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Human Lymphocyte Proliferation Responses following Primary Immunization with Rabies Vaccine as Neoantigen

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Evaluation of the T-cell immune response following primary antigenic challenge with a neoantigen is a critical aspect of assessment of the cellular immune response. While many antigens can be used to accurately assess in vitro T-cell proliferation to a recall antigen, only a few neoantigens have been tested for their capacities to measure T-cell responses in vitro to a primary immunization. Rabies vaccination is an excellent candidate for the testing of T-cell proliferation responses to a primary immunization because few individuals have been exposed to rabies virus antigens. In the present study 14 rabies vaccine-naïve, healthy adult volunteers were immunized against rabies virus, and T-cell proliferation and antibody responses were measured before and after vaccination. Optimal lymphocyte proliferation to soluble rabies virus antigen occurred after 8 days in culture. The average level of uptake of tritiated thymidine postimmunization was 29,620 ± 4,448 cpm, whereas preimmunization levels were 12,660 ± 3,448 cpm ($P = 0.002$). All individuals showed increases in rabies virus antibody titers from <0.05 to 5.59 ± 1.64 IU/ml. The degree of proliferation to tetanus toxoid as a recall antigen was similar to the response to rabies virus antigen among the cohort. Due to high levels of preimmunization proliferation, four subjects failed to demonstrate a twofold increase in response to rabies virus antigen. The high levels of T-cell responses may be due to a viral superantigen effect in some individuals. Rabies vaccination offers a safe and effective means for measurement of both T- and B-cell immune responses to a neoantigen in healthy and immune suppressed individuals.

Evaluation of the T-cell immune response following primary antigenic challenge with a neoantigen is a critical aspect of assessment of cellular immunity. Primary sensitization and restimulation of human lymphocytes can be measured by using soluble antigen in vitro (8). While many antigens have been used to accurately assess in vitro T-cell proliferation in response to a recall antigen (12, 15), only a few neoantigens have been tested for the capacity to measure the primary T-cell response in healthy and immune-deficient individuals. Keyhole limpet hemocyanin and bacteriophage ϕX174 are used to assess antibody responses to a neoantigen, but both have limitations when T-cell proliferation responses are measured in vitro (12, 14). In addition, neither of these agents is approved for use in humans by the Food and Drug Administration. A licensed vaccine that has great potential as a novel neoantigen is the rabies vaccine. Very few individuals have previously been immunized with the rabies vaccine, and the vaccine can be safely administered to both healthy and immunocompromised individuals (3, 6, 18). There is evidence that rabies virus can be used to induce in vitro proliferation responses by using human lymphocytes (19, 20). However, changes in T-cell proliferation responses prior to and following a series of immunizations with rabies vaccine have not been measured in healthy subjects. In the present study we evaluated pre- and postimmunization lymphocyte proliferation responses using a cohort of healthy, rabies vaccine-naïve individuals. We found that soluble rabies virus antigen elicits a vigorous postimmunization proliferation response in human lymphocytes. This finding indicates that this vaccine can be used to assess primary antigen-specific T-cell responses by an in vitro assay.

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MATERIALS AND METHODS

Subjects. Study subjects included 14 healthy volunteers who had not been immunized with the rabies vaccine. All subjects had received an immunization with tetanus toxoid within the previous 2 years before enrollment in the study, and informed consent was obtained by a protocol approved by the Institutional Review Board of the University of Florida. Rabies vaccine (Imovax IM; Connaught Laboratories, Inc., Swiftwater, Pa.) was administered as a single intramuscular injection on day zero and was readministered 1 and 4 weeks after the initial immunization. This vaccine is a derivative of rabies virus strain PM-L503–3 M. It is a whole virus consisting of the nucleocapsid complex surrounded by a lipoprotein envelope. The virus is grown in human diploid cells (MRC-5 strain), concentrated by ultrafiltration, and inactivated with beta-propiolactone. Each dose of the vaccine contains 5% human albumin, phenolsulfonphthalein, and neomycin sulfate (<150 μg) as an antibiotic. The vaccine contains no preservative or stabilizer (7, 13). Blood samples were collected from the volunteers prior to the initial vaccination and 4 weeks following the final rabies vaccination.

