks in generations past the parent population. For two rabies vaccinated subjects represented in Fig. 3E and F, similar proliferation into the fifth and sixth generation is evident, but the subject in Fig. 3F had more blasts (35%) compared with the subject in Fig. 3E (2%). The differences in the peak heights illustrate the utility of using the percentage of lymphocytes moving from the "resting" gate into the "blast" gate, defined as percent blast transformation (%BT) as a weight factor in the final calculation of the measure of proliferation. The UGPI of the inactivated RV-

stimulated lymphocytes of vaccinated subjects was significantly higher than that of the non-vaccinated subjects ($p = 0.006$). In addition, there was a greater difference between vaccinated and non-vaccinated subjects using the WtUGPIs ($p = 0.003$) (Fig. 4). Eight of the ten vaccinated subjects had positive UGPI proliferation values and all the vaccinated subjects had WtUGPI values greater than the cutoff values of 1.2 and 1.3, respectively, following inactivated RV stimulation. In contrast, none of the non-vaccinated subjects had positive proliferation values by the CFSE assay. The weighted *kappa* statistic test (0.73) indicated good agreement between the WtUGPI of the CFSE assay and SI of the [^{3}H]thymidine assay (19).

Antibody Response to Rabies Vaccination is Not a Factor of Age, Sex, Vaccine or Time Since Vaccination

