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## Kinetics of the in vitro antibody response to transmissible gastroenteritis (TGE) virus from pig mesenteric lymph node cells, using the ELISASPOT and ELISA tests

P. Berthon<sup>1</sup>, S. Bernard<sup>1</sup>, H. Salmon<sup>1</sup> and R.M. Binns<sup>2</sup>

<sup>1</sup> INRA, Laboratoire de Pathologie Porcine-Immunologie, 37380 Nouzilly, France, and <sup>2</sup> AFRC, Institute of Animal Physiology and Genetics Research, Babraham Hall, Cambridge CB2 4AT, U.K.

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A method is described for in vitro studies of viral humoral immune responses in the pig. After oral immunization with transmissible gastroenteritis (TGE) coronavirus, antibody production from primed mesenteric lymph node cells was revealed by an in vitro boost with viral antigen. For the latter the leukocytes were co-cultured with UV-inactivated virus using a variety of different methods of antigenic stimulation. Enumeration of specific antibody-secreting cells (ASC) and titration of secreted anti-virus antibodies were performed with ELISASPOT (using 3-amino 9-ethyl carbazole as the peroxidase chromogen) and ELISA tests respectively, according to the Ig isotype. The results showed a close relationship between ASC numbers and secreted antibody titres. The best in vitro antibody synthesis was observed when the sensitized cells were maintained in contact with virus during the whole culture period. Antibody responses were defined by a kinetic profile characterized by a narrow peak, with a maximum occurring after 4 and 6 days of culture and with the IgA response appearing earlier than the IgG. This methodology, which analyses specific antibody responses at the cellular level, may permit studies on the mechanisms of Ig isotype regulation. Extended to leukocytes from other organs of the immune system, it may also constitute an in vitro model to study antibody responses expressed in different lymphoid tissues of the pig.

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**Key words:** ELISASPOT; ELISA; Antibody-secreting cell; Mesenteric lymph node; Coronavirus; (Pig)

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**Correspondence to:** P. Berthon, INRA, Laboratoire de Pathologie Porcine-Immunologie, 37380 Nouzilly, France.

**Abbreviations:** ASC, antibody-secreting cells; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GALT, gut-associated lymphoid tissue; Ig, immunoglobulin; mAb, monoclonal antibody; MLN, mesenteric lymph node; MRBC, mouse red blood cells; NPS, normal pig serum; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline; p.f.u., plaque-forming units; SFC, spot-forming cells; ST, swine testis; TGE, transmissible gastroenteritis; TGEV, transmissible gastroenteritis virus.

### Introduction

Transmissible gastroenteritis (TGE) is a highly contagious enteric disease of swine caused by a coronavirus. It usually results in only a transient diarrhea in adults but is a major cause of death in piglets under two weeks of age (Bohl, 1981; Garwes, 1982). Lactogenic immunity, induced in sows after natural infection or oral immunization, appears to be the most important mechanism protecting newborn piglets against TGE infection

(Hooper and Haeltermann, 1966; Bohl et al., 1974; Aynaud et al., 1990). Viral stimulation of the gut-associated lymphoid tissue (GALT) of sows leads to the production in mammary secretions of antibodies (secretory IgA) which are protective for the piglet intestinal mucosa (Bohl et al., 1972a; Saif and Bohl, 1979, 1981). Our research program is mainly focused on improving protection of suckling piglets through milk-borne immunity. Although the presence of secretory IgA antibodies in milk is linked to the immune status of the intestinal mucosa, the mechanisms of regulation of this local antibody production are still unclear.

The enteric tropism of the TGE coronavirus permits studies on the induction and modulation of the local IgA response. In order to better understand the mechanisms operating in the regulation of the antigen-specific IgA production in the secretory immune system at the cellular level, it was necessary to establish both an *in vitro* model for antigenic stimulation of pig leukocytes using TGE virus (TGEV) and a technique for enumerating resulting specific antibody-secreting cells (ASC), according to their Ig isotype. The methods used *in vitro* for antigenic stimulation were essentially those developed for study of *in vitro* primary and secondary plaque-forming cell responses following stimulation of pig blood mononuclear cells with mouse red blood cells (MRBC), subsequently also applied to the response to ovalbumin (OVA) (Binns, 1982; Berthon and Binns, manuscript in preparation). Since the hemolytic plaque assay (Jerne and Nordin, 1963; Jerne et al., 1974) did not appear to be appropriate for the determination of numbers of specific ASC in the responses of pig leukocyte cultures to viral particles, their enumeration was undertaken with an ELISASPOT test. Although originally described for the investigation of ASC responding to the protein antigen OVA (Czerkinsky et al., 1983; Sedgwick and Holt, 1983, 1986; Holt et al., 1984; Bianchi et al., 1986) the ELISASPOT test has been used with viral antigens (Russell et al., 1987). With TGE virus, the test was performed using virus-infected swine testis (ST) cell monolayers as antigen (Grom and Bernard, 1985; Russell et al., 1987; Cubero Pablo et al., 1988) and 3-amino 9-ethyl carbazole as the chromogen for the peroxidase reaction (Pauli et al., 1984).

