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INDUCTION OF ANTIGEN-SPECIFIC ANTIBODY RESPONSE IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES *IN VITRO* BY A DOG KIDNEY CELL VACCINE AGAINST RABIES VIRUS (DKCV)

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In the present report an *in vitro* method for obtaining a secondary human antibody response to a dog kidney cell vaccine against rabies virus (DKCV) is described. Cultures of peripheral blood mononuclear cells from normal rabies-immune and nonimmune donors were stimulated *in vitro* by DKCV. The production of virus-specific antibody in supernatant fluids was monitored by ELISA. Antibody was produced by lymphocytes from rabies-immune individuals, whereas those of nonimmune subjects consistently failed to produce anti-rabies antibodies after *in vitro* stimulation with DKCV. The generation of the anti-rabies virus antibody response of lymphocytes stimulated with DKCV was shown to be an antigen-dependent, as well as an antigen-specific process. Optimal antigen-specific responses were observed at relatively low concentrations of antigen (10^{-1} to 10^{-2} μg/culture). At increasing concentrations of antigen in culture (>1 μg/culture), the anti-rabies virus response was suppressed. Antibody produced upon stimulation was capable of neutralizing rabies virus. The response to rabies virus requires T cell help because lymphocytes depleted of SE rosetting cells did not respond to an antigenic stimulus. Studies in which the same individuals were followed over time showed a sequential development of circulating B cell subsets. The system may provide a model for the study of human B cell differentiation *in vivo* and *in vitro* and may be valuable for testing the potency of rabies vaccines *in vitro*.

Polyclonal B cell activators (PBA)[1] have been a useful probe to study activation and regulation of B lymphocyte responses in man. Antigen-specific antibody responses, however, that result from exposure of human B cells *in vitro* to PBA, such as pokeweed mitogen, are part of a polyclonal immunoglobulin (Ig) response. The latter type of response may greatly differ from B cell responses triggered by antigen itself (1–3).

Several investigators have recently established techniques that permit analysis of either the primary or secondary *in vitro* antibody response by human B cells after antigen stimulation. Antigens applied in these studies included particulated antigens, i.e., sheep red cells (SE) (4–7), haptenated polyacrylamide beads (8), and soluble antigens, such as ovalbumin (4, 5), haptenated ovalbumin (9), keyhole limpet hemocyanin (KLH) (10, 11), hemocyanin of helix pomatia (12), and tetanus toxoid (13–15). Furthermore, systems have been described that have enabled the measurement of specific antibody in cultures of human B cells stimulated with influenza virus (16, 17) and varicella zoster virus (18).

In this paper, we report the *in vitro* induction of a secondary antigen-specific antibody response of human peripheral blood lymphocytes (PBL) by a dog kidney cell vaccine against rabies virus (DKCV). This vaccine has proven to be a potent inducer of neutralizing antibody in man (19). In addition, it has been shown to protect animals against an otherwise lethal challenge with live rabies virus (19). The kinetics of this response, "the quality" of antibody produced, as well as the cellular requirement for this response, have been investigated.

MATERIALS AND METHODS

Immunizations. Normal healthy volunteers received a primary intramuscular immunization of one dose of DKCV followed by a 1- and 6-mo booster immunization or by immunization on days 7 and 21 as indicated in *Results*. Before immunization, anti-rabies antibodies were not present in the sera of the subjects as has been reported (19). After completion of the vaccination schedule, neutralizing anti-rabies antibodies were present in sera of all individuals. *In vitro* studies were performed at times after immunization as indicated in *Results*.

Lymphocyte isolation. Mononuclear cells were isolated from heparinized blood by density gradient centrifugation on Ficoll-Isopaque ($\rho = 1.077$ g/cm^3) at 1000 × G for 20 min and then were washed twice with RPMI 1640 (GIBCO, Grand Island, NY) supplemented with penicillin, streptomycin, and Fungizone (100 IU, 100 μg, and 25 μg/ml, respectively).

Monocyte depletion. Mononuclear cells were depleted of monocytes by adherence to plastic Falcon flasks at 37° C in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS). After 60 min, supernatants containing lymphocytes were harvested and washed twice in RPMI 1640 medium. After the adherence procedure, the cell suspension usually contained between 5 and 10% monocytes determined by morphology and nonspecific esterase staining.