Sera from the ten vaccinated and the five non-vaccinated subjects were tested for the presence of RVNA. All vaccinated subjects had neutralizing antibodies titers ≥0.5 IU/mL even after as long as 19 years. Titers ranged from 0.8 to 960.0 IU/mL (Table IV). None of the five non-vaccinated subjects had detectable serum RVNA; all having titers of <0.05 IU/mL.
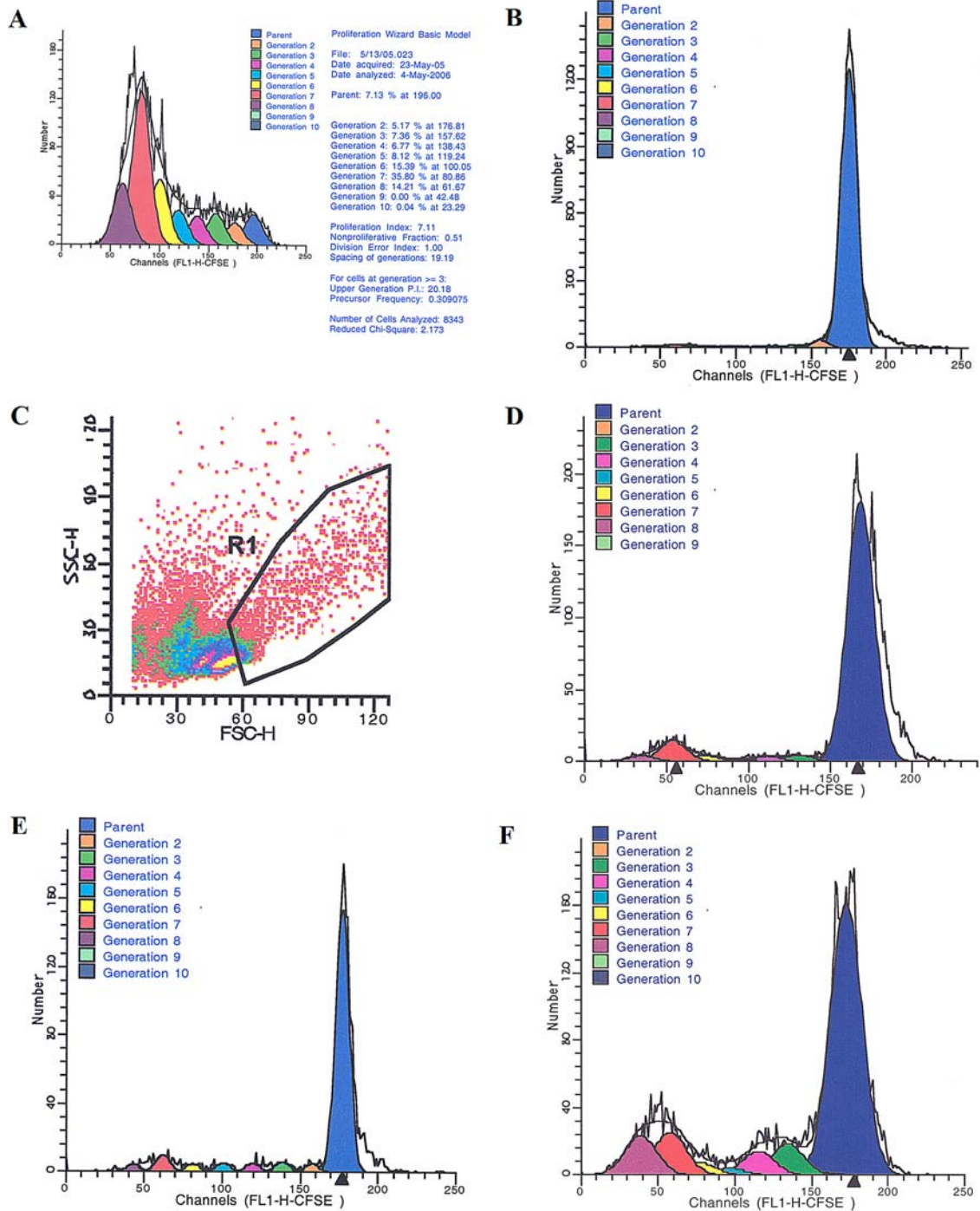
Fig. 3. Typical flow cytometry proliferation data of CFSE stained lymphocytes stimulated with mitogen (A) or rabies antigen (B–F) following 7 days of culture. PHA-stimulated lymphocytes proliferate out to eight generations with the peak occurring at 7 days of culture (A). Only two generations are evident in rabies-stimulated lymphocytes from a vaccinated human subject (B). By using a gating strategy to select the blasting population (based on size or increased forward scatter) in rabies-stimulated lymphocytes of human subject represented in B (C), the proliferating generations are more evident in the histograms (D). The number of lymphocytes proliferating to inactivated rabies virus in each generation varied between subjects with similar proliferation index values, compare (E) with (F).
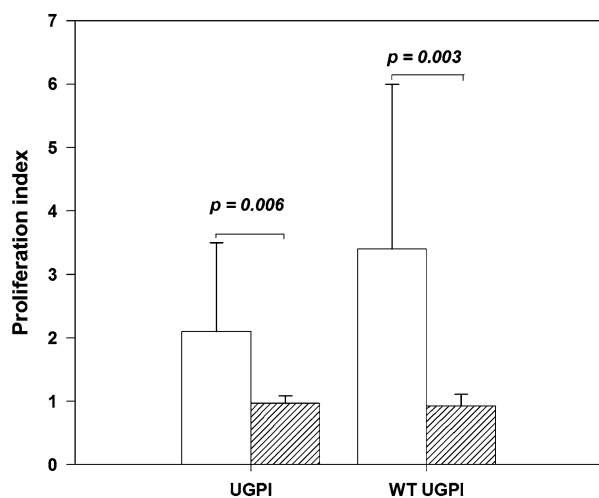
Fig. 4. Inactivated rabies virus-stimulated proliferation values of vaccinated ($n = 10$, white bars) versus non-vaccinated ($n = 5$, striped bars) subjects using the upper generation proliferation index (UGPI) and the weighted UGPI (WtUGPI). Significant differences between the two groups were determined using the Mann–Whitney rank sum test at $p < 0.05$.