This report describes a method to study *in vitro* antibody responses of the pig GALT to TGE virus at the cellular level. The effect of various *in vitro* antigenic stimulation methods on the humoral immune response from *in vivo* primed mesenteric leukocytes was studied and specific antibody production measured, according to Ig isotype, using two techniques: the enumeration of anti-coronavirus ASC (ELISASPOT test) and the titration of specific antibodies secreted in culture supernatants (ELISA test).

## Materials and methods

### *Animals*

Two Meishan piglets were orally infected with the virulent GEP II strain of TGEV (Aynaud et al., 1985, 1990) and orally boosted 3 days before slaughter, 3 months later, with  $5 \times 10^7$  viral plaque-forming units (p.f.u.) of the vaccinal attenuated Nouzilly strain (Aynaud et al., 1985, 1990; Nguyen et al., 1987a,b,c) of TGEV.

### *Source and preparation of leukocytes*

Leukocytes were prepared from the piglet mesenteric lymph node (MLN) as described previously (Salmon, 1979, 1983) except that the washing medium used was the Ely medium (ELAC medium (Flow Laboratories) supplemented with 0.1% yeast extract (Difco)) and that this medium and the culture medium (RPMI 1640, Boehringer Mannheim) were respectively supplemented with 2.5% and 10% of heat-inactivated normal pig serum (NPS) instead of 10% and 20% of fetal calf serum (FCS). Cell viability was assessed by the trypan blue dye exclusion test and the cell suspension adjusted to a final concentration of  $5 \times 10^7$  viable leukocytes per ml of culture medium (10% NPS-RPMI medium).

### *Viral antigen preparation for the in vitro leukocyte stimulation*

The high-passaged Purdue 115 strain (Bohl et al., 1972b; Frederick and Bohl, 1976; Laude et al., 1981; Nguyen et al., 1986) was used as the source of cultured viral antigenic material. The virus was propagated in monolayers of the ST cell line (McClurkin and Norman, 1966), supplied by Dr. E.H. Bohl (Wooster, OH, U.S.A.), as described by

Aynaud et al. (1985) and Nguyen et al. (1987a). After incubation at 37°C for 18 h, the infected ST cell cultures were frozen at -20°C and thawed. The culture fluid was collected, cell debris removed by centrifugation at 2000 × g for 15 min at 4°C, and the infectious viral particles inactivated by a short exposure to UV light (90 s, 2 mW/cm<sup>2</sup>). The viral titer was adjusted to the equivalent of 10<sup>7</sup> p.f.u./ml with 10% NPS-RPMI medium. Supernatants from uninfected ST cell cultures were similarly treated and used as controls. These 'antigen preparations' were stored at -80°C.

*Leukocyte cultures and antigenic stimulation in the in vitro antibody response*

In each well of 24-well tissue culture plates (Linbro, Flow Laboratories), 0.1 ml of leukocyte suspension (5 × 10<sup>6</sup> cells/well) was mixed with 0.3 ml of 10% NPS-RPMI medium and 0.1 ml of UV-inactivated virus preparation (10<sup>6</sup> p.f.u./well). Cultures were performed for 10 days and every 2 days, supernatant samples were taken from each well (0.1 ml for the first sample and 0.4 ml for the others) and fresh culture medium (0.5 ml/well) was added. In control cultures, the 'antigen' used was 0.1 ml of UV-treated supernatant of uninfected ST cell cultures (*control (ST)*). Three methods were used for the in vitro antigenic stimulation:

*Viral stimulation (V)*. Mesenteric leukocytes were normally mixed with viral antigen only at the beginning of the culture, as described above, and then fresh culture medium was added without virus.