Preparation of PBMCs. Whole blood was collected in tubes containing 1 ml of acid citrate dextran. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll (Histopaque 1077; Sigma Diagnostics, St. Louis, Mo.) gradient density centrifugation by a previously described protocol (1, 16). PBMCs were resuspended at a concentration of 10^6 cells per ml in RPMI 1640 medium (Gibco BRL, Grand Island, N.Y.) supplemented with 2 mM L-glutamine and 100 U of penicillin per ml, 100 μg of streptomycin (Gibco BRL) per ml, and 10% heat-

inactivated fetal calf serum (Gibco BRL). A portion of the cells was cryopreserved and stored in liquid nitrogen for future study, according to our previously described protocol (16).

Lymphocyte proliferation assays. Lymphocyte proliferation assays were carried out in triplicate by using in each well 10^5 cells cultured in 96-well round-bottom microtiter plates (Costar; Corning Glass Works, Corning, N.Y.). The cells were stimulated with phytohemagglutinin (PHA; 10 μg/ml; Sigma Chemical Company, St. Louis, Mo.) for 3 days, tetanus toxoid (8 μg/ml; Wyeth Ayerst Pharmaceuticals, Marietta, Pa.) for 6 days, and rabies virus antigen (Connaught Laboratories, Inc.) at 1/20, 1/40, and 1/80 dilutions for intervals of 3, 4, 6, 8, and 10 days. The cells were incubated in a humidified 37°C incubator with 5% CO_2. At each respective time point the cells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech Limited, Little Chalfont, England) per ml, cultured for an additional 18 h, and harvested with a PHD cell harvester (Cambridge Technology Inc., Cambridge, Mass.). The level of incorporation of [^{3}H]thymidine was determined with an LS-250 scintillation counter (Beckman Instruments, Inc., Van Nuys, Calif.). The counts per minute obtained for each triplicate set were averaged, and the data were quantified by subtracting the counts per minute obtained for PBMCs cultured in medium alone (unstimulated cells) from the counts per minute for the stimulated cells.

Antibody responses to rabies vaccination. Antibody responses to rabies vaccination were measured by comparing titers in serum prior to and 4 weeks after the final rabies vaccination. Tests for rabies virus neutralizing antibody using the rapid fluorescent focus inhibition technique were carried out at Kansas State University by previously described methods (3, 17). Assays were performed in parallel with stored sera from subjects obtained pre- and postimmunization. Results are reported in international units by using World Health Organization guidelines, in which a titer of 0.5 IU designates an adequate protective antibody response to the vaccination (5).

Statistical analysis. Statistical comparisons of the changes in lymphocyte proliferation before and after immunization were carried out by using the computer software program SigmaStat (Jandel Scientific, Jandel Corporation, San Rafael, Calif.). Analysis of multiple rabies virus dilutions, data from various times points, and pre- and postimmunization results for lymphocyte proliferation and antibody titer were compared by the paired *t* test. Proliferation between cryopreserved and fresh PBMCs and proliferation of PBMCs cultured with tetanus toxoid versus rabies virus antigens were also compared by the paired *t* test. Statistical comparisons between responders and nonresponders were carried out by the Student *t* test. Significance was defined as a *P* value of <0.05. Values are expressed as the means ± standard error of the means.

RESULTS

Lymphocyte proliferation response to rabies antigen. Study subjects included 14 healthy adults who had not previously been immunized with the rabies vaccine and who had received tetanus toxoid booster immunizations within the past 2 years. The cohort contained 1 man and 13 women with a median age of 25 years (age range, 23 to 33 years). None of the vacinees reported any adverse reactions to the rabies immunization series.

