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Induction of Transmissible Gastroenteritis Coronavirus-neutralizing Antibodies *in vitro* by Virus-specific T Helper Cell Hybridomas

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SUMMARY

Three transmissible gastroenteritis (TGE) virus-specific T helper (Th) cell hybridomas have been generated from virus-primed BALB/c mice, by fusion with the thymoma BW5147. The hybridomas responded to purified u.v.-inactivated TGE virus with interleukin production and growth inhibition. TGE virus recognition by the hybridomas was restricted by the major histocompatibility complex: only splenocytes from syngeneic or semi-syngeneic mice were able to recognize the antigen. The three hybridomas were Thy 1.2^+, but did not express detectable levels of Lyt 1 or Lyt 2 antigens by fluorescent cell sorting analysis. Only one hybridoma (T.1J.B5) expressed the L3T4 marker. These hybridomas had helper activity, as they were able to reconstitute *in vitro* the synthesis of TGE virus-specific antibodies by Th cell-depleted spleen cells from immune BALB/c mice. The antibodies that they induced specifically neutralized by 10^3- to 10^4-fold the infectivity of TGE virus, ruling out the possibility of inhibition of virus replication by interferon. These hybridomas could be very useful for identifying antigenic domains in TGE virus recognized by Th cells, which cooperate with B cells in the synthesis of neutralizing antibodies.

INTRODUCTION

Transmissible gastroenteritis (TGE) is a highly contagious enteric disease generally fatal in newborn piglets. The causal agent, TGE virus, is a member of the *Coronaviridae* family, and has a positive-stranded RNA genome of M_r $6{\cdot}8 \times 10^6$ (Siddell *et al.*, 1983; Sturman & Holmes, 1983; Brian *et al.*, 1980) which includes gene sequences coding for three structural proteins: a surface glycoprotein (E2), a transmembrane matrix protein (E1) and a nucleoprotein (N) comprising 1447, 262 and 382 amino acids, respectively (Garwes *et al.*, 1978–1979; Kapke & Brian, 1986; Laude *et al.*, 1987; Rasschaert & Laude, 1987). The E2 protein contains the main critical epitopes in TGE virus neutralization (Laude *et al.*, 1986; Jiménez *et al.*, 1986).

Passive immunity is of primary importance in providing newborn piglets with immediate protection against TGE virus infection. This immunity can be transferred by maternal antibodies from immune sows. Unfortunately, none of the commercially available vaccines induces protective maternal antibodies. In addition, virulent field virus is frequently used to immunize pregnant mothers in order to induce protective IgA to be transferred to the progeny, a procedure which can result in all the farm animals being persistently infected with the virus (Saif & Bohl, 1986).

To design new vaccines it is useful to define the antigenic determinants recognized by B and T cells. The antigenic structure of the E2 protein has been defined for the B cell compartment. Four antigenic sites have been described, one of which (site A) is dominant and contains conserved epitopes involved in virus neutralization (Jiménez *et al.*, 1986; Laude *et al.*, 1986). This site has been divided into three antigenic subsites and eight critical epitopes (Correa *et al.*, 1988). Although this information was originally obtained with monoclonal antibodies (MAbs) of

murine origin, it was shown that, in swine, neutralizing antibodies also reacted with the same antigenic sites (Correa *et al.*, 1988). In contrast, very little information is available on the antigenic regions recognized by the T cell compartment.

In this manuscript we describe the isolation of TGE virus-specific murine T helper (Th) cell hybridomas, which cooperated with B cells in the synthesis *in vitro* of virus-neutralizing antibodies. These hybridomas could be very useful in at least two areas: (i) the identification of T cell epitopes relevant in the induction of TGE virus-neutralizing antibodies and which could be included in a subunit vaccine containing B and T cell epitopes and (ii) the study of the antigenic variability of coronavirus with probes recognizing T cell epitopes.

METHODS

Virus. Clone PUR 46-C1.P1 formerly designated PUR 54-C1.P1 (Jiménez *et al.*, 1986) of TGE virus, originally obtained from E. H. Bohl (Ohio Agricultural Research and Development Center, Wooster, Ohio, U.S.A.) was used. The Victoria (H3N2) strain of influenza A virus was kindly provided by W. Gerhard (Wistar Institute, Philadelphia, Pa., U.S.A.). Moloney leukaemia virus (MoLV), produced by the cell line NIH-MoLV, was kindly provided by J. N. Ihle (Frederick Cancer Research Center, Frederick, Md., U.S.A.). Vesicular stomatitis virus (VSV), New Jersey strain, was obtained from the American Type Culture Collection (VR 159), and adapted to grow in swine testicular (ST) cells by three passages in cell culture.

Cells. The epithelial ST cell line developed by McClurkin & Norman (1986) was obtained from Dr L. J. Kemeny (National Animal Disease Center, Ames, Iowa, U.S.A.). A subclone (BW.EHK 5147) of BW5147 thymoma isolated by E. Heber-Katz was kindly provided by F. Melchers (Basel Institute for Immunology, Basel, Switzerland). The influenza A virus-specific T cell hybridoma P1.6 was kindly provided by W. Gerhard.

Animals. BALB/c mice, originally obtained from the Frederick Cancer Research Center, were used for immunization and as a source of splenocytes, when they were approx. 10 weeks old. F1 (BALB/c × C3H) hybrids were obtained by breeding BALB/c with C3H mice, kindly provided by M. Nabholz (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). DBA and C57BL/6 mice were purchased from IFFA-CREDO. B10.BR, B10.D2, B10 and B10.A/4R mice were kindly provided by F. Garrido (Hospital Virgen de las Nieves, Granada, Spain). BALB/k mice were kindly provided by I. Barasoain (Centro de Investigaciones Biológicas, Madrid, Spain).

MAbs. The TGE virus-specific MAb 6A.C3 was obtained and characterized as described previously (Jiménez *et al.*, 1986; Correa *et al.*, 1988). As a control MAb, culture medium from P3-X63-Ag8 myeloma cells (Kearney *et al.*, 1979) was used.

T cell growth factor (TCGF). TCGF was obtained by incubating BALB/c spleen cells with concanavalin A, as described by Gillis *et al.* (1978). Purified recombinant interleukin 2 (IL-2) of human origin was kindly supplied by J. L. Barbero (Antibióticos S.A.). TCGF and IL-2 activity was evaluated in the cytotoxic T cell line CTLL-2 (Gillis & Smith, 1977) as previously described (Gillis *et al.*, 1978). Briefly, CTLL-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% TCGF (concanavalin A-conditioned medium) and 5×10^{-5} M-2-mercaptoethanol. Forty-eight h after the last addition, the cells were washed, and 15×10^3 cells per well were resuspended in 50 µl of DMEM with 4% foetal calf serum (FCS) and 50 µl of the supernatant containing the interleukin to be evaluated. The cells were incubated for 24 h and 1 µCi of [^{3}H]thymidine (sp. act. 2 Ci/mmol; Amersham, TRA.310) was then added. The cells were incubated for a further 16 h, collected onto glass filters with a cell harvester (Skatron) and the radioactivity incorporated was determined by standard procedures. All experiments were repeated at least three times. In addition, evaluations employing cells in tissue culture were done in triplicate, and the standard error of the mean (SEM) was $<15\%$ in all cases.