*Pulsed viral stimulation (PV)*. Leukocyte cultures were washed after a 24 h incubation with viral antigen. Supernatants were replaced by fresh culture medium without virus.

*Repeated viral stimulation (RV)*. In this antigenic stimulation protocol, the level of antigen was kept more constant throughout the experiment by repeated addition of viral antigen (concentration: 10<sup>6</sup> p.f.u./0.5 ml) with each addition of fresh culture medium.

*ELISASPOT test for counting antibody-producing cells*

Microtiter plates with 96 flat-bottomed wells (Falcon, Becton Dickinson) were coated with TGE

viral cellular antigens as follows. ST cell line was grown to confluence in the wells and uniformly infected overnight with the Purdue 115 strain of TGEV. Before cytopathic effects occurred, the infected cell cultures were washed carefully with cold phosphate-buffered saline (PBS) three times and fixed for 20 min at -20°C with 0.2 ml/well of a cold 80% (v/v) acetone-water solution. The plates were stored at -20°C until use. Before the test, antigen-coated wells were washed three times with tap water and then with PBS to remove the acetone. The protein adhesiveness of the wells was then blocked with 1% bovine skimmed milk (Regilait, Francelait) in PBS for 30 min at 37°C followed by two washes of PBS.

After every 2 days of leukocyte culture, the contents of six or eight wells were harvested and the cells washed with Ely medium supplemented with 5% FCS to remove pig serum and free antibodies. After centrifuging at 400 × g for 15 min at 4°C the cell pellets were resuspended in 10% FCS-RPMI medium and suspensions prepared containing 10<sup>7</sup>, 10<sup>6</sup> and 10<sup>5</sup> viable leukocytes/ml. The ELISASPOT test was performed by layering 0.1 ml of each leukocyte dilution from each culture sample on antigen-coated wells. Wells with uninfected ST cells were used as controls. The plates were centrifuged at 1500 × g for 5 min at room temperature and incubated at 37°C for 3 h. Then the leukocytes were removed from the plates with three rinses of tap water and three washes with PBS containing 0.05% Tween 20 (Serva) and replaced by 0.1 ml/well of an optimal concentration of a peroxidase-labelled anti-Ig. These were either rabbit anti-pig IgG (H + L chains) polyclonal antibodies (Cappel, Organon Teknika, France) or a mouse anti-pig IgA monoclonal antibody (mAb K61 kindly supplied by Prof. F.J. Bourne, Dr. C.R. Stokes and K. Stevens (Langford, Bristol, U.K.) and covalently labelled with peroxidase as described by Nakane and Kawavi (1974)), diluted in PBS containing 0.05% Tween 20 and 1% skimmed milk. After a further incubation period (90 min at 37°C), the plates were washed three times with PBS-0.05% Tween 20. The enzymic reaction step was performed by incubation at room temperature with 3-amino-9-ethyl carbazole (Sigma)/H<sub>2</sub>O<sub>2</sub> substrate solution (Pauli et al., 1984). After 30 min, secreted anti-

body 'plaques' were revealed as red spots on the virus-infected ST cell monolayers. The reaction was stopped by washing the plates with PBS and the plates stored in PBS at 4°C until reading. The numbers of spots per well were counted using a Leitz-Diavert inverted microscope (100-fold magnification). When the leukocytes were incubated for 3 h, only spots covering more than three ST cells were considered to result from antibody secretion. After an overnight incubation period, the size of the secreted antibody spots increased and the direct immunoperoxidase staining tended to approach confluence, making reading unreliable.

Since assays were performed on pools of six or eight wells, they represented averages of several cultures. As the numbers of spots were directly proportional to leukocyte concentration, accurate spot numbers were determined from at least two viable cell dilutions of each sample and expressed as the means  $\pm$  SEM. The number of virus-specific spot-forming cells (SFC) was determined by the difference between the number of spots observed in viral antigen-coated wells and the number in uninfected ST cell-coated wells. The results were expressed as the number of virus-specific SFC per  $10^5$  leukocytes initially put in culture.