T cell isolation and depletion. Mononuclear cells were separated into T cell and non-T cell populations by methods previously described (5). Briefly, T cells were isolated by incubation of lymphocytes with a 50-fold number of SE that had been treated with neuraminidase. After rosette formation, cells were centrifuged on a density gradient of Ficoll-Isopaque ($\rho = 1.077$ g/cm^3) for 30 min at 1200 × G. The resulting T cell sediment was freed of contaminating SE by lysis with cold buffered NH_4Cl. The cells from the interface of the gradient that were enriched for B cells were cycled again. Non-T cell preparations used in the experiments contained ≦2% SE rosetting cells.

Antigens. Rabies vaccine used for stimulation *in vitro* was produced by the propagation of rabies virus strain Wistar PM/WI 38-1503-3M in primary dog kidney cells (DKCV) and was inactivated with β-propiolactone as described elsewhere (20). One dose of lyophilized vaccine containing 300 μg protein was dissolved in 1 ml phosphate-buffered saline (PBS) and was further diluted in culture medium. Rabies vaccine produced in human diploid cells was obtained from Mérieux (France). Measles virus (Edmonston strain) was grown on Vero cells in microcarrier cultures and was purified according to methods used for the purification of rabies virus (20). Polio virus was produced and purified according to methods described by van Wezel *et al.* (20).

Culture conditions. Lymphocytes were suspended in RPMI 1640 medium containing 25 mM HEPES,[1] 2 mM L-glutamine, penicillin, streptomycin, and Fungizone, 100 IU, 100 μg/ml, and 25 μg/ml, respectively, 10^{-5} M 2-mercaptoethanol, 1 mM Na pyruvate, and 10% horse serum. Cells were cultured in

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[1] Abbreviations used in this paper: DKCV, dog kidney cell vaccine; RFFIT, rapid *fluorescent* focus inhibition test; SE, sheep erythrocytes; KLH, keyhole limpet hemocyanin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay.

the presence of various concentrations of DKCV. Cultures were kept in 24-well, flat-bottomed plates (Costar Data Packaging, Cambridge, MA) at a cell density of 2×10^6/ml in a final volume of 1 ml at 37° C in a humidified atmosphere of 5% CO_2 in air. After 6 days the cells were harvested, washed twice, and recultured for 5 days in culture medium containing 5% FCS (Armour Pharmaceutical Company, Kankakee, IL) without antigen. Supernatant fluids were then harvested and assayed for anti-rabies antibody, for antibody to polio virus, and for total Ig.

Enzyme-linked immunosorbent assays (ELISA). Antibodies to rabies virus in culture supernatants were detected by solid-phase immunoassay with the use of a modification of the method of Zanders *et al.* (21). Flat-bottomed microtiter plates (Titertek Type III, Flow Laboratories Inc., McLean, VA) were coated with 100 μl of 12 μg/ml DKCV in PBS, pH 7.4, for 1 hr at 37° C in a humidified atmosphere. Plates were washed twice with PBS, incubated with PBS containing 1% bovine serum albumin (BSA) for 30 min at 37° C to block remaining binding sites, and washed again with PBS containing 1% BSA. This procedure consistently resulted in low background values. After washing, 60 μl of culture supernatant and dilutions thereof in PBS 0.5% BSA were applied to the wells and were incubated for 1 hr at 37° C. Undiluted culture supernatant, as well as three serial fivefold dilutions, were run for each sample. Each plate also contained four serial fivefold dilutions of a reference serum composed of pooled serum of a hyperimmune donor. The plates were washed with PBS containing 0.05% Tween by using a semi-automatic washing device and were incubated at room temperature for 2 hr with antihuman Ig conjugated with peroxidase; goat anti-μ chain (Cappel Laboratories, Downingtown, PA), sheep anti-human γ-chain, and polyspecific sheep anti-human Ig were used. The production of the latter two conjugates has been described elsewhere (22). The conjugates were diluted in PBS containing 0.5% BSA. After incubation with conjugated antisera the plates were washed with PBS containing 0.05% Tween, and 100 μl of the substrate were added to each well. The substrate consisted of 1.67 ml tetramethyl-benzidine in dimethylsulfoxide (6 mg/ml) in 100 ml 0.1 M sodium acetate buffer, pH 5.5, and 10 μl of 30% solution of H_2O_2 (Perhydrole, Merck, Darmstadt). After 10 min, the reaction was stopped by the addition of 100 μl/well 1 N H_2SO_4 (23). Absorbence at 450 nm was measured by using a Titertek Multiskan automatic plate reading machine connected with a PDP 1170 computer system. To calculate the concentration of IgM and IgG in supernatant fluids, the optical densities of samples and reference serum were compared. For this purpose we developed a line comparison method. To find out which combination of configurations would give the best correlation, a computer program was used (van Asten *et al.*, unpublished data). Configurations that had been programmed were: linear, logarithm, reciprocal, probit square, and square root. On the basis of data obtained with 72 samples, the combination of logarithm of concentration *vs* linear extinction was selected. Next, the actual concentration in nanograms of Ig in a sample was calculated by the determination of the slope ratio of the slopes of the best-fitting line of both the reference and sample. By using this method, slight differences in background values did not influence the final results. Large differences in background values, however, were noticeable by poor correlations that automatically indicated the necessity to repeat the test.

