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# Rabies Virus-specific Human T Cell Clones Provide Help for an *in vitro* Antibody Response against Neutralizing Antibody-inducing Determinants of the Viral Glycoprotein

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## SUMMARY

Human T cell clones were prepared from peripheral blood mononuclear cells from a vaccinated human donor and kept in culture in the presence of rabies virus antigen and growth factors. Phenotypic analysis of the T cell clones revealed expression of the CD3 and CD4 cell surface markers, but not of CD8, consistent with a phenotype of helper/inducer T cells. The rabies virus specificity of the T cell clones was established by virus-specific proliferation in response to the rabies virus Pitman-Moore strain (PM) produced in three different cell substrates. The clones also responded to the rabies virus strains Evelyn-Rokitnicki-Abelseth (ERA) and challenge virus standard (CVS), but not to the rabies virus-related Mokola and Duvenhage-6 virus strains. Proliferative responses of T cell clones required rabies virus antigen to be presented by autologous antigen-presenting cells in association with HLA class II molecules. When cultured with rabies virus antigen, but in the absence of growth factors, some of the T cell clones provided help for an antibody response of rabies virus immune B lymphocytes. Analysis of culture supernatant fluids showed that at least a part of this antibody response was directed against neutralizing antibody-inducing determinants of the viral glycoprotein.

## INTRODUCTION

Rabies virus is a neurotropic virus that can cause fatal disease associated with virus replication in the central nervous system. Both virus-neutralizing antibodies and cell-mediated immunity appear to be involved in the protection against rabies virus (Wiktor *et al.*, 1974, 1977; Turner, 1985). The glycoprotein is generally regarded as the most relevant protein for protection, since only this protein was shown to induce virus-neutralizing antibodies (Wiktor *et al.*, 1973, 1984*a*, *b*; Cox *et al.*, 1977). Recently, data have been presented showing that the glycoprotein contains at least six distinct antigenic sites that induce virus-neutralizing antibodies (Lafon *et al.*, 1983*a*, *b*; Bunschoten *et al.*, 1989). Furthermore, the glycoprotein has been shown to elicit cytotoxic T lymphocytes that may directly eliminate rabies virus-infected cells (Wiktor *et al.*, 1977; Cho *et al.*, 1987; Celis *et al.*, 1988). Evidence that the development of humoral immunity against rabies virus depends on the activity of T lymphocytes was first demonstrated in athymic nude mice (Mifune *et al.*, 1981; Smith, 1981), in cyclophosphamide-treated mice (Smith, 1981) and by adoptive transfer experiments in mice (Prabhakar *et al.*, 1981). Previously, we have shown that also in man T helper cells are required for the induction of a rabies virus-specific antibody response *in vitro* (UytdeHaag *et al.*, 1983) and we have established human T cell clones specific for idiotypes of autologous anti-rabies virus antibody, the regulatory role of which remains to be defined (UytdeHaag *et al.*, 1987).

To investigate further the regulatory role of T lymphocytes in the establishment of rabies virus

immunity in man, we have generated antigen-specific T cell clones from a vaccinated human donor and analysed their specificity and function. Here we report on T cell clones that recognize rabies virus antigen in a major histocompatibility complex (MHC)-restricted manner, which assist rabies virus-immune B cells in the production of specific antibodies including those which have the ability to neutralize the rabies virus.

## METHODS

*Antigens.* Rabies virus Pitman-Moore (PM) strain (Wistar PM/wl-38-1503-3m) was propagated in dog kidney cells, purified and inactivated with $\beta$-propiolactone to produce dog kidney cell vaccine (PM-DKCV), as described previously (van Wezel *et al.*, 1978). The rabies virus PM strain was propagated in human diploid cells for the production of PM-HDCV, also in Vero cells for PM-VRV (gifts of Dr L. M. Lombard, Institut Mérieux, Lyon, France), both followed by inactivation, and in BHK-21 cells for PM-BHK (gift of Dr B. Dietzschold, Wistar Institute, Philadelphia, Pa., U.S.A.). The potency of rabies virus vaccines was 3·10 international units (IU)/dose for PM-DKCV, 3·73 IU/dose for PM-HDCV and 3·16 IU/dose for PM-VRV. Rabies virus Evelyn-Rokitnicki-Abelseth (ERA) and challenge virus standard (CVS) strains and rabies virus-related Mokola and Duvenhage-6 virus strains were propagated in BHK-21 cells, purified by gradient centrifugation, inactivated with $\beta$-propiolactone and adjusted to a protein concentration of 100 μg/ml (gifts of Dr B. Dietzschold). For use in cell cultures, antigen preparations were diluted in RPMI 1640 tissue culture medium. Unless otherwise stated the preparations were used at final concentrations of 0·01 IU per culture, corresponding to 1 μg per culture of PM-DKCV.

Rubella vaccine was prepared from the rubella HPV-77 strain (gift from Dr A. Plantinga, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). Dog kidney cells were washed and subjected to three cycles of freezing and thawing. The clarified supernatant fluid was used as dog kidney cell extract (DKCE).