#### *ELISA test for the Ig isotype of synthesized antibody*

Supernatants from leukocyte cultures were analyzed to determine the Ig isotype of the TGEV-specific antibodies. An ELISA immunocapture technique was used (Bernard et al., 1986). Briefly, 96-well microtiter plates (Nunc immunoplates, 4-42404) were precoated with a mixture of monoclonal antibodies specific for structural proteins of TGEV (Delmas et al., 1986; Laude et al., 1986) and diluted in carbonate buffer, pH = 9.6 (2 h, 37°C). After washing, the plates were blocked overnight at 4°C with 1% skimmed milk in PBS. Viral antigens were bound to the wells of precoated plates by incubating supernatants from ST cell cultures infected with the viral Purdue 115 strain, diluted in PBS containing 0.05% Tween 20 and 1% skimmed milk (2 h, 37°C). Uninfected ST cell culture supernatants were used as controls. Samples containing unknown antibodies were added after dilution (1/3) in the same buffer and

incubated for 2 h at 37°C. Immune complex conjugates (peroxidase-labelled pig IgG (or IgM)/rabbit anti-pig IgG (or IgM) polyclonal antibodies) (Bernard et al., 1984; Bernard and Lantier, 1985) were added for a further 2 h at 37°C. TGEV-specific IgA antibodies were revealed on Purdue 115-infected ST cell monolayers with peroxidase-conjugated mAb K61, using the same conditions as in the ELISASPOT test. The enzymic reactions were developed with 2,2'-azino-bis-(3-ethyl benzthiazoline-6-sulfonic acid) (Boehringer Mannheim)/H<sub>2</sub>O<sub>2</sub> substrate solution and read at 415 nm (Bernard et al., 1986). The results, which are the averages of duplicates, were expressed as the optical density (OD) observed with 0.1 ml of 1/3 diluted culture supernatants.

## Results

The kinetics of the *in vitro* antibody response obtained with the MLN cells from different animals were similar but differed in their magnitude. In this report, we present data from a piglet in whom the magnitude was close to the mean value determined for eight animals:  $240.1 \pm 76.4$  total Ig-ASC per  $10^5$  leukocytes with  $26.1 \pm 14.2$  IgA-ASC.

#### *Cell culture recoveries under in vitro antigenic stimulation*

Preliminary studies on the *in vitro* boost carried out with blood leukocytes and measured by ELISA tests, showed that the amount of specific antibody secreted into the culture supernatant was proportional to the concentration of viral antigen added at the beginning of the cultures (from  $10^4$  to  $10^7$  p.f.u./well). However, a higher antigen concentration did not induce a great increase of the detected OD: 7% from  $10^6$  to  $10^7$  p.f.u./well versus 17% from  $10^5$  to  $10^6$  p.f.u./well, at day 7 of the cultures. On the basis of the viral titers usually reached by the Purdue 115 strain in the ST cell line,  $10^6$  p.f.u./well was chosen as the antigen concentration for subsequent experiments.

Mesenteric leukocyte cultures continuously stimulated with TGEV (V and RV samples) showed higher recoveries of living cells than control (ST) or pulsed stimulated (PV) cultures ( $P < 0.005$ ), especially on the 6th and 8th days of

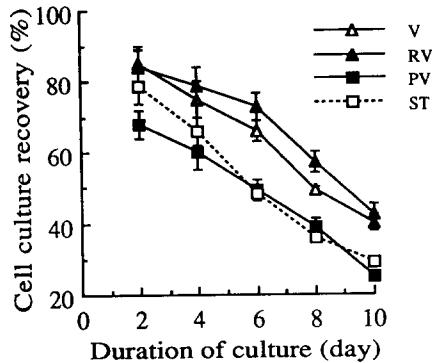


Fig. 1. Cell culture recoveries of pig mesenteric lymph node cells stimulated *in vitro* with TGE virus. Antigenic stimulation was carried out for 10 days. Every 2 days, cell viability was assessed by trypan blue dye exclusion test. For each culture sample, the number of viable cells was expressed as the percentage (mean value  $\pm$  SD) of the number of living cells initially put in culture ( $5 \times 10^6$  cells/well). Statistical analyses were performed using Student's *t* test on paired groups. Data represented by triangular symbols are significantly different ( $P < 0.005$ ) from those symbolized by squares.  $\square$ , ST: control;  $\blacksquare$ , PV: pulsed viral stimulation;  $\triangle$ , V: viral stimulation;  $\blacktriangle$ , RV: repeated viral stimulation (see materials and methods section).

culture (Fig. 1). Highest cell recoveries were obtained from cultures performed with repeated addition of virus (RV), though these were not significantly different from normal stimulated cultures (V) ( $P > 0.05$ ). Thus *in vitro* antigenic stimulation seemed to induce some cell proliferation (e.g., T cells, plasma blasts) from the *in vivo* primed cells. On the other hand, compared to controls, pulsed antigenic stimulation (PV) tended to lead to lower cell recoveries until the 6th day of culture (not significantly different:  $P > 0.05$ ), probably because of a loss of leukocytes during the washing step used to remove free viral antigens.