Virus titration, neutralization and purification. TGE virus was grown and titrated on ST cells as previously described (Jiménez *et al.*, 1986). Plaque reduction assays and purification of the virus were performed as described (Jiménez *et al.*, 1986; Correa *et al.*, 1988). The neutralization index was determined by dividing the p.f.u. of virus per ml mixed with control antibody by the p.f.u. of virus per ml in the presence of the given antibody, and was expressed as the $\log_{10}$ of this ratio.

MoLV was produced and purified as described by Ihle *et al.* (1973). Influenza virus was grown in MDCK cells and purified by a procedure similar to one previously described (Pons, 1977).

Radioimmunoassay (RIA). For RIA, TGE virus particles (0·25 µg of protein/well in 50 µl of phosphate-buffered saline) was adsorbed to polyvinyl chloride disposable U-bottomed plates (Dynatech Laboratories) by overnight incubation at 37 °C. The subsequent steps of the assay were performed as described previously (Sanz *et al.*, 1985; García-Barreno *et al.*, 1986), using a second antiserum (mouse immunoglobulin-specific rabbit antiserum) to amplify the reaction and ^{125}I-labelled Protein A in the final stage of the assay. Evaluations were done in triplicate, and the SEM was $<15\%$ in all cases.

Isolation of T cell hybridomas. The procedure used was derived from a method described by Hurwitz *et al.* (1984). BALB/c mice were immunized via the rear footpads with u.v.-inactivated, purified TGE virus emulsified in complete Freund's adjuvant (20 μg in 0·04 ml per footpad). After 7 days popliteal and inguinal lymph nodes were excised, single cell suspensions were prepared, and the cells were resuspended in DMEM supplemented with 10% FCS. T cells were selected for on nylon wool columns (Julius *et al.*, 1973) and were incubated for 3 days (37 °C, 9% CO_2), at a density of 2×10^6 cells/ml, in DMEM supplemented with 1% BALB/c mouse serum, 2 mM-glutamine, 1 mM-pyruvic acid, and 10 mM-HEPES in the presence of 4 μg of TGE virus per ml and 2×10^6 2000 rads (R)-irradiated spleen cells from non-immune mice per ml.

T cells were fused to the azaguanine-resistant tumour line BW5147 originally isolated by R. Hyman (Salk Institute, La Jolla, Ca., U.S.A.). To perform the fusion a mixture of T cells and tumour cells (3:1) was incubated with 50% polyethylene glycol (PEG) (Koch-Light) at 37 °C for 1 min. The PEG was then diluted for 8 min with culture medium not containing serum. The cells were left 10 min at room temperature, sedimented, resuspended ($7{\cdot}5 \times 10^5$ lymphocytes per ml) in DMEM supplemented with 15% FCS, 2 mM-glutamine, 1 mM-sodium pyruvate, 20 mM-HEPES, $6{\cdot}6 \times 10^{-5}$ mM-sodium selenite, $5{\cdot}3 \times 10^{-5}$ mM-D-biotin, $9{\cdot}6 \times 10^{-6}$ mM-vitamin B12 and 50 μg/ml of gentamicin (T cell growth medium), and 0·3 ml aliquots per well were grown in 48-well microculture plates (Costar), in the presence of 6×10^6 syngeneic spleen feeder cells (2000 R-irradiated) per ml. After 24 h hybridomas were selected for by adding T cell growth medium with hypoxanthine, aminopterin and thymidine (HAT medium) (Jiménez *et al.*, 1986). Half the medium was changed every 5 days, and 15 to 20 days after fusion the HAT medium was replaced by HT medium (HAT medium without aminopterin). The HT medium was removed 15 days later. Hybridoma growth was detected 7 to 15 days after fusion. Growth-positive cultures were harvested and recultured at a cell density between 10^5 and 10^6 cells/ml.

The hybrid cell lines were then tested for the ability to secrete interleukin in response to antigenic (TGE virus) challenge. To perform this assay 50×10^3 hybridoma cells were cultured at 37 °C for 48 h in 160 μl (final volume) of T cell growth medium. The u.v.-inactivated TGE virus (4·0 μg/ml, unless indicated otherwise) was presented by 2×10^5 2000 R-irradiated spleen cells from BALB/c mice per well. The supernatants were then harvested and the presence of interleukin was evaluated on CTLL-2 cells (Gillis *et al.*, 1978; Kupper *et al.*, 1987). TGE virus antigen-reactive cell lines were then cloned by limiting dilution in the presence of feeder splenocytes (2000 R-irradiated). The cell hybrids were seeded (0·3 to 1 cells/well) in flat bottom 96-well microplates (Costar), and postive clones were selected from plates that showed growth in fewer than 30% of the wells.

H-2 restriction of T cell hybridomas. To determine the major histocompatibility complex restriction of the isolated hybridomas, the induction of interleukin synthesis by antigen (u.v.-inactivated virus) and presenting cells (2×10^5 2000 R-irradiated spleen cells) from the indicated mouse strains was studied in an assay similar to the one used in the evaluation of the T cell hybridomas.

Analysis of cell surface antigens by immunofluorescence. Cells were washed once at 4 °C with phosphate-buffered saline and aliquots of 5×10^5 to 10×10^5 cells per tube were incubated at 4 °C for 30 min in the presence of 20 μl of undiluted hybridoma supernatant of the indicated specificity. Cells were then washed twice with DMEM by centrifugation at 1500 r.p.m. (Sorvall HL-4 rotor) at 4 °C for 4 min. The cell pellet was resuspended in 20 μl of the second antibody, a fluorescein isothiocyanate-labelled $F(ab')_2$ rabbit anti-rat immunoglobulin or a fluorescein isothiocyanate-labelled $F(ab')_2$ goat anti-mouse immunoglobulin, and incubated at 4 °C for 30 min. The cells were washed twice as before, resuspended in 0·5 ml of DMEM and immediately analysed in a fluorescence-activated cell sorter (EPICS; Coulter). MAbs specific for Thy 1.2 (30-H12, rat IgG2b) (Ledbetter & Herzenberg, 1979), Lyt 1 (53-7.313, rat IgG2a) (Ledbetter & Herzenberg, 1979), Lyt 2 (53-6·72, rat IgG2a) (Ledbetter & Herzenberg, 1979) and $H\text{-}2^d$ (34-1-2, mouse IgG2a) (Ozato *et al.*, 1982), purchased from the American Type Culture Collection, were used in the analysis. The MAbs specific for L3T4 (GK1.5, rat IgG2b) (Wilde *et al.*, 1983). MAb J11d (a B and T cell marker) (rat IgM) (Crispe & Bevan, 1987) and $H\text{-}2^k$ (11-4-1, mouse IgG2a) (Oi *et al.*, 1978) were also used and were kindly provided by B. Regueiro (University of Santiago de Compostela, Spain).