*Proliferative responses of peripheral blood mononuclear cells (PBMC) to PM-DKCV.* Humans received a primary intramuscular vaccination with one dose of PM-DKCV, followed by two further immunizations on days 7 and 21. PBMC were obtained from seven donors by the sedimentation of heparinized blood on Ficoll-Isopaque (density 1·0779 g/ml) at 100 ***g*** for 20 min. PBMC were cultured in 96-well round-bottomed microtitre plates (Greiner & Söhne) at a concentration of $10^5$ cells per well in 150 μl RPMI 1640, supplemented with 10% (v/v) pooled human blood group AB serum, 2 mM-L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and $10^{-5}$ M-2-mercaptoethanol (referred to as complete medium). PM-DKCV was added in doses ranging from $10^{-2}$ to 10 μg per well. PBMC were incubated for 5 days at 37 °C and pulse-labelled with 0·5 μCi [$^3$H]thymidine over the last 16 h of culture incubation. Cells were harvested and the incorporated radioactivity was counted in a liquid scintillation counter. Results are expressed as the mean ± S.D. of triplicate cultures.

*T cell cloning.* PBMC were cultured in 24-well flat-bottomed microtitre plates (Costar) at a concentration of $2 \times 10^6$ cells per well in 1 ml complete medium, supplemented with 1 μg PM-DKCV. After 7 days in culture at 37 °C, the cells were harvested, washed twice and cloned by limiting dilution (0·3 cell/well) in 96-well round-bottomed microtitre plates. Each well received $4 \times 10^4$ of irradiated (1500 rads) autologous PBMC, 1 μg PM-DKCV and 10% (v/v) conditioned medium from MLA 144 cells containing 200 U/ml of interleukin 2 (IL2). Ten to 14 days later, growing clones were expanded, kept at a concentration of $1 \times 10^4$ cells per well and restimulated with PM-DKCV every 7 to 14 days of culture.

*Proliferation assays for T cell clones.* T cell clones were harvested 14 days after the last stimulation with PM-DKCV and washed three times. Cloned T cells ($10^4$) were cultured in round-bottomed wells in the presence of $4 \times 10^4$ autologous irradiated (1500 rads) PBMC and antigen preparations in complete medium. In proliferation assays to determine HLA restriction of antigen presentation, anti-HLA class I and class II monoclonal antibodies (MAbs) were added to the culture. The MAbs used were: B9.12.1 monomorphic anti-HLA class I (gift of B. Malissen), PdV5.2 monophorphic anti-HLA class II (gift of F. Koning) and B8.11.2 anti-HLA-DR at concentrations of approximately 1 μg per well. Furthermore, HLA restriction was studied using PBMC of different HLA-typed donors (HLA typing was performed at the Department of Immunology and Bloodbank, University Hospital, Leiden, The Netherlands). Cloned T cells ($10^4$) were cultured in round-bottomed wells in the presence of $4 \times 10^4$ irradiated (1500 rads) PBMC and 1 μg PM-DKCV. For all assays T cell clones were incubated for 3 days at 37 °C and pulse-labelled with 0·5 μCi [$^3$H]thymidine over the last 16 h of incubation. Cells were harvested and the incorporated radioactivity was determined in a liquid scintillation counter. Results are expressed as the mean ± S.D. of triplicate cultures.

*Helper activities of T cell clones.* Autologous PBMC were depleted from T cells and adherent cells by methods previously described (UytdeHaag *et al.*, 1983). Additional T cell depletion was achieved by incubation with a MAb to CD3 (anti-Leu 4, Becton-Dickinson) for 1 h at 4 °C, followed by incubation with 10% Low-Tox rabbit complement (Cedarlane) for 30 min at 37 °C. Non-T cell suspensions were shown to contain approximately 5% monocytes as determined by morphology and contained less than 1% $CD3^+$ T cells as determined by fluorescent antibody cell sorting (FACS) analysis. Non-T cells ($2 \times 10^5$) were incubated in complete medium in 96-well flat-

bottomed microtitre plates (Greiner & Söhne) together with cloned T cells ($10^3$ per well) and PM-DKCV ($10^{-2}$ to 1 μg per well). After 6 days of culture at 37 °C, the cells were washed four times and incubated for another 6 days. Supernatant fluids were harvested after 12 days of culture and then assayed for PM-DKCV-binding IgG in a solid-phase immunoassay (ELISA) as previously described (UytdeHaag *et al.*, 1983). The titre was calculated as the reciprocal of the highest dilution giving more than three times background value. Results are expressed as the mean value obtained with cultures tested in triplicate.

Competitive binding experiments were carried out by ELISA. Briefly, anti-glycoprotein MAbs 3-7B6 and 6-15C4 were coupled to horseradish peroxidase (HRP, Type VI, Sigma) according to standard methods (Nakane & Kawaoi, 1974). Supernatant fluids of the cultures were added to PM-DKCV-coated wells (600 ng/well). After incubation and washing, 100 μg/well anti-glycoprotein HRP-conjugated MAbs were added at dilutions that were known to give 90% of the maximum absorbance value on PM-DKCV. Results are expressed as the mean absorbance value of triplicate samples. Virus-neutralizing activity was determined in a rapid fluorescent focus inhibition test as previously described by Wiktor & Koprowski (1978).