Standardization of the reference serum. The reference serum was standardized according to the method described by Stevens and Saxon (24), which provides an estimate of the antibody content in nanograms per milliliter. The amount of rabies-specific Ig in the reference was estimated by the comparison of the amount of Ig reference serum bound to microtiter wells coated with DKCV with the amount of an Ig standard bound to microtiter wells coated with anti-Ig antibody. The plates were coated with 150 μl/well, 5 μg/ml polyspecific sheep anti-human Ig antibody, or 12 μg/ml DKCV in carbonate buffer, pH 9.6, by incubation at 20° C for 24 hr. After washing, wells coated with anti-Ig antibody were filled with 100 μl of dilutions of an Ig standard preparation, whereas wells coated with virus received 100 μl of serial fivefold dilutions of the reference serum. After 2 hr at 37° C, the plates were washed, and 100 μl of purified peroxidase-conjugated polyspecific sheep anti-human Ig (22) were applied to each well. After 2 hr at 37° C, plates were developed as described above. The amount of rabies-specific Ig in the reference was estimated by comparison of the optical densities obtained with virus-coated wells with the curve obtained with anti-Ig-coated wells. The determination of rabies-specific IgM and IgG in the reference was estimated in an identical manner. Goat anti-human μ-chain-specific (Cappel Laboratories) and sheep anti-human γ-chain-specific (22) antisera were used to coat microtiter wells. Purified human IgG and IgM were used as standard preparations. To develop the plates, a polyspecific sheep anti-human Ig conjugated with peroxidase was used (22).

Virus-neutralizing antibody determination. The neutralizing capacity of culture supernatants on rabies virus (CVS strain) was evaluated by the rapid fluorescent focus inhibition test (RFFIT) (25).

RESULTS

Antigenic requirements. Anti-rabies antibody responses were seen at concentrations of DKCV in lymphocyte cultures as low as 10^{-4} μg protein/culture. Peak responses were observed at concentrations ranging from 10^{-2} to 10^{-1} μg/culture of DKCV. The further increase of antigen concentration in culture (>1 μg/culture) resulted in the abrogation of antibody formation (Fig. 1). This has been a consistent finding with cells from 25 rabies-vaccinated individuals who were studied. Lymphocytes from nonimmunized subjects failed to respond to rabies vaccine at all concentrations that were tested.

To determine whether the antibody generated by DKCV *in vitro* resulted from *de novo* protein synthesis, antigen-stimulated cells were cultured in the presence of 100 μg/culture cycloheximide. The addition of cycloheximide 5 days before the harvesting of culture supernatants reduced antibody production to less than 10% of values found in supernatants of cells cultured without this drug (Fig. 1). Furthermore, no antibody production could be detected in cultures of cells with medium alone (0 μg/culture of DKCV) (Fig. 1).

Kinetics of the in vitro anti-rabies response. Time course studies were performed to define the *in vitro* kinetics of the anti-rabies antibody production. Cells were cultured for various periods after which the supernatants were tested for the presence of specific antibody in the ELISA. Specific antibody production was not detected through day 3 of culture. Then antibody production increased to a maximum by day 10 or 11 (Fig. 2). To avoid residual antigen present in the culture supernatant affecting the detection of specific antibody, the following experiments were performed. After an incubation period of 4 days in the presence of antigen, cells were washed free of antigen and were cultured again in fresh media for an additional period of 6 to 7 days. The supernatants were then assessed for antibodies against rabies virus. The results of these experiments showed again that maximal and constant production of specific antibody was detected after a period of culture of 10 days (data not shown). These results argue against an assay artifact related to antigen in culture and confirm the data obtained with cycloheximide studies that indicate that *in vitro* protein synthesis is required for this response.