Synthesis of TGE virus-specific antibodies in vitro. The method followed was derived from one used by Folks & Sell (1983), with minor modifications. As a source of B cells, splenocytes from TGE virus-immune BALB/c mice were used. Best results were obtained with mice immunized twice intraperitoneally, with 40 μg of u.v.-inactivated, purified TGE virus. The first dose was administered in complete Freund's adjuvant, the second was administered without adjuvant 4 weeks later, and the splenocytes were isolated 2 to 4 weeks after the last immunization. After single cell suspensions had been prepared, the erythrocytes were lysed with ACK buffer (0·155 M-NH_4Cl, 0·1 mM-disodium EDTA, 0·01 mM-$KHCO_3$). The cells were washed three times with 2% FCS in DMEM, resuspended (10^7 cells/ml) in culture medium (DMEM supplemented with 5% FCS, 10 mM-HEPES, 5×10^{-5} M-2-mercaptoethanol and 50 μg/ml gentamicin) and incubated (10 to 15 ml per 100 mm diameter Petri dish) for 45 min at 37 °C to effect partial removal of the macrophages. The unbound cells were collected by gently swirling with 2% FCS in DMEM. When indicated, the cells were depleted of Th cells by resuspending 10^7 cells in 125 μl of a mixture (1:1) of undiluted culture medium of the hybridomas secreting the MAbs GK1.5 and 53-7-313, specific for the cell surface markers L3T4 and Lyt 1, respectively, and 25 μl of rabbit serum, reconstituted following the

manufacturer's (Cedarlane Laboratories) instructions and used without further dilution as a source of complement. Cells were washed, and the incubation was repeated. There was 25% cell lysis. The cells were then washed three times with 2% FCS in DMEM and resuspended in culture medium.

T cells were prepared from immunized BALB/c mice as indicated above, by passage over nylon wool columns (Julius *et al.*, 1973; Enjuanes *et al.*, 1979) and were resuspended in culture medium.

TGE virus-specific T cell hybridomas were 2000 R-irradiated, washed once with 2% FCS in DMEM and resuspended in culture medium.

To study the synthesis of TGE virus-specific antibodies, spleen cells from BALB/c mice (7×10^5 cells/well), almost totally depleted of macrophages (by removal of adherent cells after incubating at 37 °C for 45 min on plastic dishes), were used as a source of B cells. The cells were seeded in flat bottom 96-well microplates in 200 µl/well. To evaluate the helper activity, Th cell-depleted spleen cells ($4{\cdot}5 \times 10^5$ cells/well) were cultured in the presence of nylon wool-bound T cells ($3{\cdot}5 \times 10^5$ cells/well) or the indicated T cell hybridoma (5×10^3 to 10×10^3 cells/well). After the first incubation of 3 days in the presence of u.v.-inactivated purified TGE virus, 30 µl of culture medium was added to each well and the incubation was continued for 2 or 3 days. Part of the medium was then changed by centrifuging the plates at 1000 r.p.m. in an HL-4 Sorvall rotor for 5 min at room temperature, collecting 180 µl of the supernatant, and adding an identical volume of fresh culture medium. After 5 days of additional incubation (or when indicated) samples of 100 µl were collected and stored at −20 °C until evaluation.

RESULTS

Isolation of TGE virus-specific T cell hybridomas

Fusions were performed between the lymphoma BW5147 and T cells from TGE virus-immune BALB/c mice to obtain T cell hybridomas. Clones of the BW5147 thymoma from various sources were tested, and the subclone BW.EHK 5147 was found to provide the best results. Two fusions were performed, with this clone, and a total of 500 wells were seeded, of which 290 showed growth and 135 were positive in the first screening. The cells in a well were considered positive when, after incubation in the presence of TGE virus, their supernatants induced proliferation in CTLL-2 cells with a stimulation index >3 in relation to control medium. The 30 hybridomas giving the highest response to the virus were selected, but in subsequent cloning steps most lost their ability to produce interleukin. After 12 cloning steps only three hybridomas retained a high stimulation index (ranging between 7 and 15) and showed reproducible production of interleukin when incubated in the presence of TGE virus (Table 1).

In order to find out whether the hybridomas were specific for TGE virus, the production of interleukin in the presence of TGE virus and of antigenically unrelated viruses (influenza virus and MoLV) was studied. The results (Fig. 1) indicated that the T.1C.D7, T.1P.D1 and T.1J.B5 hybridomas secreted interleukin in the presence of TGE virus, but not in the presence of influenza virus, MoLV or tissue culture medium (Fig. 1*a*, *b* and *c*, respectively). In contrast, the T cell hybridoma P1.6 secreted interleukin in the presence of influenza virus, but not when it was incubated in the presence of TGE virus or MoLV (Fig. 1*d*). As expected, the parental thymona BW5147 did not respond to any of the viruses tested (Fig. 1*e*). These results indicate that the three selected hybridomas were TGE virus-specific.

Table 1. *Cloning of TGE virus-specific T cell hybridomas*

	Fraction (%) of positive wells* (S.I.)† Cloning number			
Hybridoma	1	4	8	12
T.1C.D7	37 (12)	100 (13)	70 (10)	94 (8)
T.1P.D1	100 (9)	86 (10)	83 (9)	100 (15)
T.1J.B5	76 (9)	96 (11)	88 (7)	92 (10)

* Percentage of positive wells, i.e. hybridoma subclones producing interleukin, after stimulation with TGE virus, that induced proliferation in the CTLL-2 cell line, measured by [^{3}H]thymidine incorporation as described in Methods, with a stimulation index >3.

† S.I., Mean value of the stimulation index of positive clones.