## RESULTS

### *Establishment and specificity of T cell clones*

PBMC from seven vaccinated donors proliferated significantly in response to PM-DKCV in a dose-dependent manner. Table 1 shows the results of these responses after stimulation of PBMC with 1 μg/well PM-DKCV (0·01 IU/well). T cell clones were generated from lymphoblast-containing cultures of PBMC from donor 6. Of 2000 wells plated at 0·3 cell/well from PM-DKCV-stimulated bulk cultures, 2·6% yielded growing clones. Seven continuously growing, antigen (PM-DKCV)- and growth factor (IL2)-dependent T cell clones, designated RUR 8, RUR 23, RUR 25, RUR 33, RUR 34, RUR 60 and RUR 71, were further characterized for their phenotype, specificity and function. In FACS analysis all clones showed a homogeneous cell surface phenotype ($CD3^+$, $CD4^+$, $CD8^-$), consistent with T helper/inducer T cells (data not shown).

In the absence of exogenous IL2, all clones displayed a strong proliferative response upon stimulation with a wide range of concentrations (0·01 μg to 10 μg per well) of PM-DKCV. The optimal concentration of 1 μg per well was used in subsequent experiments. To determine the specificity of this response, proliferation was measured in response to antigen prepared from the PM strain of rabies virus produced in three different cell lines and in response to control antigen preparations. Specific responses were obtained for RUR 8, RUR 23, RUR 33, RUR 34, RUR 60 and RUR 71 with all antigen preparations tested of the rabies virus PM strain vaccines, PM-DKCV and PM-VRV, but not with the control vaccine (rubella vaccine) nor the antigen preparation, DKCE, containing dog kidney cell components present in PM-DKCV (Table 2). In contrast, T cell clone RUR 25 was found to exhibit specificity for a cell antigen since it proliferated in response only to PM-DKCV and DKCE antigen preparations (Table 2).

The rabies virus specificity of T cell clones RUR 8 and RUR 34 was further analysed in detail with antigen preparations of different rabies virus strains and rabies virus-related virus strains, propagated in BHK-21 cells. Both clones RUR 8 and RUR 34 responded to antigen

Table 1. *Antigen-specific proliferation of PBMC**

| Donor | Medium only (c.p.m.) | With PM-DKCV† (c.p.m.) |
|---|---|---|
| 1 | 719 ± 96 | 5172 ± 382 |
| 2 | 359 ± 43 | 14437 ± 1057 |
| 3 | 874 ± 64 | 7238 ± 102 |
| 4 | 769 ± 83 | 12019 ± 1266 |
| 5 | 298 ± 55 | 3267 ± 719 |
| 6 | 996 ± 209 | 34663 ± 4886 |
| 7 | 759 ± 128 | 16970 ± 645 |

* PBMC ($10^5$/well) were cultured for 72 h and then pulsed-labelled with 0·5 μCi/well [$^3$H]thymidine. Results are expressed as mean c.p.m. ± S.D. of triplicate cultures.

† 1 μg PM-DKCV/well.

Table 2. *Antigen specificity of T cell clones*

| T cell clones | Medium only | Antigen used for stimulation* PM-DKCV | PM-HDCV | PM-VRV | Rubella | DKCE |
|---|---|---|---|---|---|---|
| RUR 8 | 202 ± 126 | 9156 ± 632 | 6217 ± 517 | 6866 ± 832 | 314 ± 84 | 181 ± 72 |
| RUR 23 | 203 ± 59 | 14476 ± 1832 | 15160 ± 1604 | 5719 ± 447 | 278 ± 73 | 236 ± 22 |
| RUR 25 | 251 ± 156 | 9283 ± 703 | 305 ± 96 | 334 ± 75 | 264 ± 32 | 5972 ± 998 |
| RUR 33 | 114 ± 32 | 6565 ± 1449 | 4087 ± 1391 | 3049 ± 583 | 180 ± 72 | 112 ± 56 |
| RUR 34 | 359 ± 100 | 11388 ± 893 | 8039 ± 1047 | 6118 ± 769 | 493 ± 144 | 420 ± 38 |
| RUR 60 | 309 ± 162 | 7638 ± 742 | 8388 ± 885 | 7049 ± 443 | 378 ± 71 | 350 ± 41 |
| RUR 71 | 139 ± 74 | 4831 ± 602 | 3978 ± 663 | 3327 ± 501 | 162 ± 27 | 129 ± 47 |

* T cells ($10^4$) were cultured for 72 h in the presence of $4 \times 10^4$ irradiated autologous APC and antigen and then pulse-labelled with 0·5 μCi/well [$^3$H]thymidine for 16 h, as described in Methods. Results are expressed as mean c.p.m. ± S.D. of triplicate cultures. Antigen concentrations: PM-DKCV, 1 μg/well or 0·01 IU/well; PM-HDCV, 0·01 IU/well; PM-VRV, 0·01 IU/well; rubella virus vaccine, 1 μg/well; DKCE, 30% (v/v).