Antigen-specific nature of the response to rabies vaccine. The

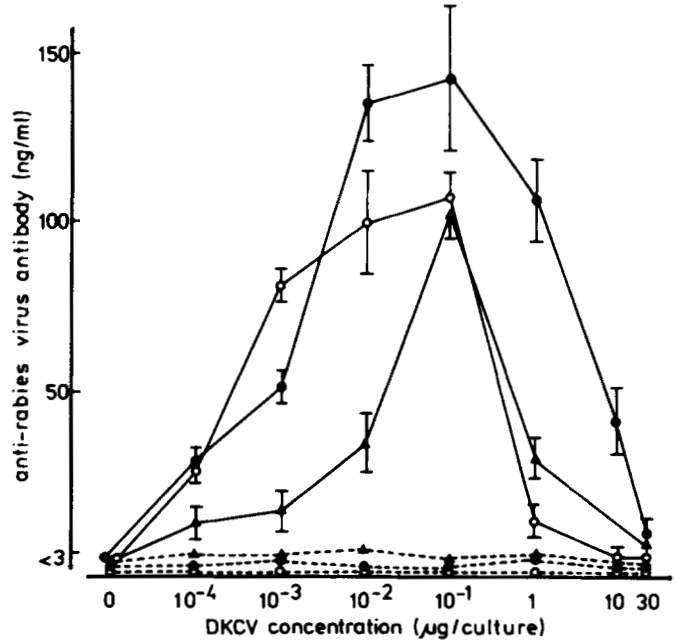

Figure 1. Antigen dose dependency of anti-rabies response. PBL were cultured in the presence of different concentrations of DKCV. Specific antibodies in the culture supernatant fluid were determined on day 11. Three representative experiments from different donors are shown. Values are expressed as mean ng Ig/ml ± SE of triplicate determinations.

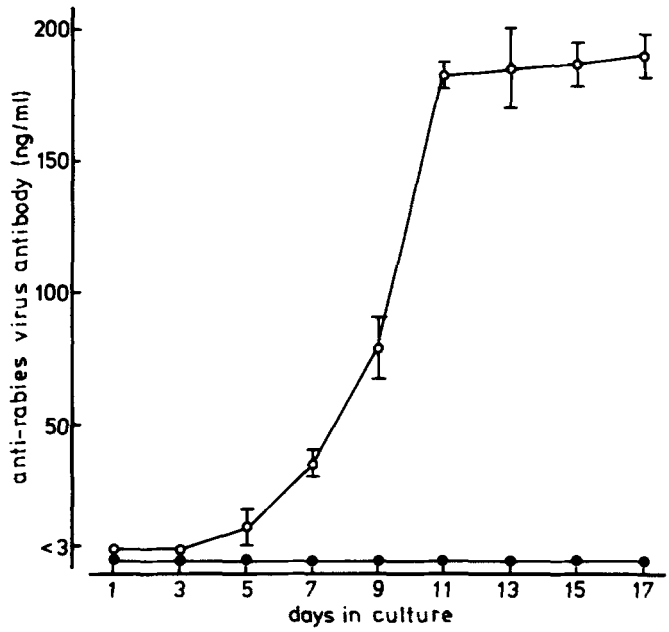

Figure 2. The kinetics of anti-rabies antibody formation in cultures of PBL. Cells were cultured for various periods in the presence of 0.1 μg/culture DKCV. One representative experiment is shown. Values are expressed as mean ng Ig/ml ± SE of triplicate determinations.

TABLE I

Specificity of anti-rabies response

Culture Stimulus	Supernatant Fluid Adsorbed with[a]	Specific Antibody[b] ELISA coat		
		DKCE[c]	DKCV	Polio virus
None			0.111 ± 0.017	0.094 ± 0.023
DKCV			1.699 ± 0.013	0.070 ± 0.021
Polio virus[d]			0.065 ± 0.026	1.494 ± 0.013
DKCV		0.131 ± 0.049	1.477 ± 0.154	
DKCV	DKCV		0.330 ± 0.012	
DKCV	Measles virus		1.620 ± 0.022	
DKCV	Polio virus		1.660 ± 0.034	

[a] Supernatant fluids were adsorbed with 1 vol highly concentrated antigen: DKCV, 300 μg/ml; measles virus, 150 μg/ml; polio virus, 33 μg/ml, for 1 hr at 37°C. After centrifugation supernatant fluids were tested for specific antibody.

[b] Data expressed as mean OD 450 nm ± SE. One representative experiment out of three is shown.

[c] Dog kidney cells were cultured as described in Reference 20. The noninfected cells were washed free of serum and were subjected to three cycles of freezing and thawing. The clarified supernatant fluid was diluted with PBS, pH 7.4, to a protein content of 12 μg/ml. One hundred microliters of this material was used as coat in ELISA DKCE, dog kidney cell extract.