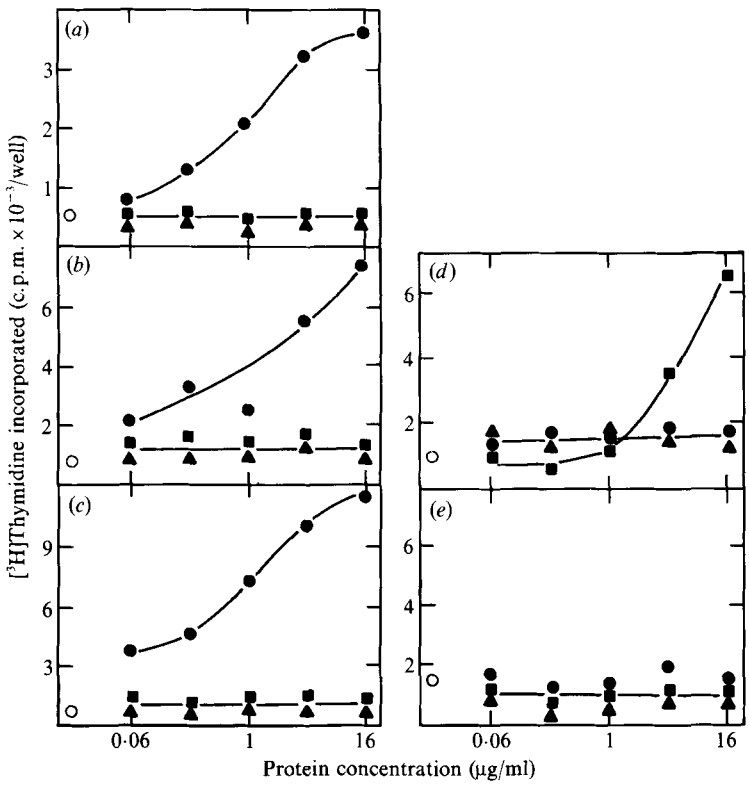

Fig. 1. Specificity of the T cell hybridomas. The induction of interleukin synthesis in the TGE virus-specific hybridomas T.1C.D7, T.1P.D1 and T.1J.B5 (*a*, *b* and *c*, respectively), in the influenza virus-specific hybridoma P1.6 (*d*) or in the parental thymoma BW5147 (*e*), by TGE virus (●), influenza virus (■), MoLV (▲) or culture medium (○) is shown. To perform the assay 5 × 10^4 hybridoma cells per well were cultured for 48 h in T cell growth medium (see Methods) in the presence of the indicated amounts of TGE virus per ml and 2000 R-irradiated spleen cells from BALB/c mice. The presence of interleukin was then evaluated in the supernatants using a bioassay based on the incorporation of [^{3}H]thymidine by CTLL-2 cells (Gillis & Smith, 1977; Kupper *et al.*, 1987).

Specific growth inhibition of the T cell hybridomas by antigen

In preliminary experiments, the three hybridomas yielded a higher number of growing clones and of interleukin producers when they were maintained and cloned in the absence of the antigen (results not shown). Therefore, we specifically studied the effect of u.v.-inactivated TGE virus on the cloning efficiency of these T cell hybridomas and of a TGE virus-specific B cell hybridoma. The three T cell hybridomas and the B cell hybridoma had a lower cloning efficiency in the presence of TGE virus than they had in its absence (Fig. 2*a*, *b*, *c* and *d*, respectively). In contrast, the negative variant T.1C.D7$^-$ (a subclone of T.1C.D7 that had lost its capability to secrete interleukin after TGE virus stimulation), BW5147, P1.6 and a non-TGE virus-specific B cell hybridoma were not inhibited by TGE virus (Fig. 2*e*, *f*, *g* and *h*, respectively), confirming that the growth of specific hybridomas was inhibited by the presence of the antigen. Furthermore, more positive hybridoma clones grew in the absence of antigen (80 to 100%) than in its presence (15 to 70%) (results not shown).

When the selected clones were passaged *in vitro* without cloning, they maintained their activity for at least 4 months. When the hybridomas were maintained by continuous cloning, between 70 and 100% of the wells contained positive cells (cells that released interleukin in presence of the virus) (Table 1). These results indicate that the hybridomas were not completely stable, and that it was advisable to reclone them in order to maintain their full activity.

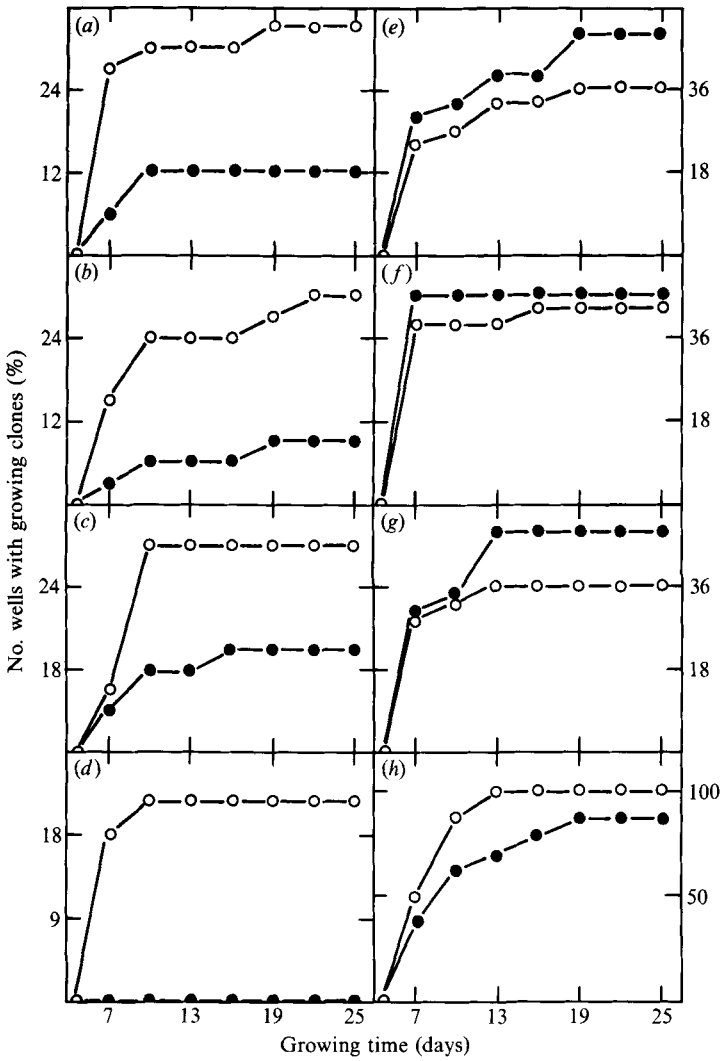

Fig. 2. Growth inhibition of TGE virus-specific T and B cell hybridomas by u.v.-inactivated virus. The percentage of wells with growing clones of T and B cell hybridomas in the absence (open circles) or presence (closed circles) of non-infectious TGE virus is shown (*a* to *d*, left-hand ordinates; *e* to *h*, right-hand ordinates). One cell of the indicated hybridoma was seeded per well in the presence of 2×10^5 2000 R-irradiated spleens cells from BALB/c mice and 4 μg of TGE virus per ml. At the indicated times, 96 wells of each type were screened for cell growth. (*a*, *b*, *c*) Results with the TGE virus-positive T hybridomas T.1C.D7, T.1J.B5, and T.1P.D1, respectively. (*e*, *f*, *g*) Results for the control T cell hybridoma T.1C.D7$^-$ (a negative clone derived from T.1C.D7), for the parental thymoma BW5147 and for P1.6 (an influenza virus-specific T cell hybridoma), respectively. (*d*, *h*) Percentage of wells with growing colonies of the TGE virus-specific B cell hybridoma 6A.C3 (Jiménez *et al.*, 1986) and of a negative control B cell hybridoma, respectively.