Table 3. *Specificity of T cell clones for rabies virus and rabies virus-related viruses*

| Antigen used for stimulation† | T cell clone* RUR 8 | RUR 34 |
|---|---|---|
| Medium only | 1075 ± 221 | 797 ± 268 |
| PM-BHK | 7689 ± 1505 | 7947 ± 543 |
| ERA | 4269 ± 980 | 4146 ± 587 |
| CVS | 8058 ± 778 | 7249 ± 129 |
| Mokola | 1570 ± 540 | 1198 ± 129 |
| Duvenhage-6 | 1036 ± 99 | 973 ± 157 |

* T cells ($10^4$) were cultured for 72 h in the presence of $4 \times 10^4$ irradiated autologous APC and antigen and then pulse-labelled with 0·5 μCi/well [$^3$H]thymidine for 16 h, as described in Methods. Results are expressed as mean c.p.m. ± S.D. of triplicate cultures.

† Rabies virus strains PM, ERA or CVS and the rabies virus-related viruses, Mokola and Duvenhage-6 (0·25 μg/well).

preparations of the rabies virus PM strain (PM-BHK), the rabies virus ERA strain and the rabies virus CVS strain (Table 3). However, these clones did not react upon stimulation with antigen preparations of the rabies virus-related Mokola and the Duvenhage-6 virus strains (Table 3).

### *MHC-restricted recognition of antigen by T cell clones*

MHC restriction of antigen recognition of the rabies virus-specific T cell clones was analysed with autologous irradiated PBMC as antigen-presenting cells (APC) and anti-HLA-specific MAbs. In the presence of an anti-HLA class I-specific MAb (B9.12.1) the proliferative response of clones RUR 8, RUR 23, RUR 33, RUR 34, RUR 60 and RUR 71 to PM-DKCV was not significantly reduced ($\leqslant 25\%$ inhibition, Fig. 1). Significant reduction in the proliferation of clones RUR 8, RUR 23, RUR 33, RUR 34 and RUR 60 to PM-DKCV ($\geqslant 40\%$ inhibition) was obtained in the presence of an anti-HLA class II-specific MAb, PdV5.2, and an anti-HLA-DR-specific MAb, B8.11.2. However, MAbs PdV5.2 and B8.11.2 were unable to reduce the proliferation of RUR 71 ($\leqslant 25\%$ inhibition) significantly upon stimulation with PM-DKCV in this assay (Fig. 1).

Using a different approach, with PBMC of an HLA-typed panel of donors as APC, specific proliferation of clones RUR 8, RUR 23, RUR 34 and RUR 71 to PM-DKCV could be demonstrated only when APC were matched for HLA-DR2 (Table 4).