Comparison of the Humoral and Cellular Immune Response to Rabies Vaccination

Of the ten vaccinated subjects, the level of RVNA in serum ranged from 0.8 to 960 IU/mL, with no apparent association noted between demographic factors or vaccine history (Table I). The cellular response as measured with the CFSE assay ranged from 1.7 to

Table IV. Comparison of Humoral and Cellular Immune Response to Rabies Vaccination

RVNA response category	RVNA IU/mL	WtUGPI
Low	0.8	7.8
Low	1.2	1.8
Low	2.1	2.3
Low	3.4	2.5
Low	4.2	8.8
Mean	2.3	4.6
Intermediate	13	2.0
Intermediate	17.4	2.6
Mean	15.2	2.3
High	31.3	2.7
High	46.1	2.2
High	960	1.7
Mean	348.8	2.2

Notes. Sera from all subjects were tested for the presence of RVNA by RFFIT. Adequate protection was defined as greater than complete neutralization at the 1/5 dilution (ACIP) or as ≥ 0.5 IU/mL (WHO). The ten vaccinated subjects were apportioned into categories based on their RVNA level: low <5.0 IU/mL, intermediate >5.0 to <20.0 IU/mL, high >20.0 IU/mL. Proliferation values are expressed as WtUGPI. Means for each group were calculated.

8.8 WtUGPI values (Table IV). The WtUGPI values were compared to the RVNA levels and although no statistically significant association was determined, the general trend of higher RVNA levels with lower WtUGPI values was noted (Table IV). The vaccinated subjects were separated into response groups based on individual titer results (expressed in IU/mL): <5, low; >5 to <20, intermediate; and >20, high. By graphing the mean values of the humoral (RVNA titers expressed as log dilution) and cellular response (WtUGPI) for each response group together, an inverse relationship between RVNA titers and WtUGPI was noted (Fig. 5).

CD4 is the Major Responding Subset of Lymphocyte to Inactivated Rabies-Stimulation

To determine whether the proliferative response to inactivated RV in the vaccinated subjects was due primarily to the CD4$^+$ or CD8$^+$ subset, the numbers of cells were calculated by gating on the CD4$^+$ or CD8$^+$ cells in the transformed blast gate. Using these values, seven of ten subjects had higher percentages of CD4$^+$ cells than CD8$^+$ cells (Table V). Two subjects had similar proportions of CD4$^+$ cells and CD8$^+$ cells, whereas one subject had greater percentages of CD8$^+$ cells than CD4$^+$ cells (Table V). By comparing the ratio of the number of proliferating CD4$^+$ cells to the number of proliferating CD8$^+$ cells, seven subjects demonstrated a CD4$^+$ bias with ratios ranging from 1.5 to 6.3. Additionally, three subjects had
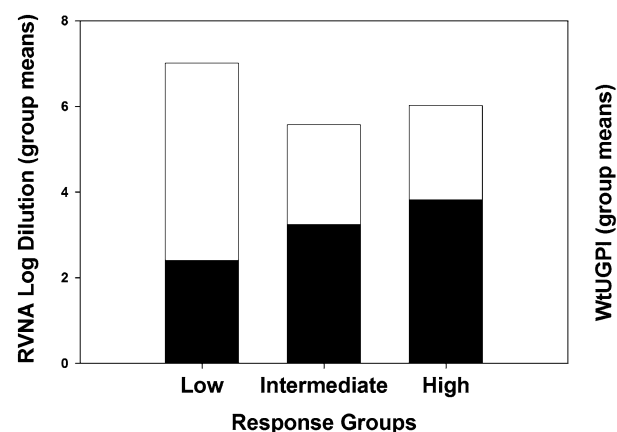

Fig. 5. The ten rabies vaccinated subjects were categorized based on their rabies virus neutralizing antibody (RVNA) levels as determined by serum testing in the rapid fluorescent foci inhibition test: low is <5.0 IU/mL, intermediate is >5.0 to < 20.0 IU/mL, high is >20.0 IU/mL. Each subject's corresponding cellular proliferation value (WtUGPI) was grouped in the same manner. Mean RVNA titer (black bars) expressed in log dilution and mean WtUGPI (white bars) were compared.

Table V. Comparison of CD4$^+$ and CD8$^+$ Subsets Reactive to Rabies Antigens

RVNA response category	% CD4 blasts	% CD8 blasts	CD4/CD8
Low	60	40	1.5
	61	39	1.6
	86	14	6.3
	44	56	0.8
	50	50	1.0
Intermediate	86	14	6.2
	49	51	1.0
High	78	22	3.5
	65	35	1.9
	63	37	1.7

Notes. The ten vaccinated subjects were separated into categories based on their RVNA level: low <0.5 IU/mL/mL, intermediate >5.0 to <20.0 IU/mL, high >20.0 IU/mL. Number of blast cells of each lymphocyte subset was obtained, and the percent CD4 and CD8 reactive cells were determined. Ratios of CD4 to CD8 reactive cells were calculated.

nearly equal number of cells in each subset. Moreover, high RVNA values were associated with higher percentage of CD4$^+$ proliferating cells (Table V).