#### *Kinetics of the numbers of cells secreting antibody against TGE virus (ELISASPOT)*

Comparisons of the jejunal Peyer's patch and MLN have shown that the latter is the more important of these two main organized lymphoid tissues of the pig's small intestine as a source of cells secreting *in vitro* anti-TGEV IgA. For example, after 7 days MLN and Peyer's patch cell cultures from a sow orally infected with the virulent strain of TGEV showed  $76.5 \pm 5.3$  ASC ( $41.8 \pm 0.3$  IgA-ASC) and  $7.6$  ASC ( $1.3$  IgA-ASC) per  $10^5$  leukocytes, respectively. From another sow,

$240.5$  ASC/ $10^5$  leukocytes with  $45.7 \pm 6.0$  IgA-ASC were detected in the MLN cell cultures on day 4, while  $120.7$  ASC with  $19.1 \pm 1.9$  IgA-ASC/ $10^5$  leukocytes were observed from the Peyer's patch cell cultures. These studies of *in vitro* antigenic stimulation methods have been focused on pig mesenteric leukocytes. After *in vivo* priming and the *in vitro* boost, ASC were revealed as SFC on TGEV-infected cell monolayers. Immunoperoxidase staining showed that secreted antibodies bound to viral antigens expressed on the surface membrane and in the cytoplasm of acetone-fixed ST cells.

No antibody secretion was detected when *in vivo* primed leukocytes were cultured *in vitro* without viral antigens (ST) (Fig. 2). However, following antigenic stimulation, the kinetic profiles of the numbers of TGEV-specific IgG ASC (Fig. 2A) showed quite narrow peaks between the 4th and 8th day of culture, with maximum numbers on day 6. Cultures carried out with repeated addition of virus (RV) showed greater numbers of anti-TGEV IgG ASC (max:  $401.0 \pm 0.6$  SFC/ $10^5$  leukocytes) than those with virus introduced only at the beginning of culture (V) (max:  $304.1 \pm 0.1$  SFC/ $10^5$  leukocytes). Using a 24 h pulse of stimulation (PV), the peak IgG response was very low ( $68.0 \pm 4.4$  SFC/ $10^5$  leukocytes) and SFC had disappeared by the 8th day of culture. No IgM-secreting cell was detected in this experiment (data not shown). The profiles of the changes in numbers of anti-TGEV IgA SFC were broadly similar to those in the IgG responses but were at a lower level (Fig. 2B). However, in all of the antigenic stimulation protocols, the numbers of specific IgA-secreting cells reached a maximum at an earlier stage, on the 4th day of culture. As with the IgG response, exposure to virus only during the 1st day of culture (PV) induced a poor IgA response ( $8.9 \pm 0.2$  SFC/ $10^5$  cells on day 4) which then decreased rapidly. Although RV stimulation generated larger numbers of anti-TGEV IgA SFC than V stimulation ( $25.8 \pm 0.5$  to  $16.5 \pm 0.8$  SFC/ $10^5$  leukocytes), the ratio of IgA:total Ig SFC was not altered (19.1–17.2%).

#### *Kinetics of the in vitro synthesis of anti-TGEV antibody (ELISA)*

The amounts of secreted antigen-specific antibody in the supernatants of the mesenteric leuko-