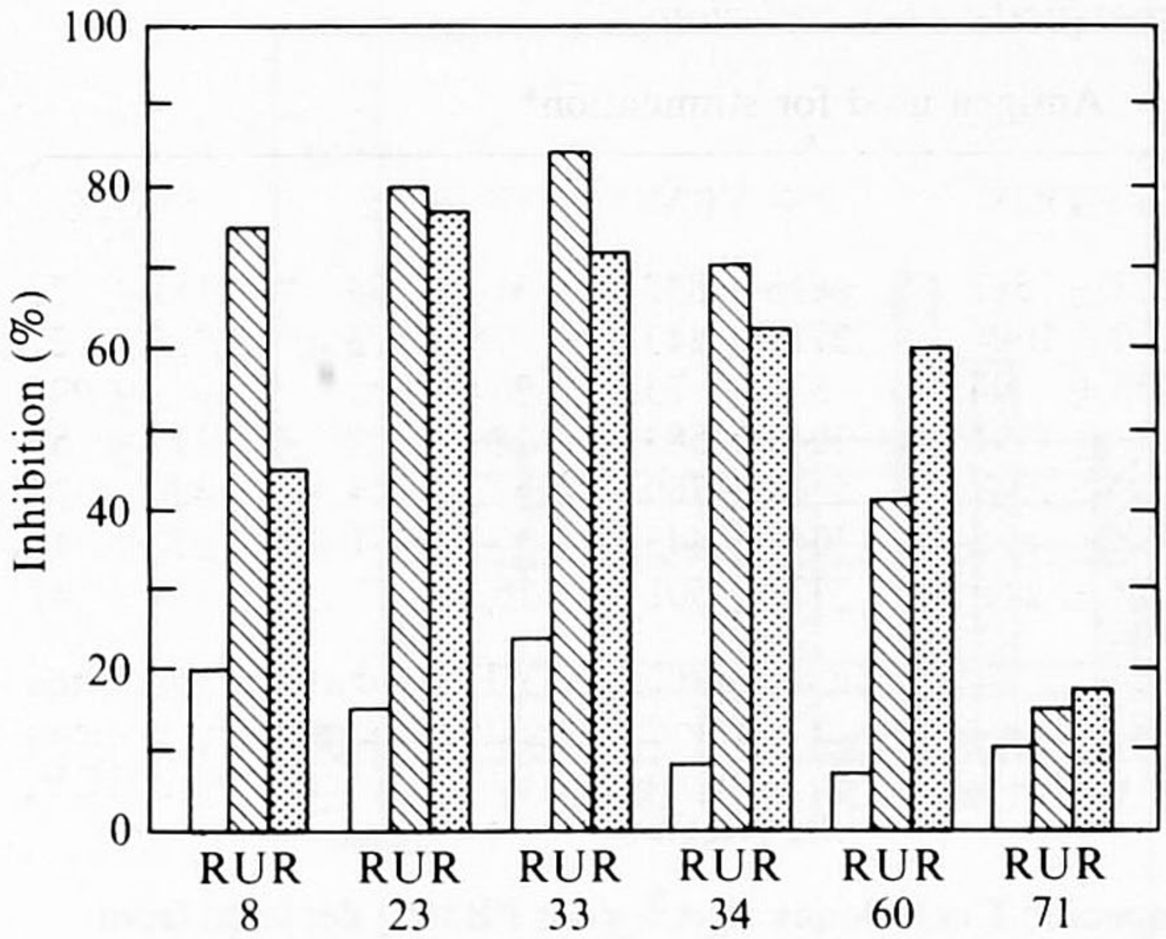


Fig. 1. MHC-restricted recognition of antigen by rabies virus-specific T cell clones. T cell clones RUR 8, RUR 23, RUR 33, RUR 34, RUR 60 or RUR 71 were cultured as described in Methods in the presence of an anti-HLA class I-specific MAb (□), an anti-HLA class II-specific MAb (▨) or an anti-HLA-DR-specific MAb (⊡). The results are expressed as the percentage inhibition of maximum proliferation to rabies virus antigen of triplicate cultures.

Table 4. *HLA-DR2-restricted antigen recognition of rabies virus-specific T cell clones*

| T cell clone* | Antigen used for stimulation | Antigen-presenting cells: DR*2*, 4† DQ1, 3 | DR3, 4‡ DQ2, 3 | DR*2*, 3‡ DQ1, 2 | DR1, *2*‡ DQ1 | DR1, 7‡ DQ1, 2 |
|---|---|---|---|---|---|---|
| RUR 8 | Medium only | 312 ± 175 | 218 ± 109 | 124 ± 55 | 447 ± 130 | 115 ± 17 |
| | PM-DKCV | 3004 ± 436 | 318 ± 205 | 2090 ± 220 | 4927 ± 68 | 424 ± 115 |
| RUR 23 | Medium only | 60 ± 7 | 131 ± 42 | 101 ± 18 | 204 ± 117 | 139 ± 12 |
| | PM-DKCV | 1868 ± 392 | 197 ± 90 | 1453 ± 362 | 4400 ± 476 | 261 ± 45 |
| RUR 34 | Medium only | 67 ± 37 | 199 ± 43 | 143 ± 32 | 430 ± 225 | 357 ± 136 |
| | PM-DKCV | 5330 ± 145 | 602 ± 323 | 1465 ± 425 | 3671 ± 416 | 586 ± 172 |
| RUR 71 | Medium only | 111 ± 18 | 184 ± 84 | 149 ± 7 | 185 ± 39 | 77 ± 15 |
| | PM-DKCV | 2861 ± 601 | 220 ± 33 | 2368 ± 238 | 6488 ± 2463 | 245 ± 73 |

* T cells ($10^4$) were cultured for 72 h with 1 μg/well PM-DKCV or in medium alone in the presence of $4 \times 10^4$ irradiated autologous (†) or HLA-typed allogeneic (‡) antigen-presenting cells and then pulse-labelled with 0·5 μCi/well [$^3$H]thymidine for 16 h, as described in Methods. Results are expressed as mean c.p.m. ± S.D. of triplicate cultures.

### *Functional analysis of T cell clones*

T cell clones were examined for their ability to assist in a secondary antibody response involving autologous B cells *in vitro*. PBMC, depleted of T cells (further referred to as non-T cells) were reconstituted with T cell clones and cultured in the presence of PM-DKCV. The non-T cells were found to produce PM-DKCV-binding IgG (Fig. 2) when cultured in the presence of any one of clones RUR 8, RUR 23, RUR 33, RUR 34, RUR 60 (1000 cells/well) using optimal doses of PM-DKCV (0·01 to 1·0 μg/well). However, clone RUR 71 failed to assist the non-T cells in the production of PM-DKCV-binding IgG, at either of the PM-DKCV concentrations tested (Fig. 2). Clearly, no PM-DKCV-binding IgG could be detected, when the non-T cells were cultured either with PM-DKCV in the absence of T cell clones, or without PM-DKCV and in the presence of T cell clones (Fig. 2).

We further investigated whether the antibodies produced showed specificity for rabies virus glycoprotein. Supernatant fluids from cultures containing PM-DKCV-binding IgG were tested for the ability to inhibit the binding of virus-neutralizing MAbs 3-7B6 (specific for antigenic site I) and 6-15C4 (specific for antigenic site VI) to plate-bound PM-DKCV. Fig. 3 shows that supernatant fluids from cultures of non-T cells that had been stimulated in the presence of antigen and clones RUR 8 or RUR 34 inhibited the binding of both MAbs 3-7B6 (Fig. 3*a*) and