[d] The antibody response induced by polio virus *in vitro* will be described in detail elsewhere (UytdeHaag *et al.*, manuscript in preparation). Background values in ELISA for determination of anti-polio virus antibody were usually below 0.100 absorbence.

specificity of the response was assessed by culturing cells of individuals who were double immune to rabies virus and to polio virus with DKCV or polio virus and by testing supernatant fluids for antibodies against rabies virus and polio virus. The outcome of such experiments revealed evidence that antibody generated by DKCV *in vitro* was specific for the inducing antigen, because no significant production of antibodies against polio virus was observed (Table I). On the other hand, supernatant fluids of cultures stimulated with polio virus did not bind to DKCV used in the ELISA. In the latter cases, specific antibodies to polio virus could be detected by using the homologous antigen in the ELISA.

The specificity of the response could be further established by the absorption of the culture supernatant fluids with the immunizing antigen or an unrelated antigen. The addition of excess rabies vaccine to culture supernatant fluids of the rabies vaccine-stimulated cells before determination of the antibody content in the ELISA resulted in a diminished antibody response. Absorption with measles virus or polio virus did not significantly change the level of antibodies in supernatant fluids of rabies vaccine-stimulated cultures (Table I).

Although DKCV used to stimulate the cultures is relatively pure and antibodies to dog kidney cells have never been found in individuals immunized with DKCV (unpublished observation), the generation of antibody against dog kidney cell antigens was considered after stimulation of lymphocytes with DKCV. The supernatant fluids from cultures of different individuals containing rabies-specific antibody were tested in ELISA against a coat of noninfected dog kidney cell extracts. None of these supernatant fluids showed a significant reaction in ELISA (Table I).

The nonpolyclonal nature of DKCV. The possibility that an antigen may behave like a polyclonal activator cannot be excluded (26). This possibility was tested by culturing PBL with various concentrations of DKCV or in the absence of antigen. As can be seen in Figure 3, the characteristic bell-shaped dose-response curve was obtained for rabies-specific antibody when PBL were exposed to various concentrations of DKCV. Total Ig production, however, did not vary significantly among stimulated and non-stimulated cultures indicating that, at least in this system, DKCV does not provide a polyclonal stimulatory signal to PBL.

In vivo kinetics of rabies-specific antibody production. In PBL cultures from six individuals who were followed over time, an *in vitro* antigen-inducible antigen-specific IgM antibody response was observed 1 wk after primary immunization. When taken before immunization with DKCV, PBL of these subjects did not respond to antigenic stimulation *in vitro* (Fig. 4). Maximal *in vitro* antigen-induced, rabies-specific IgM responses were observed in PBL cultures of all individuals 2 to 5 wk after the first booster immunization (Fig. 4). When examined 1 wk after the primary immunization, PBL of some subjects produced rabies-specific IgM antibody when cultured in the presence of very low, otherwise nonstimulatory concentrations of antigen; moreover, this

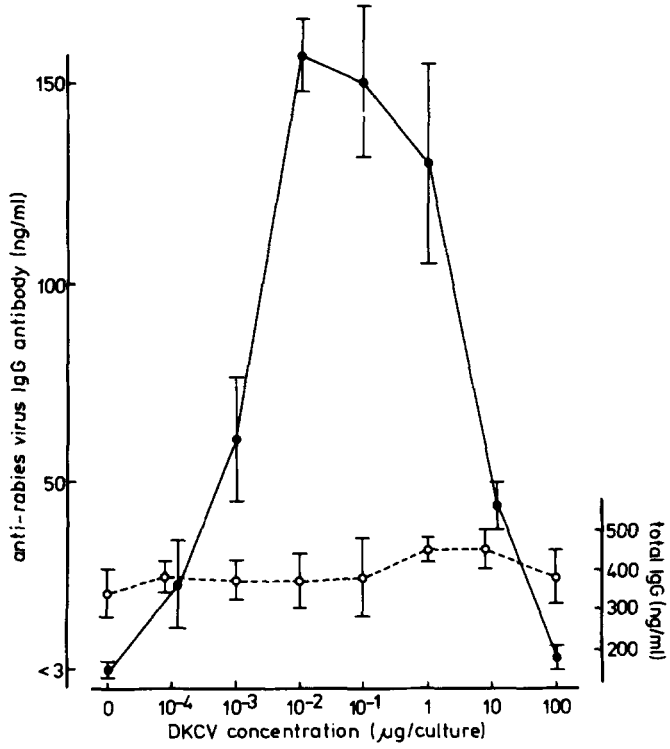

Figure 3. The nonpolyclonal nature of antibody responses of PBL of five individuals induced by DKCV *in vitro*. Data are expressed as geometric means ± SE. ●, specific anti-rabies virus IgG; ○, total IgG.