Cytokine Production Differences Between High Humoral and High Cellular Responding Subjects

Four of the ten vaccinated subjects were selected for cytokine production measurement based on their RVNA level and WtUGPI in response to inactivated rabies virus. Two of the subjects (S4 and S5) had high RVNA levels (960 and 46.1 IU/mL) but low to moderate WtUGPI values (1.7 and 2.2); while the other two (S2 and S7) had low RVNA levels (0.8 and 4.2 IU/mL) but high WtUGPI (7.8 and 8.8). After 7 days of culture, the supernatants from both of the subjects (S2 and S7) with high WtUGPI contained significantly higher quantities of TNF-α when stimulated with inactivated rabies virus than the supernatant of cells in media only ($p < 0.001$ and $p = 0.002$) and one of these two subjects had significant quantities of IFN-γ (S7) also ($p < 0.001$) (Fig. 6). For all four of the subjects tested, IL-2, IL-4, IL-5, and IL-10 concentrations were undetectable (data not shown).

DISCUSSION

The administration of rabies vaccine has dramatically reduced the incidence of human rabies deaths after exposure to infected animals. After more than 3 decades of use, it is clear that cell culture rabies vaccines are among the world's safest and most effective vaccines. Induction of long-term immunological memory as well as high titers of protective RVNA is the hallmark of a safe
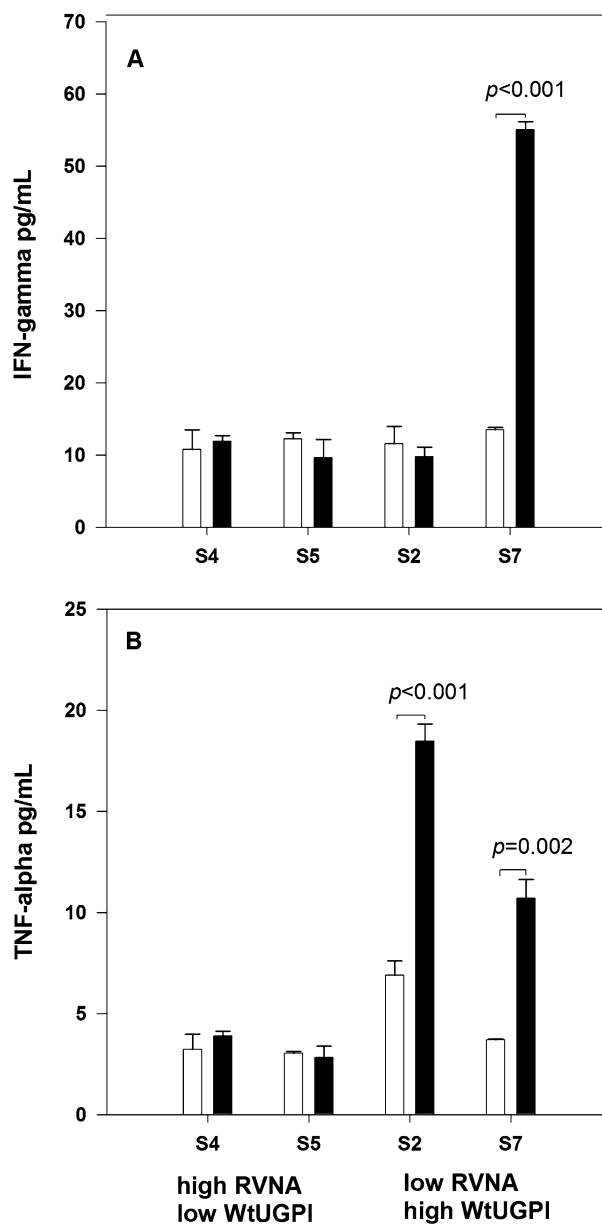