The differences in tritiated thymidine uptake for PBMCs between unstimulated cells in medium and cells stimulated with rabies virus antigen over 3 to 10 days in culture are shown in Figure 1. Maximum postimmunization lymphocyte proliferation of fresh and cryopreserved cells occurred at 8 days in culture. Tritiated thymidine incorporation by cryopreserved cells rose significantly, from 758 ± 156 cpm at 3 days to 29,620 ± 4,448 cpm by 8 days ($P = 0.001$), and fell to 24,500 ± 3,736 cpm by day 10 in culture. Surprisingly, for days 6, 8, and 10, preimmunization cryopreserved PBMCs incubated with rabies virus antigen demonstrated a more significant degree of proliferation than PBMCs incubated in medium alone ($P = 0.001$, 0.007, and 0.013, respectively), and the proliferation values on those days of culture were higher than the proliferation values on day 3 of culture. However, mean thymidine uptakes as a measure of postimmunization proliferation responses to rabies virus antigen on day 8 of culture were three- to fivefold higher than the preimmunization thymidine uptake ($P = 0.002$). There were no significant differences in proliferation among the various dilutions of rabies virus antigen for samples obtained either preimmunization or postimmunization (data not shown).

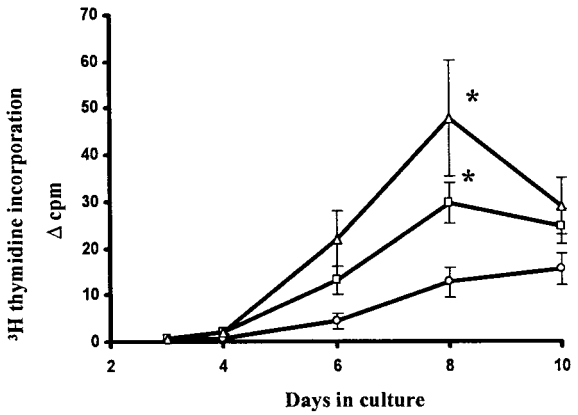

FIG. 1. Comparison of proliferation responses of cryopreserved PBMCs obtained prevaccination (circles), cryopreserved PBMCs obtained postvaccination (squares), and fresh PBMCs obtained postvaccination (rectangles). Values represent the mean ± standard error of the mean for each day in culture. Cells were cultured in the presence or absence of rabies virus antigen, and the values shown are the differences in mean tritiated thymidine update ([Δ cpm], change in counts per minute) between the stimulated and unstimulated cells for 14 individuals. The number of days in culture is shown on the *x* axis, and tritated thymidine uptake (counts per minute [in thousands]) is shown on the *y* axis. An asterisk indicates a statistically significant difference ($P < 0.05$) for postimmunization values compared to preimmunization values for the same day. There was no significant difference in postimmunization proliferation between cryopreserved and fresh PBMCs.

Differences in mean postimmunization proliferation responses between freshly isolated and cryopreserved PBMCs are also shown in Figure 1. At the 6- and 8-day time points the mean tritiated thymidine uptakes by fresh PBMCs were 68 and 61% higher, respectively, than those by cryopreserved PBMCs from the same individuals, although the differences were not significantly different ($P = 0.14$ and 0.10, respectively). Both cryopreserved and fresh PBMCs demonstrated significantly higher responses than samples obtained preimmunization ($P = 0.002$ and 0.002, respectively).

Comparison of lymphocyte proliferation to rabies virus and tetanus toxoid antigens. All subjects enrolled in the study had received a tetanus toxoid immunization within 2 years prior to enrollment in the study. The relative degree of proliferation to a recall antigen (tetanus), a neoantigen (rabies), and to a mitogen (PHA) were compared and are shown in Figure 2. Reponses in the same individuals were measured by using cryopreserved PBMCs. The results indicate that proliferation responses to tetanus toxoid were similar to those to rabies virus antigen (23,136 ± 7,501 cpm and 29,620 ± 4,448 cpm, respectively) ($P = 0.475$, paired *t* test). Fresh and cryopreserved PBMCs from samples from all subjects obtained pre- and postimmunization demonstrated a vigorous response to PHA after 3 days in culture (data not shown).