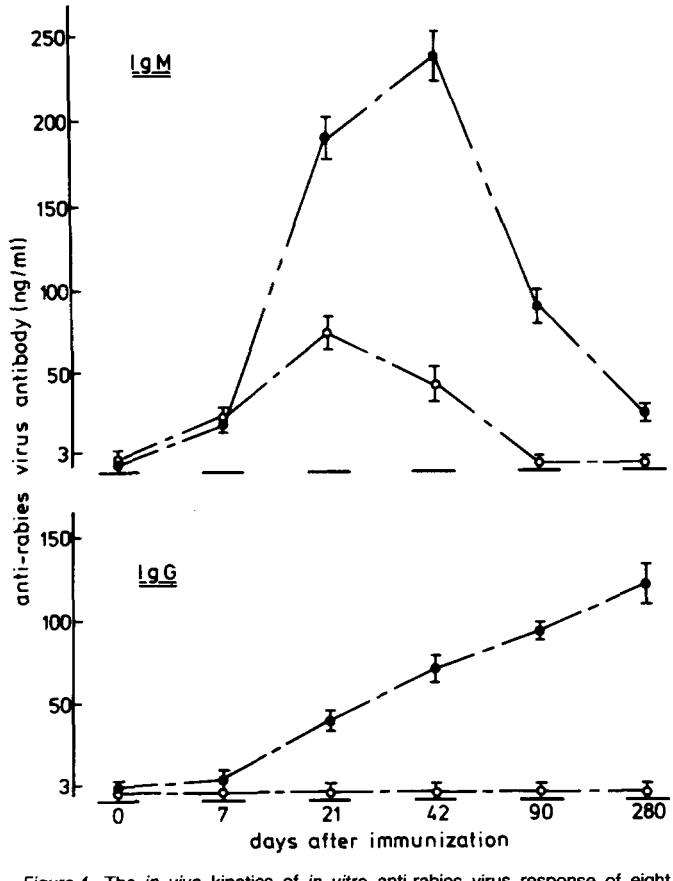

Figure 4. The *in vivo* kinetics of *in vitro* anti-rabies virus response of eight individuals. *Upper panel;* O, rabies virus-specific non-antigen-induced IgM antibody; ●, rabies virus-specific DKCV-induced IgM antibody. *Lower panel;* O, rabies virus-specific non-antigen-induced IgG antibody; ●, rabies virus-specific DKCV-induced IgG antibody. Data are expressed as mean responses of eight individuals in ng Ig/ml ± SE.

response was also seen in the absence of antigen. This phenomenon of nonantigen-induced rabies virus-specific IgM production was markedly expressed in cultures of PBL of most individuals when tested 3 wk after primary immunization, i.e., 2 wk after repeat immunization, and it was no longer detectable 13 wk after primary immunization (Fig. 4). When tested 40 wk after primary immunization, PBL of most individuals showed a decreased antigen-inducible specific IgM response, whereas PBL of other individuals became unresponsive. This phenomenon occurred irrespective of persistent levels of circulating neutralizing antibodies. Cells from other subjects, however, could be stimulated by DKCV to produce specific IGM antibody *in vitro* up to 40 wk after the primary injection (data not shown).

Low levels of rabies virus-specific IgG antibody were found in cultures of PBL of some subjects as early as 1 wk after primary immunization. The cells of all but one individual could be triggered by antigen to produce rabies virus-specific IgG antibody between 2 and 40 wk after the first booster immunization (Fig. 4). The magnitude of those responses was highly variable. PBL of one subject could not be stimulated by rabies virus to produce IgG antibody despite the ability of those cells to secrete specific IgM spontaneously, as well as in response to antigen (data not shown).

Neutralizing antibody activity in culture supernatant fluids. Culture supernatant fluids containing antigen-binding rabies virus-specific antibodies shown by ELISA were examined for the presence of neutralizing antibodies to rabies virus. In most supernatant fluids that were tested, neutralizing antibodies to rabies virus could be demonstrated. Some culture supernatants, however, lacked detectable amounts of neutralizing antibody activity. In the sera of all subjects, neutralizing antibodies were found (Table II).