Characterization of T cell hybridomas

The restriction by the H-2 locus of the major histocompatibility complex in antigen presentation to these hybridomas was studied. The three hybridomas were H-2-restricted (Fig. 3), as they produced interleukin in response to the antigen presented by spleen cells with an H-2^d haplotype (BALB/c, DBA and B10.D2), but not with an H-2^k (BALB/k and B10.BR) or H-2^b (C57BL/6 and C57BL/10) haplotype. As expected, the three hybridomas responded to the semi-

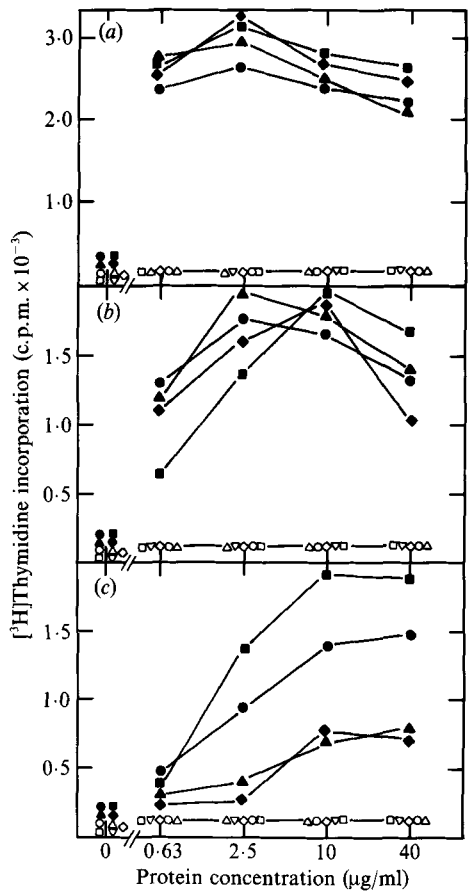

Fig. 3. Major histocompatibility complex restriction in interleukin production by T cell hybridomas stimulated with TGE virus. The production of interleukin by the T cell hybridomas T.1C.D7, T.1P.D1 and T.1J.B5 (*a*, *b* and *c*, respectively), after TGE virus presentation by 2000 R-irradiated spleen cells from mice with different H-2 backgrounds, was evaluated by determining the amount of [³H]thymidine incorporated in CTLL-2 cells as indicated in Methods. The accessory cells were from the following mouse strains: BALB/c (●), DBA (■), B10.D2 (▲), F1 (BALB/c × C3H) (◆), B10.BR (○), B10 (△), BALB/k (▽), B10.A/4R (◇) and C57BL/6 (□).

syngeneic antigen-presenting cells with H-$2^{d/k}$ (BALB/c × C3H), but not to antigen-presenting cells from the recombinant B10.A/4R mice with the genetic background H-2 K^kD^b (Fig. 3).

The phenotype of the hybridomas was studied by immunofluorescence using a cell sorter. All three hybridomas were Thy 1.2$^+$ (Fig. 4), in contrast to the B cell hybridoma 6A.C3, which was negative for this marker. In addition, T.1J.B5 was L3T4$^+$ (a marker for Th cells), unlike the other two hybridomas, T.1C.D7 and T.1P.D1, which did not express this marker. None of the B or T cell hybridomas expressed detectable levels of Lyt 1 or Lyt 2 antigen, in contrast to the positive control cell lines EL-4 (Freeman *et al.*, 1983) and CTLL-2 (Gillis & Smith, 1977), respectively. The marker defined by the MAb J11d was not expressed by any of the three T cell hybridomas (Fig. 4).

TGE virus is a T cell-dependent antigen

To study the functional activity of the T cell hybridomas, a system for TGE virus-specific antibody synthesis was established using spleen cells from virus-immune BALB/c mice. The antibody produced *in vitro* reacted with TGE virus, as determined by RIA and neutralization, with titres of 800 and 1800, respectively (results not shown). This synthesis was dependent on Th cells, as depletion of this subset of cells inhibited the response to TGE virus (Table 2). Although the specific depletion of T cell subsets with either Lyt 1- or L3T4-specific MAbs plus complement significantly reduced the antibody synthesis, a combination of both MAbs provided the highest inhibition of the TGE virus-specific antibody synthesis. Similar reduction of this synthesis was obtained with the MAb specific for Lyt 1 antigen (53.7-313) providing that this MAb was present during the antigenic *in vitro* stimulation (Table 2).