Fig. 6. Cytokine levels in the supernatant of PBMCs of two vaccinated subjects (S4 and S5) with high RVNA titers and low WtUGPI values and two subjects (S2 and S7) with low RVNA titers and high WtUGPI values in the presence of either medium alone (white bars) or inactivated rabies virus (black bars) after 7 days of culture. Significant differences between cytokine concentrations in the presence of inactivated rabies virus and medium alone for each subject were determined using the *t*-test at $p < 0.05$.

and effective rabies vaccine. Historical data proves that appropriate vaccination with modern cell culture rabies vaccines produces a wide range of RVNA titers from 0.5 to >70.0 IU/mL that can persist for more than 20 years in some individuals. Although the role of cellular immunity

in effective protection from rabies after vaccination has been previously recognized, the ability to detect and measure the T cell response to recall antigens has been a serious problem due to the lack of an available, practical, and sensitive assay. Typically, vaccination generates antigen-specific effector T cells of which over 90% die by apoptosis and the remaining 10% become either central (residing in the lymphoid organs) or effector memory cells (residing in the peripheral tissues) (20). As only effector memory cells circulate in the periphery, an assay designed to detect these few cells (estimated to be from 1/200 to 1/50,000) requires a very sensitive assay (20). The traditional, FDA approved method commonly used to quantitate lymphocyte proliferation is the measurement of $[^3H]$thymidine incorporation into the DNA of proliferating cells during the final hours of cell culture. The utility and practicality of this assay is reduced by the fact that it requires radioisotopes. In addition, the assay measures of all cell subsets in culture at one specific time point. Using this traditional assay, we found that eight of ten rabies vaccinated subjects had proliferative responses to inactivated RV either on day 3 and/or day 7 of culture. On both days there were significant differences noted between the SIs of the vaccinated and the non-vaccinated subjects ($p < 0.05$). The observation that the peak response of PMBC from some subjects to inactivated RV occurred at day 3 rather than at day 7 could be due to the fact that rabies N protein has a superantigen effect on Vβ8-specific T cell receptors which has been reported previously (21).

In this report, a novel nonradioactive assay to measure the cellular immune response to rabies vaccination was developed as an alternative to the $[^3H]$thymidine assay and was used to evaluate the proliferative response of PMBC from vaccinated and non-vaccinated subjects. Prior studies have shown CFSE to be a useful tool in the detection of lymphocyte proliferation in response to mitogens and recall antigens (13, 14). Using this new assay, the ability to detect the two-fold reduction of CFSE intensity as a measure of proliferation in mitogen-stimulated lymphocytes proved to be relatively easy compared to the detection of the much smaller population of rabies-specific T cells. The typical proliferation pattern of dye intensity reduction for inactivated RV-stimulated cells was greatly enhanced by initially gating on the cells which had undergone blast transformation prior to software analysis of proliferation. Because blast transformation alone is not a good measure of proliferation, a weighted proliferation index combining blast transformation and cellular proliferation proved to be a more specific measure (13). The use of the upper generation proliferation index (UGPI) eliminated the interference of non-specific proliferation factors by excluding those cells having undergone only three