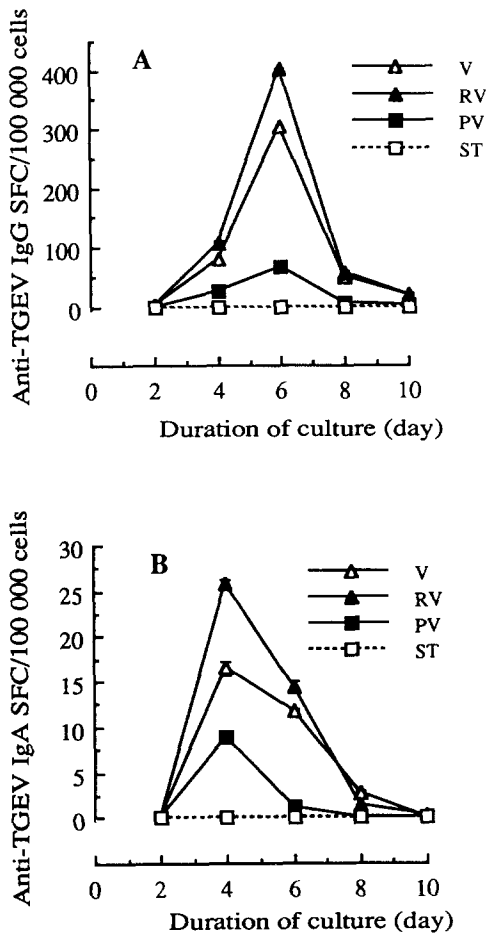


Fig. 2. Kinetics of the numbers of anti-TGEV spot-forming cells from pig mesenteric leukocytes cultured with TGEV virus. TGEV-specific SFC were revealed on Purdue 115-infected ST cell monolayers using polyclonal anti-pig Ig and monoclonal anti-pig IgA peroxidase-conjugated antibodies. Few or no spots appeared on uninfected ST cells. The results were expressed as numbers of antigen-specific SFC ( $x \pm \text{SEM}$ ,  $n = 2$ ) per  $10^5$  leukocytes initially put in culture. *A*: IgG-SFC: since no IgM-secreting cell was detected, the results were obtained from the difference between the number of the whole of anti-TGEV SFC (total Ig) and the number of TGEV-specific IgA SFC; *B*: IgA-SFC. Abbreviations as for Fig. 1.

cyte cultures were measured using ELISA tests (Fig. 3). No IgM antibody was found (data not shown). Although not detected on day 2, IgG anti-TGEV appeared in the supernatants after antigenic stimulation from the 4th day of culture (Fig. 3A), increased rapidly and reached a maximum on day 8. The largest IgG antibody responses were detected when virus was added only at the beginning of cell culture (V sample) rather

than when virus was added repeatedly (RV sample). Little or no TGEV-specific IgG was detected in samples from cultures without virus (ST) or following short antigenic stimulation (PV).

The profile of the IgA anti-TGEV response after the *in vitro* boost (Fig. 3B) was similar to the IgG response, though the highest level of antibody was observed after 6 days of culture. In this response too, pulsed viral stimulation produced less antigen-specific IgA synthesis, increasing to a lower peak level from a high average background.

Comparison of the responses *in vitro* shown by counting anti-TGEV ASC and by measurement of

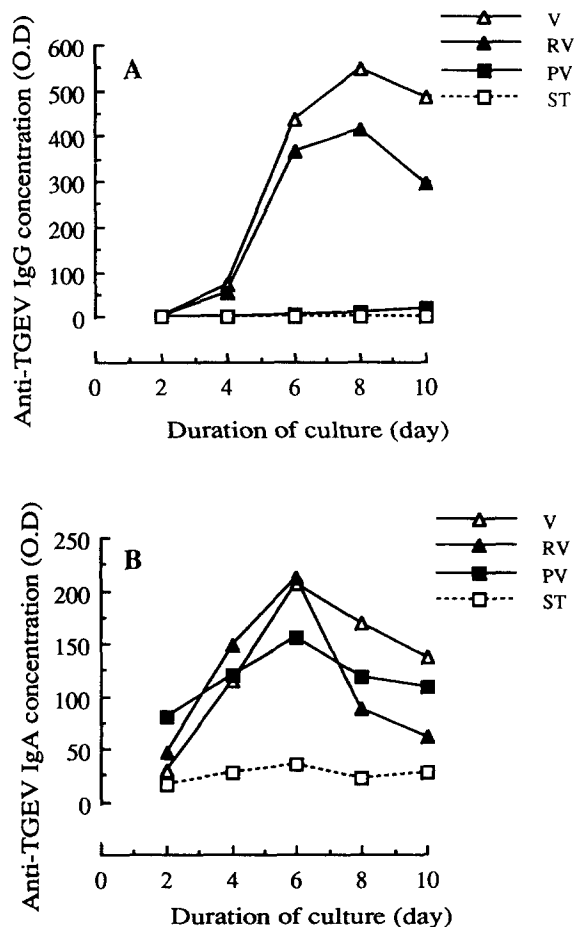


Fig. 3. Kinetics of *in vitro* secretion of anti-TGEV antibody. ELISA titration of anti-TGEV antibodies secreted into the supernatants of leukocyte cultures. The results were expressed as the optical density (OD) obtained with 0.1 ml of 1/3 diluted sample. *A*: IgG responses using an ELISA immunocapture technique. *B*: IgA responses measured on Purdue 115-infected ST cell monolayers. Abbreviations as in Fig. 1.