TABLE II

Rabies virus-neutralizing antibodies produced in vitro

Experiment	Days after Booster[a]	Anti-rabies Ig Culture Supernatant Fluids		Anti-rabies Serum
1	18	173 ± 14[b]	1.2[c]	11.4[c]
2	18	174 ± 31	1.0	14.3
3	18	93 ± 17	<0.1	3.5
4	18	161 ± 8	0.3	14.0
5	250	127 ± 29	0.2	5.0
6	146	106 ± 9	<0.1	7.0

[a] Peripheral blood was obtained from six different donors after booster immunization at the times indicated. After stimulation of the cells with DKCV, culture supernatant fluids were tested for antigen binding in the ELISA and for the presence of neutralizing antibodies as described in *Materials and Methods*.

[b] Values are expressed as ng Ig/ml ± SE of duplicate determinations in ELISA.

[c] International units (IU) determined in RFFIT. Essentially the same results were obtained in a mouse protection test.

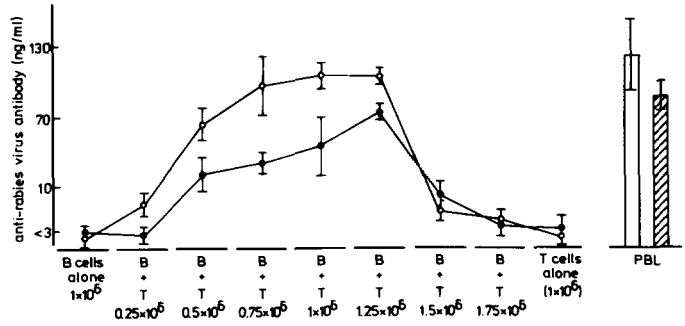

Figure 5. T cell dependency of anti-rabies antibody production. Human PBL were separated in T and non-T cells. 1 × 10^6 non-T cells were cultured with various numbers of T cells and in the presence of 0.1 μg DKCV/culture. Two representative experiments with two different donors are shown. Antibody responses of separated reconstituted cells and unseparated PBL are expressed as mean ng Ig/ml ± SE of triplicate determinations.

Cellular requirement. To investigate whether there was a T cell dependency of the human anti-rabies response *in vitro*, human PBL were separated into T cells and non-T cells by rosetting of T cells with neuraminidase-treated SE. The T cells and non-T cells obtained were cultured with rabies vaccine separately or were reconstituted at various T to B ratios. As shown in Figure 5, neither T cells nor non-T cells alone responded after stimulation with rabies vaccine, whereas combined T cells and non-T cells responded almost as well as unseparated PBL. Maximal anti-rabies antibody formation by 1 × 10^6 non-T cells was obtained by the addition of 0.75 × 10^6 to 1.25 × 10^6 T cells. These results indicate that the anti-rabies response is T cell dependent. When more than 30% monocytes were present in cultures, specific antibody production decreased. To standardize the system, monocytes were always removed by adherence to plastic, and the percentage of monocytes in cultures was adjusted to 5 to 10% (data not shown).

DISCUSSION

Thus far studies on the host immune response to viruses have been performed largely in experimental animals. In the present paper, we have described a simple and reproducible culture and assay system for the specific stimulation of unselected human PBL with rabies virus and for the subsequent detection of rabies virus-specific antibodies in culture supernatant fluids by ELISA. Some investigators have recently reported the successful activation of human B lymphocytes *in vitro* with influenza virus (16, 17) and varicella zoster virus (18) as well as the measurement of virus-specific antibodies in culture supernatant fluids.

In these systems, however, as well as in most other human models described thus far, PBL were taken from individuals who had been previously primed for the antigens that have been used to trigger antibody responses *in vitro* judged by serum antibody (14, 15, 24, 26). As a consequence it is reasonable to assume that reexposure to these antigens may occur at any moment during a lifetime. Because the kinetics of the circulating B cell repertoire is greatly influenced upon contact of the immune system with these recall antigens (24, 27–29), those systems may not be the most ideal tools to study B cell activation in man. In the present study a vaccine against rabies virus has been used to trigger an *in vitro* immune response of PBL from subjects after *in vivo* primary sensitization with the same vaccine. Rabies is usually always fatal unless immediate local wound treatment is done and postexposure prophylaxis is administered. Consequently this virus, apart from being of biologic importance, must be considered to be a new antigen for most individuals. Indeed, rabies virus serum antibodies have not been demonstrated thus far in individuals who have not experienced vaccination or infection and postexposure treatment.