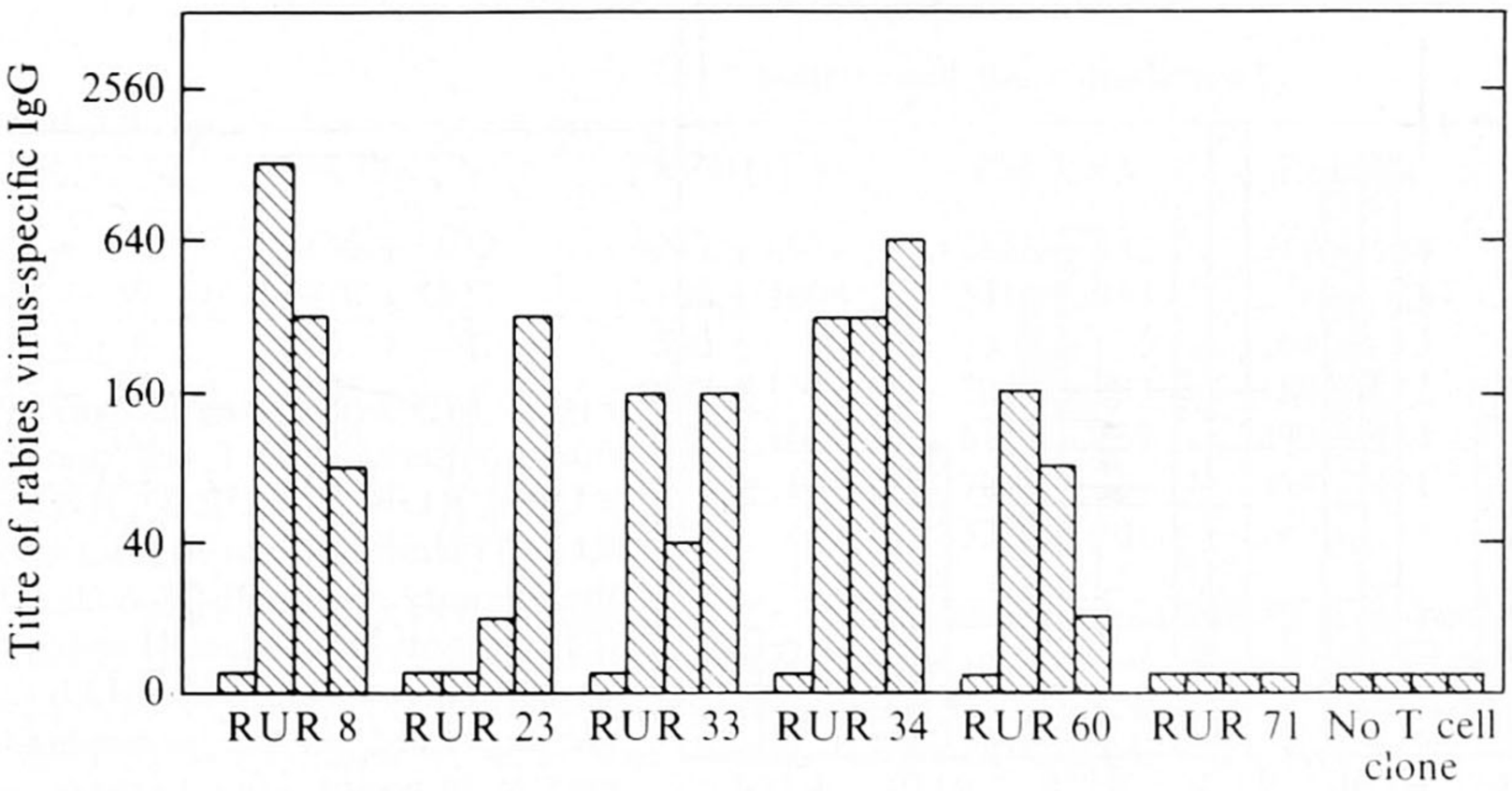


Fig. 2. *In vitro* helper function of rabies virus-specific T cell clones. Autologous PBMC, depleted from T cells and adherent cells, were cultured at $2 \times 10^5$ cells/well with T cell clones RUR 8, RUR 23, RUR 33, RUR 34, RUR 60, RUR 71 ($10^3$ cells/well) or no T cells and with PM-DKCV (0·01, 0·1 and 1·0 μg/well in the second, third and fourth columns, respectively for each clone), or no rabies virus antigen (0 μg/well in the first column) for 12 days. Thereafter, the supernatant fluids of cultures were harvested and assayed for PM-DKCV-binding IgG in ELISA. The results represent the mean antibody titre of supernatant fluids obtained from triplicate cultures.

Table 5. *T cell help for a neutralizing antibody response*

| Sample | Culture conditions | Titre* |
|---|---|---|
| Culture supernatant | Non-T cells + PM-DKCV + RUR 34 | 16 |
| Culture supernatant | Non-T cells + PM-DKCV | ⩽2 |
| MAb 3-7B6 | | 4096 |
| MAb 6-15C4 | | 2048 |

* Titre in rapid fluorescent focus inhibition test.

6-15C4 (Fig. 3*b*), and supernatants from cultures that had been stimulated in the presence of antigen and clone RUR 33 inhibited only the binding of MAb 3-7B6 to plate-bound antigen (Fig. 3). In contrast, supernatant fluids from cultures that had been stimulated with antigen and clone RUR 60 inhibited only the binding of MAb 6-15C4 to plate-bound antigen (Fig. 3). In spite of the presence of the PM-DKCV-binding IgG, the supernatants from cultures that had been stimulated with antigen and clone RUR 23 inhibited the binding of neither MAb 3-7B6 nor MAb 6-15C4 to plate-bound antigen (Fig. 3).