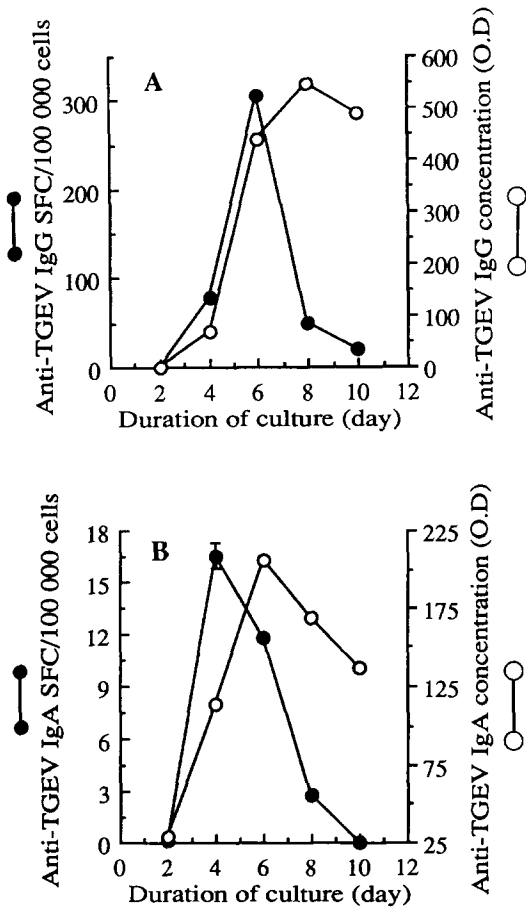


Fig. 4. Comparison between the numbers of antigen-specific antibody-producing cells (ELISASPOT) and the titers of secreted anti-TGEV virus antibodies (ELISA) from *in vivo* primed mesenteric lymph node cells cultured *in vitro* according to the normal viral stimulation (V) method (see legends of Figs. 2 and 3). A: IgG immune response; B: IgA immune response.

the amount of secreted antigen-specific antibodies in culture supernatants showed that the two methods gave similar results (Fig. 4) as illustrated in data obtained from the normal viral stimulated (V) sample. Similar correlations were found in the responses following the other forms of the antigenic stimulation (data not shown). In both Ig isotype responses (IgG (Fig. 4A), IgA (Fig. 4B)), the evolution of the antibody response was defined by a narrow peak. In both cases, the maximum titer of secreted anti-TGEV antibodies occurred 2 days after the maximum number of spe-

cific antibody-forming cells. Moreover using both ELISASPOT or ELISA tests, the IgA response against TGEV seemed to take place earlier than the IgG response with a two day interval between the maxima in the IgA and IgG immune responses.

## Discussion

Previous studies on the *in vitro* immune response of pig leukocytes to TGEV have focused on antigen-induced lymphocyte proliferation (Welch et al., 1988) and on the production of virus-specific neutralizing antibodies by primed lymphocyte cultures stimulated with purified TGEV (Wesley et al., 1986). Compared to this last report, our *in vitro* antigenic stimulation methods permit both the use of crude viral antigen preparations and the titration of secreted anti-TGEV antibodies without any treatment of the culture supernatant samples. Since the protective mechanisms against TGE infection do not seem to be only provided by anti-TGEV neutralizing antibodies (Shirai et al., 1988; Wesley et al., 1988), the humoral immune responses induced in pig MLN by TGEV have been studied by estimating both total TGEV-specific antibody-producing cells and their secreted product, according to their Ig isotype and regardless of their neutralizing activity.

Specific ASC were revealed with an ELISASPOT technique using 3-amino 9-ethyl carbazole as the chromogen for the peroxidase reaction (Pauli et al., 1984) to give well-contrasted red spots without any background on ST cell monolayers. As with the *o*-dianisidine chromogen used by Russell et al. (1987), the immunoperoxidase staining was stable and the plates could be stored for 2 or 3 weeks before reading. Furthermore, this chromogen did not need to be mixed with agarose, unlike paraphenylenediamine which is used for the peroxidase reaction (Czerkinsky et al., 1983) or 5-bromo-4-chloro-3-indolyl phosphate which is used with alkaline phosphatase conjugates (Sedgwick and Holt, 1983, 1986; Holt et al., 1984; Bianchi et al., 1986).