This study clearly demonstrates that *in vitro* synthesis of rabies virus-specific IgM antibody triggered by DKCV *in vitro* requires primary immunization. The rabies virus-specific IgM antibody response induced by DKCV *in vitro* seems to follow the same pattern of responsiveness as has been found for anti-KLH IgM antibody response of PBL of immunized subjects induced by KLH *in vitro* (11). In contrast, however, to the results reported by Lane *et al.* (11), unresponsiveness to antigen after a 4-wk booster immunization was not observed in the course of a rabies virus-specific IgM antibody response. When followed over time, rabies virus-specific IgM antibody was produced *in vitro* by PBL of most individuals at a rather constant quantity until at least 12 wk after the first booster immunization.

The temporary appearance in the circulation of cells that secrete rabies virus-specific antibody without *in vitro* exposure to DKCV may reflect the presence of *in vivo* primed rabies virus-reactive cells that *in vitro* can be induced easily by nonspecific factors generated in the culture system to differentiate to antibody-secreting cells (30). Another possibility may be that this transient phenomenon represents the appearance in the circulation of a subset of antigen-reactive B cells that spontaneously synthesize specific IgM antibody without the need for an additional signal *in vitro.* From the kinetics of appearance in the circulation, as well as the differential requirements for an antigenic stimulus *in vitro* to differentiate to antibody-secreting cells, it cannot be deduced at present whether spontaneously IgM-secreting cells and *in vitro* DKCV-induced IgM-producing cells represent different maturational stages of the same B cell subset or if they developed independently. With regard to the decline of the *in vitro* DKCV-triggered specific IgM response between 6 and 13 wk after primary immunization, the depletion of circulating long-term memory cells soon after *in vivo* immunization has been reported in rat and mouse (31, 32). The assumption that anti-rabies virus IgM-producing cells represent long-term memory B cells is supported by the observation that specific IgG production becomes detectable after the first booster immunization and continuously increases after repeat booster immunization 3 wk later. Saxon *et al.* (28) and Stevens *et al.* (27), however, reported data to suggest that long-term memory cells may be processed by lymph nodes to become short-term memory cells that are large lymphoblastoid B cells spontaneously secreting specific IgG.

In the present study, spontaneous rabies virus-specific IgG production has not been observed. Short-term memory cells have been shown to be only demonstrable in circulation between days 5 and 12 after booster immunization (27). The time intervals for collecting blood samples in our study were 2 or 3 wk. As a consequence, the phenomenon of spontaneous IgG production by short-term memory cells may not have been detected in the present study.

The antibody response observed here requires T cell help, as well as the presence of antigen in the induction phase. This observation is compatible with results obtained in similar systems in which influenza virus and varicella zoster virus were used as inducing antigens (16–18). There was a diminution in the response to rabies virus at high antigen concentrations in culture. Although the induction or activation of suppressor T cells could be an explanation for this phenomenon, the tolerization of B cells by antigen cannot be excluded (11, 33–36). The T cell dependency of the anti-rabies antibody response has also been documented in experimental animals by using nude mice and cyclophosphamide-treated animals and by adoptive transfer studies (37–40). The role of T cells in the regulation of the human anti-rabies response *in vitro* is currently under study.

A number of explanations may be suggested regarding the finding that in culture supernatant fluids of PBL of some individuals, apparently only nonneutralizing anti-rabies virus antibodies were detected.

First, the ELISA must be considered to be more sensitive than RFFIT. In 10- to 100-fold concentrated PBL culture supernatant fluids of those individuals, however, neutralizing antibodies could not be found.

Another explanation for the lack of neutralizing antibodies in culture supernatant fluids of PBL of some individuals may be that B cells responsible for the production of neutralizing antibodies were not present in the circulation or had become unresponsive to antigen exposure *in vitro.* The presence of neutralizing antibodies in sera of the subjects in question need not be in contradiction with this explanation.

Kinetics studies on the appearance of circulating memory B cells for the different proteins of rabies virus combined with precursor frequency analysis of the respective subsets are in progress. Furthermore, the use of PBA, as well as subunits of rabies virus (41–45), for *in vitro* stimulation of lymphoid cells from different organ sources may help to clarify this intriguing observation.

The stimulation of human PBL with this biologically relevant antigen that is also applicable *in vivo* is potentially a valuable tool for the dissection of the function of human T, as well as B cells, in the antibody response to rabies virus. In addition, this technique, using a simple and highly reproducible method for the detection of specific antibody, may prove to be a useful system for potency testing of vaccines *in vitro*.

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