The presence of virus-neutralizing antibody in supernatant fluids was confirmed in the rapid fluorescent focus inhibition test. Supernatants from cultures that had been stimulated with antigen and RUR 34 neutralized the CVS strain of rabies virus, whereas supernatant fluids of non-T cells cultured in the absence of clone RUR 34 did not (Table 5).

## DISCUSSION

Antigen-specific T cell clones and lines, specific for rabies virus antigens, producing IL2 and exerting cytotoxic T lymphocyte functions have been previously documented for both the human (Celis *et al.*, 1986, 1988) and murine (Cho *et al.*, 1987) systems. In this report we describe a panel of rabies virus-specific, HLA class II-restricted human T cell clones which have been shown to function as helper T cells for the *in vitro* synthesis of antibodies by autologous B cells.

All clones reported in this study, except one, RUR 25, showed specificity for rabies virus antigen as demonstrated by their proliferative response to different antigen preparations of the

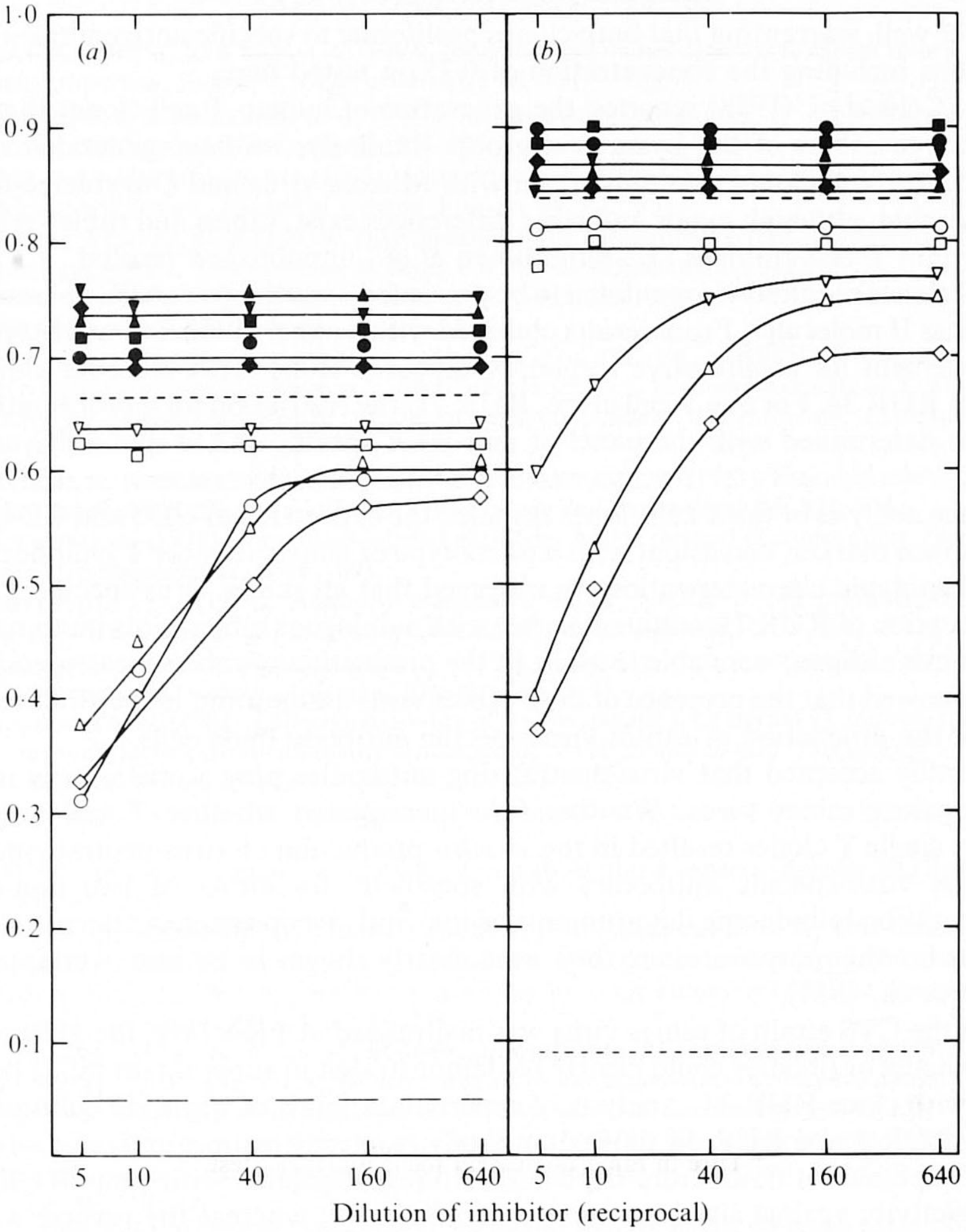


Fig. 3. *In vitro* helper function for an anti-glycoprotein antibody response of rabies virus-specific T cell clones. Autologous PBMC, depleted from T cells and adherent cells, were cultured as described for Fig. 2 with T cell clones RUR 8 (◇, ◆) RUR 23 (□, ■), RUR 33 (○, ●), RUR 34 (△, ▲) or RUR 60 (▽, ▼) in either the presence (open symbols) or absence (closed symbols) of rabies virus antigen. Supernatant fluids were tested for their reactivity against two non-overlapping antigenic sites, site I (*a*) and site VI (*b*) of the glycoprotein, by their ability to inhibit the binding of HRP-coupled rabies virus-neutralizing MAbs 3-7B6 (*a*) and/or 6-15C4 (*b*) in competitive binding assays. Reactivities of homologous uncoupled MAbs 3-7B6 (——) and 6-15C4 (– – –) were tested at a concentration of 1 mg/ml. The results are expressed as the mean absorbance value obtained from triplicate cultures.

rabies virus PM strain and also as illustrated by the response of clones RUR 8 and RUR 34 to antigen preparations of the ERA and CVS strains of rabies virus. Clones RUR 8 and RUR 34, however, did not cross-react with the rabies virus-related Mokola and Duvenhage-6 virus strains, which is consistent with the observations of others who demonstrated a high degree of antigenic variability among rabies virus isolates and other representatives of the Lyssavirus group (Wiktor *et al.*, 1984*a*, *b*; Lumio *et al.*, 1986). One could argue that the failure to demonstrate cross-reactivity of these T cell clones is related to the concentration of stimulating antigen. However, antigen preparations of the rabies virus PM, ERA and CVS strains were used at concentrations similar to those used for the antigen preparations of the rabies virus-related Mokola and Duvenhage-6 virus strains. Furthermore, RUR 8 and RUR 34 proliferated significantly in response to an antigen preparation of the rabies virus PM strain between 0·01 μg

and 10 μg per well, warranting that both clones proliferate to specific antigen in a wide range of concentrations including the concentration of 0·25 μg tested here.

However, Celis *et al.* (1988) reported the generation of human T cell clones that cross-react with different members of the Lyssavirus group. Similarly, we have generated rabies virus-specific murine T cell clones that cross-react with Mokola virus and Duvenhage-6 virus. This demonstrates that, although major antigenic differences exist, rabies and rabies-related viruses do share certain T cell epitopes (H. Bunschoten *et al.*, unpublished results).