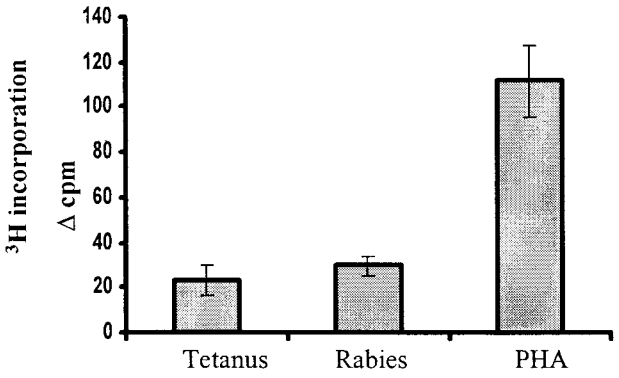

FIG. 2. Comparison of mean lymphocyte proliferation responses of cryopreserved PBMCs incubated with tetanus toxoid antigen for 6 days, rabies virus antigen for 8 days, and PHA for 3 days. PBMCs were obtained 4 weeks after immunization with rabies vaccine. The mean ± standard error of the mean tritiated thymidine uptakes for 14 individuals are shown ([Δ cpm], change in counts per minute [in thousands]). There was no statistical difference between the responses to tetanus toxoid and rabies virus antigens ($P = 0.475$, paired t test).

Comparisons of pre- and postimmunization antibody and lymphocyte proliferation responses to rabies virus antigen. Prior to immunization none of the subjects had detectable titers of antibody to rabies virus (Table 1). However, 8 weeks following immunization all subjects demonstrated protective levels of rabies virus antibody, ranging from 0.7 to 16.5 IU/ml (mean level, 5.59 ± 1.64 IU/ml) ($P = 0.001$). While all subjects demonstrated an increase in rabies virus antibody titer following immunization, not all subjects demonstrated the same degree of increase in lymphocyte proliferation responses to rabies virus antigen. PBMCs from 4 of 14 subjects (29%) failed to demonstrate greater than a twofold increase in proliferation when the pre- and postimmunization responses were compared. The mean preimmunization proliferation responses for the nonresponders (subjects 2, 5, 6, and 7, Table 1) were greater than those for the 10 responder subjects (23,677 ± 7,530 and 8,253 ± 2,430 cpm, respectively) ($P = 0.023$, t test). However, there was no difference when postimmunization responses between nonresponders and responders were compared (23,218 ± 7,280 and 32,179 ± 5,512 cpm, respectively) ($P = 0.384$, t test).

DISCUSSION

T-cell responses before and after rabies immunization demonstrated a consistent proliferation pattern. Optimal uptake of tritiated thymidine by PBMCs occurred on the day 8 of culture and displayed kinetics similar to those of other T-cell-specific proliferation responses (15). The timing of optimal proliferation was similar to that in a study that showed that soluble rabies virus antigen can selectively induce T-cell proliferation of CD45RA T cells from rabies virus-naïve individuals (20). In vitro primary sensitization and restimulation of human lymphocytes with soluble antigen have already been reported (8). Our study is the first to demonstrate clearly that rabies virus can be applied as a neoantigen for the accurate measurement of pre- and postimmunization proliferation responses in a cohort of rabies virus-naïve individuals. The magnitude of lymphocyte proliferation to soluble rabies virus antigen is adequate to accurately determine differences in tritiated thymidine uptake between unstimulated cells and rabies virus antigen-stimulated cells. The mean level of proliferation to rabies virus antigen was similar to the level of proliferation to tetanus toxoid antigen, a soluble antigen commonly used to measure antigen-specific T-cell proliferation (4, 11). Comparison of pre- and postimmunization antibody responses to T-cell proliferation showed parallel T-cell and B-cell responses to rabies virus antigen. In addition, the postimmunization proliferation obtained with cryopreserved PBMCs was similar to that obtained with freshly obtained PBMCs. These results indicate that pre- and postimmunization PBMCs can be prospectively collected, stored, and analyzed simultaneously in the same assay. The predictable and brisk lymphocyte proliferation responses, the safety of the vaccine in humans, and the capacity