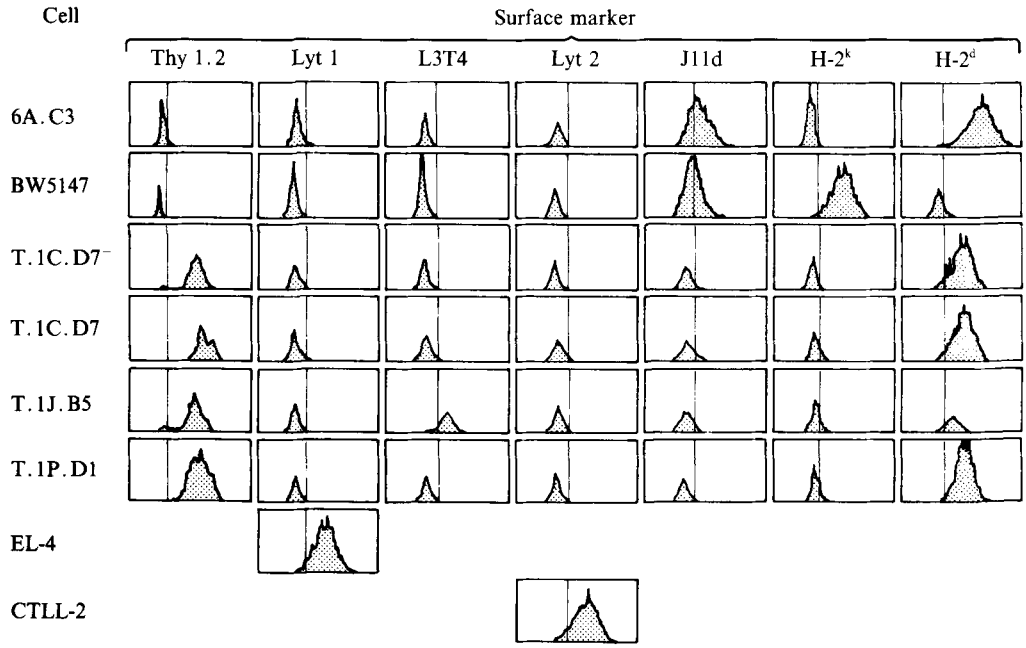

Fig. 4. Immunofluorescence determination of the expression of mouse T and B cell-associated markers. The phenotype of the hybridomas was studied by immunofluorescence in a cell sorter, using undiluted supernatants from hybridomas producing MAbs reactive to T, B and T–B cell markers (see Methods). The frequency of cells stained by the indicated MAb (ordinates) is represented as a function of the relative fluorescence intensity (abcissas), as determined in a fluorescent-activated cell sorter. A minimum of 4×10^3 cells were screened in each case. The study was performed with the TGE virus-specific hybridomas (T.1C.D7, T.1J.B5 and T.1P.D1), a negative T cell hybridoma (T.1C.D7$^-$) derived from the hybridoma T.1C.D7, and other cell lines used as controls for cell surface markers: a TGE virus-specific B cell hybridoma (6A.C3), the parental thymoma BW5147 (H-2^k), the Lyt 1$^+$ cell line EL-4 (Freeman *et al.*, 1983), and the Lyt 2$^+$ cell line CTTL-2 (Gillis & Smith, 1977).

Table 2. *T cell dependence of TGE virus-specific antibody synthesis* in vitro

Antibody*		Antibody treatment†		Antibody titre‡	
Specificity	Designation	Lysis	Culture	−TGEV	+TGEV
−	−	−	−	3	1875
Control	P3-X63-Ag8	+	−	3	1875
Lyt 1	53.7-313	+	−	3	375
L3T4	GK1.5	+	−	3	150
Lyt 1	53.7-313	+	+	3	75
Lyt 1 + L3T4	53.7-313 and GK1.5	+	−	3	75

* Spleen cells from immune BALB/c mice were incubated twice with the indicated MAb and complement.
† Cell lysis was carried out as described above (*) and, when indicated, a 1:15 dilution of supernatant from the hybridoma culture was added during the antibody synthesis *in vitro*.
‡ Determined by RIA using TGE virus-coated plates.

Activity of the TGE virus-specific T cell hybridomas

To determine whether the isolated hybridomas cooperated with B cells in the synthesis of TGE virus-specific antibodies, spleen cells from TGE virus-immune BALB/c mice, depleted of Lyt 1$^+$ and L3T4$^+$ cells, were cultured with cells from the three hybridomas studied. The results

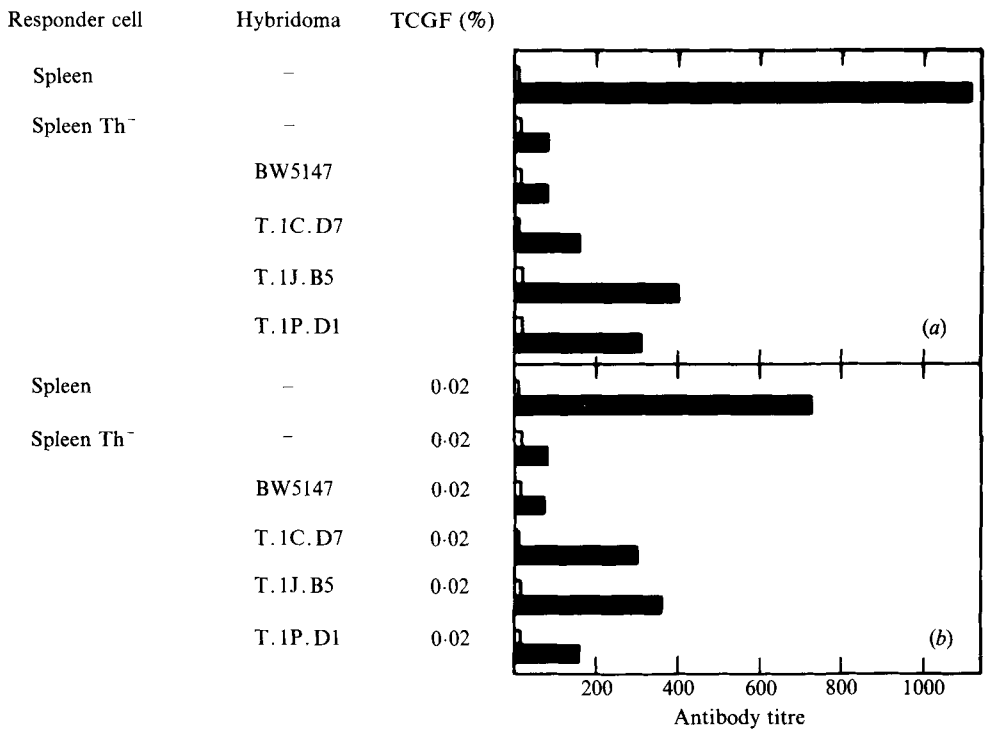

Fig. 5. Helper activity of the TGE virus-specific T cell hybridomas in the antibody synthesis *in vitro*. The figure shows the reconstitution with T cell hybridomas of the TGE virus-specific antibody synthesis by Th cell-depleted immune BALB/c mice spleen cells, after secondary stimulation. Spleen cells were depleted of Th cells (spleen Th⁻) by incubating them in the presence of MAbs specific for Lyt 1 and L3T4 cell surface markers (MAbs 53.7-313 and GK1.5, respectively) and complement (see Table 2). The cells were then incubated in the absence (open bars) or presence (closed bars) of TGE virus, and the parental thymoma BW5147 or the TGE virus-specific hybridomas (T.1C.D7, T.1J.B5 and T.1P.D1). TGE virus-specific antibodies were synthesized following the procedure described in Methods in the absence (*a*) or presence (*b*) of TCGF. The antibody titre was determined by RIA using purified TGE virus as antigen.

(Fig. 5*a*) indicated that the hybridomas T.1C.D7, T.1P.D1 and T.1J.B5 reconstituted 16%, 35% and 42%, respectively, of the response of unfractionated immune spleen cells. In contrast, similar numbers of BW5147 cells did not restore the antibody synthesis by spleen cells depleted of Th lymphocytes. The extent of reconstitution of antibody synthesis was not significantly improved by the addition of T cell growth factor (Fig. 5*b*) or recombinant IL-2 to the cultures (results not shown). The extent of the antibody synthesis restoration could be enhanced by pre-incubating the T cell hybridomas with the virus before adding these cells to the cultures. Pre-stimulation of hybridomas T.1C.D7 or T.1J.B5 with antigen for 4 days, followed by the 6 days of standard incubation in the presence of antigen, restored 33% and 53%, respectively, of the antibody synthesis by unfractionated spleen cells (Fig. 6).