divisions. Although the use of the UGPI alone allowed the classification of eight out of the ten vaccinated subjects as positive for rabies vaccine antigen-specific proliferation, inclusion of the %BT as a weighting factor was more representative of the total proliferation response in all subjects. This was illustrated by the differences in the proliferation analysis of two subjects. Although both subjects had similar proliferation values, the %BT were quite variable, 35% compared to 2%. Use of the WtUGPI as a measure of proliferative response in the CFSE assay demonstrated that all of the vaccinated subjects had positive responses ranging in value from 1.7 to 8.8 and none of the non-vaccinated subjects had a proliferation value above the cutoff of 1.3. A significant difference in the proliferative response to inactivated RV was detected between vaccinated and non-vaccinated subjects. The presence of the CFSE dye did not alter the proliferation responses as measured by the $[^3H]$thymidine assay and there was good agreement between the $[^3H]$thymidine and the CFSE assays. The difference between vaccinated and non-vaccinated proliferation values was greater in the CFSE assay than the $[^3H]$thymidine assay. The CFSE assay had a better assessment of the proliferative responses over a 4-day period because the cumulative proliferation was measured. Additionally, it has been reported that cells that have died after undergoing proliferation can still be included in the analysis of total proliferation as the dye intensity is maintained for several days after cell death (22). The CFSE assay is able to differentiate between individuals with the same degree of proliferation, but with different numbers of responding cells. Due to this fact, the CFSE assay can distinguish the quantity of rabies-specific T cell precursors from the magnitude of the proliferation response. The ability to detect these differences in response to rabies vaccination may be useful in the determination of factors involved in mounting a high RVNA response due to the importance of CD4$^+$ T cells in the production of antibody by B cells. The relative contribution of CD4$^+$ and CD8$^+$ cells in the rabies-specific proliferating cells was determined with the use of immunophenotyping and multi-parameter flow cytometry analysis. The results confirmed an early report that suggested CD4$^+$ T cells are the primary responding T lymphocyte subset (11). In the current study, one of ten vaccinated subjects demonstrated a larger number of proliferating CD8$^+$ cells compared to the number of proliferating CD4$^+$ cells.

The ability to detect rabies-specific CD4 and CD8 T cells generated after vaccination using the CFSE assay is an improvement in the measurement of the cellular response. In addition, determination of the cytokines involved in lymphocyte stimulation to rabies antigens

would identify which T helper types were involved. A flow cytometric method described by Maino and Picker to detect intracellular cytokines produced in response to antigens allows classification of the immune response into either a Th1 (IFNγ and TNFα) or Th2 (IL-4, IL-5, and IL-10) response (23). Because rabies is a Th2 healing disease, the goal of a rabies vaccine is to drive the immune response toward neutralizing antibody production and generation of Th2 CD4 effector and memory T cells. The difference in cytokine production for the four subjects selected in this study support the premise of a Th2 response resulting in a skewing toward a humoral response over a cellular immune response in that the high RVNA responders did not demonstrate Th1 cytokines, whereas the low RVNA responders had detectable Th1 cytokine responses (TNF-α and/or IFN-γ). A study examining the antigen-specific cytokine profiles demonstrated similar levels of cytokines detected in response to recall antigens, CMV and *Candida* (24). Specific assays to ascertain the preferred Th2 vaccine response as well as the ability to examine the immune responses of persons exposed to rabies through a bite exposure would add to the current knowledge of the mechanisms of rabies protection. Vaccine response or non-response can be selectively defined by criteria such as antibody titer levels. However, in reality there exists normal variation involving the spectrum of humoral and cellular immune responses. Although the number of subjects in this study was not large enough to determine statistically significant associations, the trend of subjects with higher RVNA levels having lower cellular proliferation values as measured in the CFSE assay was demonstrated. Whether this phenomenon would be present in a study with more vaccinated subjects should be confirmed. Measurement of the RVNA levels along with the proliferation response in samples taken from subjects at the same time point(s) after vaccination would allow for better associations between the humoral and cellular immune response. In addition, the lack of Th2 cytokines in the supernatants of lymphocytes cultured with inactivated rabies virus may be a function of the kinetics of cytokine production so that evaluation of supernatants at earlier time points may prove informative as to temporal relationship of cytokine production (24).

In summary, there is a need for a rabies-specific cellular immune response assay which is practical, informative and sensitive to complement the humoral measurement of RVNA levels. The CFSE assay has important advantages over the traditional assay in terms of avoidance of radioactive material and ability to identify specific subsets of lymphocytes. This is the first report to apply CFSE cell proliferation measurement to the cellular immune response to rabies vaccination. The findings in this report

of an inverse relationship between humoral and cellular response to rabies vaccination as well as the pronounced CD4 response associated with higher RVNA levels will provide us new knowledge of the subsets of cells induced by rabies vaccination and allow us to predict which individuals are "high" or "low" responders by comparing the lymphocytes to the antibody responses. Cytokine analysis of the different groups of responders may provide insight into the types of T helper subsets involved in the recall responses.

ACKNOWLEDGMENTS

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