TABLE 1. Comparison of antibody production and lymphocyte proliferation responses before and after rabies immunization

Subject	Proliferation response[a]		Level of antibody production (IU/ml)[b]	
	Preimmunization	Postimmunization	Preimmunization	Postimmunization
1	23,438	53,206	<0.05	16.5
2	36,891	21,601	<0.05	0.8
3	13,194	41,838	<0.05	4.2
4	14,141	40,478	<0.05	0.7
5	10,334	18,274	<0.05	3.3
6	10,942	9,362	<0.05	0.7
7	36,542	43,637	<0.05	12.9
8	1,448	6,005	<0.05	3.3
9	4,937	44,030	<0.05	0.8
10	892	7,319	<0.05	1.3
11	1,978	42,512	<0.05	4.2
12	3,083	9,913	<0.05	0.7
13	15,184	40,559	<0.05	12.4
14	4,232	35,938	<0.05	16.5
Mean	12,660 ± 3,224	29,650 ± 4,778	<0.05	5.59 ± 1.64

[a] The lymphocyte proliferation response is measured as the level of tritiated thymidine uptake (in counts per minute).
[b] Antibody levels are for blood samples drawn prior to and 4 weeks after completion of rabies vaccination.

to use cryopreserved PBMCs indicates that the rabies vaccine is an excellent reagent for assessment of antigen-specific T-cell responses to a primary immunization in both healthy and immune-deficient individuals.

While there are many advantages to the use of the rabies vaccine to assess primary T-cell responses to an immunization, the assay does have limitations. Four of the 14 (29%) subjects failed to demonstrate an increase in postimmunization lymphocyte proliferation. These subjects had preimmunization proliferation responses that were significantly higher than those for the rest of the cohort, even though their postimmunization responses were similar. While it is possible that these subjects were previously exposed to the rabies virus, this explanation for the high level of preimmunization response is unlikely because none of the subjects displayed detectable preimmunization antibody levels. Rabies virus nucleocapsid can serve as a Vβ8-specific exogenous superantigen for human T cells (2, 9). Among T cells from unimmunized individuals, selective expansion of Vβ8-bearing T cells was demonstrated following incubation with rabies virus nucleocapsid protein (9, 10). While these studies indicate that the rabies virus nucleocapsid binds directly to major histocompatibility complex class II determinants to activate Vβ8 T cells, our results indicate that the relative impact of this effect on PBMC proliferation in vitro was variable among the different individuals enrolled in the study. Similar to the findings in our study, Martinez-Arends et al. (10) showed that rabies virus nucleocapsid induces proliferation of human tonsil lymphocytes in a third of the healthy individuals examined. In the same study, 83% of the subjects responded to toxic shock syndrome toxin type 1, and all responded to type E *Staphylococcus* enterotoxins.

The origin of the differences between individuals who respond to rabies virus antigen prior to immunization is unknown, but it may be related to differences in the frequency of Vβ-bearing T cells within PBMCs. All subjects who failed to demonstrate a change in PBMC proliferation still showed a significant rise in rabies virus antibody titer, indicating that the vaccine can easily measure the antibody response even when the assessment of the T-cell response is masked by the superantigen effect. However, the unique aspect of rabies virus as a viral superantigen for human T cells hampers its utility in assessing T-cell proliferation in response to a neoantigen. Despite these shortcomings, immunization against rabies virus offers a unique opportunity to measure both T- and B-lymphocyte responses to a neoantigen. In over 70% of subjects there was measurable T-cell proliferation, and all subjects demonstrated a clear rise in antibody titer. The rabies vaccine can easily be applied to larger-scale clinical investigations of the immune response in healthy and immune-suppressed individuals.

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