The T cell clones required virus antigen to be presented as autologous APC in association with HLA-DR class II molecules. From results obtained with a panel of HLA class II-typed APC the restriction element for proliferative responses appeared to be HLA-DR2 for clones RUR 8, RUR 23 and RUR 34. For one T cell clone, RUR 71, the restriction for a proliferative response could not be determined with the panel of anti-HLA-specific MAbs and APC used in these studies.

Cell surface analysis of the T cell clones revealed the expression of CD3 and CD4, but not the CD8 cell surface marker, consistent with a phenotype of helper/inducer T lymphocytes. In line with this phenotypic characterization we observed that all rabies virus-specific T cell clones, with the exception of RUR 71, cultured *in vitro* with autologous rabies virus immune non-T cells and rabies virus antigen, were able to assist in the production of rabies virus-specific IgG. The results also showed that the presence of both rabies virus antigen and helper/inducer T cells are required for the production of rabies virus-specific antibody by B cells.

It is generally accepted that virus-neutralizing antibodies play a crucial role in protective immunity against rabies virus. We therefore investigated whether T cell helper activity mediated by single T clones resulted in the *in vitro* production of virus-neutralizing antibodies and/or rabies virus-specific antibodies with specificity for either of two non-overlapping neutralizing antibody-inducing determinants of the viral glycoprotein. Antigenic sites I and VI were chosen for this purpose, since they were clearly shown to be non-overlapping (Fig. 3; Bunschoten et al., 1989).

Although the CVS strain of rabies virus was neutralized at a low titre, the presence of rabies virus-neutralizing antibodies could clearly be demonstrated in supernatant fluids from cultures stimulated with clone RUR 34. Analysis of supernatant fluids of triplicate cultures stimulated with clones RUR 8 and RUR 34 showed antibody reactivity against antigenic sites I and VI. Analysis of supernatant fluids from triplicate cultures stimulated with clone RUR 33 showed antibody reactivity against site I but none against site VI, whereas the reverse was observed when supernatant fluids from cultures stimulated with clone RUR 60 were analysed. Analysis of supernatants from triplicate cultures stimulated with clone RUR 23 showed the presence of virus-specific IgG, but antibody directed to antigenic site I or VI could not be detected.

These observations demonstrate that rabies virus-reactive B cells are not activated indiscriminately, but that rabies virus-specific T cell clones mediate helper activity to defined populations of rabies virus-specific B cells. Therefore, the specificities observed of the *in vitro* produced antibodies may either be a reflection of differences in polypeptide specificities of the respective T cell clones and/or of differences in processing and presentation mechanisms of T cell epitopes by different B cell populations (Manca *et al.*, 1985; Ozaki & Berzofsky, 1987; Scherle & Gerhard, 1988). At present, studies on the polypeptide specificities of the T cell clones are in progress.

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