The critical question was whether the selected hybridomas cooperated with B cells in the production of TGE virus-neutralizing antibodies. To answer this question, the neutralizing activity of the antibodies synthesized *in vitro* with the cooperation of the T cell hybridomas was studied. The hybridomas T.1C.D7, T.1P.D1 and T.1J.B5 cooperated (Table 3) in the production of supernatants that reduced the virus titre by 0·7, 2·6 and 2·6 $\log_{10}$ units, respectively. The neutralization indices of these supernatants were increased to 1·2, 3·4 and 3·6, respectively, when the culture supernatants were present in the plaque assay medium. In contrast, the negative variant T.1C.D7⁻ did not cooperate with the same population of B cells.

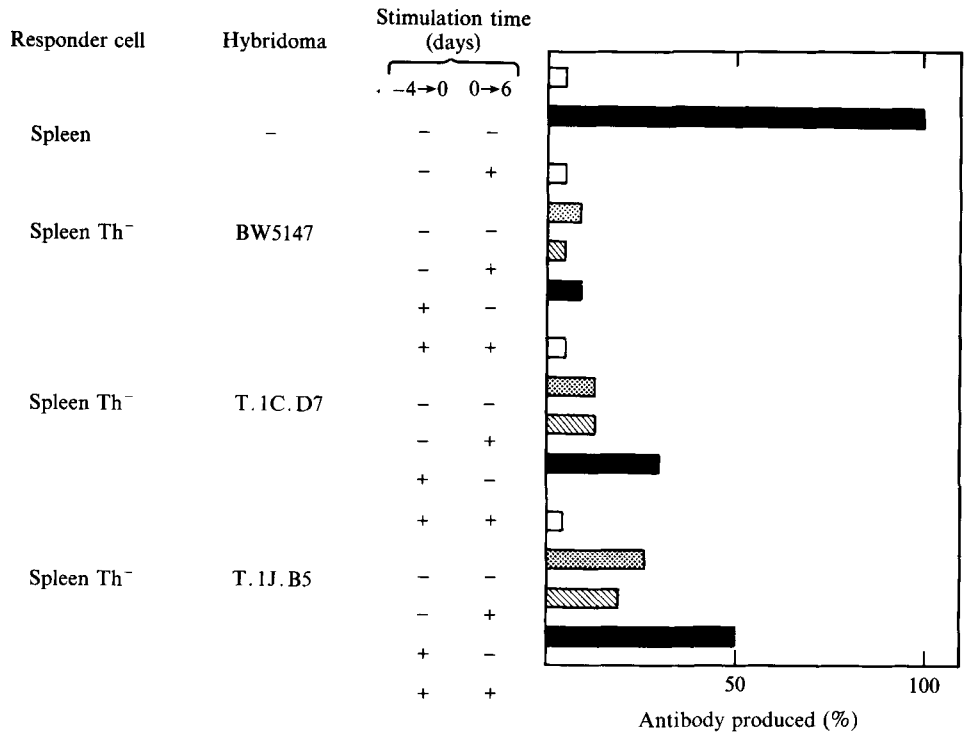

Fig. 6. Increase of the helper activity of the TGE virus-specific T cell hybridomas in antibody synthesis *in vitro*, by preincubation of the hybridomas with u.v.-inactivated TGE virus. The synthesis of TGE virus-specific antibodies by spleen cells from TGE virus-immune BALB/c mice, in the presence of the T cell hybridomas T.1C.D7 and T.1J.B5 (see legend of Fig. 5 and Methods), was determined after the T cell hybridomas or the parental thymoma BW5147, used as a control, were preincubated for 4 days (from day −4 to day 0) in the absence (−) or presence (+) of the virus, and then (day 0) added to Th cell-depleted immune spleen cells (spleen Th⁻), and incubated for 6 days in the absence (−) or presence of the antigen (+). The cultures were further incubated for 5 days and the antibodies were evaluated by RIA. The results are expressed as the percentage of the antibody synthesized in relation to the antibody produced by cultures of unfractionated immune cells.

As the TGE virus-neutralizing activity of the supernatants could have been due to the induction of interferon, we tested the neutralizing activity of these supernatants for VSV in the same cellular system. The results (Table 3) indicated that the neutralizing activity was TGE virus-specific.

DISCUSSION

In this manuscript the selection of three H-2-restricted TGE virus-specific Th cell hybridomas which cooperated with B cells in the synthesis of virus-neutralizing antibodies *in vitro* is described. Whereas the antigenic structure relating to B cells has been studied in detail in TGE virus (Delmas *et al.*, 1986; Jiménez *et al.*, 1986; Enjuanes *et al.*, 1987; Correa *et al.*, 1988), the nature of T cell recognition and activation by TGE virus antigens has received little attention. Only interferon induction by the E1 glycoprotein of TGE virus has been reported (Charley & Laude, 1988).

The specificity and activity of the three selected hybridomas have been maintained for more than 2 years, and they could be grown in culture without recloning for up to 4 months and still kept their activity. Nevertheless, as reported by others (Tada & Nonaka, 1982), when cloned, only 70 to 100% of the wells had positive hybridomas, and thus the cells had to be recloned in order to maintain their maximum activity. Their stability was much lower than that of B cell

Table 3. *Neutralization of TGE virus by antibodies synthesized* in vitro *with the cooperation of virus-specific Th hybridomas*

Antibody produced by*	Stimulation of cells with TGE virus†	Neutralization index‡ for TGE virus Supernatant in plaque assay −	Neutralization index‡ for TGE virus Supernatant in plaque assay +	Neutralization index‡ for VSV
Culture media	−	<0·1	<0·1	<0·1
B cell hybridoma 6A.C3§	−	>3·9	>3·9	<0·1
Immune spleen cells‖	−	<0·1	0·4	<0·1
Immune spleen cells	+	3·9	3·9	<0·1
Immune spleen cells Th^{-}¶ + T.1C.$D7^{-}$**	+	<0·1	0·2	<0·1
Immune spleen cells Th^{-} + T.1C.D7††	+	0·7	1·2	<0·1
Immune spleen cells Th^{-} + T.1J.B5††	+	2·6	3·6	<0·1
Immune spleen cells Th^{-} + T.1P.D1††	+	2·6	3·4	<0·1

* The neutralizing antibody present in culture supernatants was evaluated by a plaque reduction assay on ST cells.

† The cells were cultured in the absence (−) or presence (+) of TGE virus.

‡ The neutralization index is expressed as the $\log_{10}$.

§ Supernatant from the B cell hybridoma 6A.C3, TGE virus-specific.

‖ Spleen cells from TGE virus-immune BALB/c mice.

¶ Spleen cells from TGE virus-immune BALB/c mice depleted of Th cells.

** T.1C.$D7^{-}$, negative subclone of the TGE virus-specific hybridoma T.1C.D7.

†† T.1C.D7, T.1J.B5 and T.1P.D1 are TGE virus-specific T cell hybridomas.

hybridomas specific for the same antigen. To find out whether the culture of hybridomas in the presence of antigen would deliver a stimulus preferentially to those clones keeping the specificity for TGE virus, favouring their selection, we studied the cloning efficiency of the hybridomas in the presence and absence of the antigen. In the presence of antigen, the growth of specific clones was inhibited, but in the absence of antigen, both the cloning efficiency and the percentage of positive clones were higher. For normal T lymphocytes, binding of the antigen receptor usually results in a complex series of activation events that include expression of new cell surface receptors, production of lymphokines, and entry of the cell into its growth cycle (Ashwell *et al.*, 1987). In contrast, we have observed that the interaction of TGE virus-specific B and T cell hybridomas with the antigen resulted in a decrease in both growth and [^{3}H]thymidine incorporation (results not shown). Similar results have been obtained with cytochrome *c*- and hen egg lysozyme-specific hybridomas (Ashwell *et al.*, 1987). This inhibition of [^{3}H]thymidine incorporation could be used as a screening method to select antigen-specific B or T cell hybridomas.

The selection of TGE virus-specific T cell hybridomas has been based on the induction of interleukin production in these cells by the virus, which was evaluated in the interleukin-dependent CTLL-2 cell line (Gillis *et al.*, 1978). As both IL-2 and IL-4 can promote CTLL-2 cell growth, the selected T cell hybridomas could release either IL-2 or IL-4 after antigen stimulation. With MAbs specific for both interleukins we determined that the T cell hybridomas selected produced IL-2 (results not shown).

We have shown that TGE virus is a T cell-dependent antigen, as the synthesis *in vitro* of virus-specific antibodies by spleen cells from immune BALB/c mice was prevented by depleting the splenocytes of Lyt 1^{+} or L3T4^{+} cells, i.e. Th cells. The depletion of T cells was responsible for the inhibition of the TGE virus-specific antibody synthesis, as this synthesis could be restored by reconstituting the system with virus-specific T cell hybridomas that were free of other cell types. This observation classifies TGE virus with influenza and other T cell-dependent viruses (Burns *et al.*, 1975; Sullivan *et al.*, 1976).

As the three hybridomas restored the synthesis of TGE virus-specific antibodies *in vitro*, they could be classified as Th hybridomas in the classical sense (Rajewsky *et al.*, 1969; Hurwitz *et al.*,

1984). These hybridomas were able to restore the antibody synthesis *in vitro* up to 33 to 53% of the antibody levels produced by unfractionated populations of immune spleen cells. Nevertheless, these levels of reconstitution were considered significant in order to classify the hybridomas as helper cells. The synthesis of virus-specific antibodies *in vitro* has been shown in some viral systems (Tan *et al.*, 1985; Casal *et al.*, 1987) to be dependent on the presence of IL-2. However, the addition of exogenous IL-2 did not increase the level of TGE virus-specific antibodies induced by the T cell hybridomas. These results indicated that either the level of IL-2 in the *in vitro* system was saturating, or that the soluble factor active in this system was not IL-2.

The isolated T cell hybridomas cooperated in the synthesis of TGE virus-neutralizing factors. Normal T cells or T cell hybridomas, present in the culture, could produce non-specific factors, such as interferon, after antigenic stimulation (Mosmann *et al.*, 1986). The neutralizing factor synthesized in the cultures with the cooperation of the isolated T cell hybridomas was most probably an antibody, since (i) it has been shown by RIA using a second antibody to mouse immunoglobulin that TGE virus-specific antibodies were synthesized *in vitro*, (ii) that the neutralization was virus-specific as only TGE virus and not VSV was neutralized and (iii) that the neutralizing factor acted in different species, as it was produced by murine cells whereas the neutralization assay was performed on porcine ST cells. A final objective in the isolation of the Th hybridomas was to select tools to define the viral antigenic determinants involved in the induction of neutralizing antibodies. As the isolated T cell hybridomas cooperated in the synthesis of TGE virus-specific neutralizing antibodies, they should be useful in identifying the antigenic determinants of interest.

TGE virus has a dominant antigenic site (site A) critical in its neutralization. This site has been subdivided into three antigenic subsites (a, b and c). MAb-resistant mutants able to escape neutralization by MAbs specific for these subsites have been isolated (Jiménez *et al.*, 1986; Correa *et al.*, 1988), each altered at one of the subsites. As the antibodies produced *in vitro* with the cooperation of each of the T cell hybridomas were able to neutralize the three types of MAb-resistant mutants (unpublished data), the three Th cell hybridomas isolated were most probably able to cooperate in the synthesis of neutralizing antibodies of more than one specificity.

Effective protection against TGE has been provided reproducibly by infectious virus (Saif & Bohl, 1986). Reports claiming satisfactory protection against TGE with subunit vaccines (Gough *et al.*, 1983*a*, *b*) await confirmation. In the future, only vaccines based on non-infectious virus or viral subunits will probably be accepted. Effective vaccines against TGE virus should contain both B and T cell epitopes to stimulate the corresponding cell populations, in order to provide protection. T cell epitopes could mimic viral components (Perlmann *et al.*, 1987; Milich *et al.*, 1987) or have a non-viral origin (major histocompatibility complex molecules, etc.) (Sanderson, 1984). However, to ensure good boosting of a vaccination by subsequent natural infection, the B and T epitopes included in the vaccine should be part of the original virus particle. We hope that the isolated T cell hybridomas will help to identify T cell-reactive epitopes that would be included in a B–T cell vaccine (i.e. a vaccine with B and T cell determinants), or in a T cell vaccine (i.e. a vaccine containing T cell epitopes, but not epitopes inducing virus-neutralizing antibodies). This type of vaccine may be important in the future, since protection against herpes simplex virus type 1 has been induced by a glycoprotein that lacks antigenic determinants for stimulating the synthesis of virus-neutralizing antibodies (Chan *et al.*, 1985). The application of data defining T cell epitopes detected with murine hybridomas to a porcine system will require proof that the selected epitopes are relevant in the porcine system; T cell epitopes predicted by different algorithms (Berzofsky *et al.*, 1987; Rothbard, 1986), which use data based on the primary sequence of the protein and not on the species or the strain haplotype have to be tested on the basis of the prediction. The factors controlling immunodominance of T cell epitopes could be divided into two categories, those intrinsic to the structure of the antigenic site and those extrinsic to it, depending on the host, such as, for example, MHC-linked *Ir* genes (Berzofsky *et al.*, 1987). In consequence, it would be expected that, in some cases, T cell epitopes defined as major determinants in a murine system will be also immunodominant in a porcine system. In fact, that is what has been shown for the haemagglutinin (HA) of influenza virus, in which two corresponding regions of the HA

molecule are now known to be major determinants for human and murine helper T cells (Hurwitz *et al.*, 1984).

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