The antigenic spectrum provided by the virus-infected ST cells is probably wider than the one presented by the isolated viral particles. Thus,



some of the anti-TGEV antibodies which bound to infected cell monolayers may not recognize isolated virus. For example, some antibody specificities which react with virus-infected cells may be against epitopes which are either (a) not expressed at all on isolated viral particles or (b) are hidden on such particles (Bernard et al., manuscript in preparation). So, for some samples the ELISA technique performed on virus-infected cell monolayers (Grom and Bernard, 1985; Cubero Pablo et al., 1988) may be more sensitive than the ELISA immunocapture technique. Because of this, the more sensitive ELISA technique using virus-infected cell monolayers was employed in experiments studying IgA antibody secretion, while secreted IgG antibody could be determined with an immunocapture assay.

The results presented in this report showed a close correlation between the data obtained with the ELISASPOT and ELISA tests. In both cases, kinetic profiles of specific antibody production were characterized by narrow peaks (the anti-TGEV IgA response occurring earlier than the IgG), suggesting that, under these *in vitro* culture conditions, antigenic stimulation caused differentiation in only some late precursors of antibody-forming cells. According to this hypothesis, the lower total and IgA anti-TGEV antibody responses observed with leukocytes from jejunal Peyer's patches than with mesenteric cells could reflect differences in their cell content and stage of differentiation. Although good antibody responses were induced from unprimed blood lymphocytes stimulated *in vitro* with MRBC and OVA (Binns, 1982; Berthon and Binns, manuscript in preparation) using the same culture conditions, we have not yet detected an *in vitro* antibody response to the coronavirus without preliminary *in vivo* immunization of the leukocytes. These observations are in agreement with studies performed with other viral antigens (African swine fever virus) in which induction of antibody secretion from cultured pig leukocytes appeared to be dependent on growth factors (e.g., interleukin-2) which could be provided by supernatants of concanavalin A-stimulated leukocytes (Casal et al., 1987). The transient antibody response, as illustrated by the peak of the kinetic profiles, may indicate that these antigenic stimuli caused selective terminal differentia-

tion of primed precursors to synthesizing plasma cells and then cell death. No more SFC were detected after 10 days of leukocyte culture, in contrast to the response of blood lymphocytes to MRBC, which under appropriate conditions showed continuing synthesis of antibody beyond this time.

*In vitro* antibody production occurred only when primed leukocytes were maintained in contact with virus (V and RV samples) and could be detected either by ELISA or ELISASPOT tests from the 4th day of culture. No ASC and no free IgG were revealed from the culture samples on day 2, suggesting that there was no effective Ig-secreting cell in the cell population isolated from the MLN. The small amounts of free IgA detected on day 2 in the culture supernatants, in the absence of anti-TGEV IgA-SFC, may result from a spontaneous release of antibody (e.g., lysis of some IgA(+) lymphoblasts and/or release of cytophilic antibodies).

A 24 h pulse of antigenic stimulation (PV) led to much lower and briefer immune responses. Thus, under these culture conditions, the continued presence of viral antigens seemed necessary for the antibody response. This was unlike the results obtained with primed pig blood lymphocytes cultured *in vitro* with membrane components of *Haemophilus pleuropneumonia* (Borthwick et al., 1988) in which the highest immune antibody responses were observed after a pulsed antigenic stimulation for 16 h. However although these bacterial antigens are cytotoxic for lymphocytes, they are both persistent and contain lipopolysaccharide which is a B cell mitogen, so providing a more potent and complex response. The presence of viral antigen preparations in the cell cultures also induced greater cell recoveries and this may have resulted from a polyclonal proliferation of sensitized lymphocytes and/or some putative mitogenic activity of the coronavirus.

The highest numbers of anti-TGEV SFC were observed with the RV sample, performed with repeated addition of antigen throughout the culture period. This small improvement in the antibody response occurred without an increase of the virus-specific IgA:total Ig SFC ratio. Furthermore, the repeated addition of viral antigens at the same time as fresh medium resulted in the use

of large amounts of antigen preparation and so more complexing of the synthesized antibodies in the culture supernatants. Thus in comparison with the normal viral stimulation (V) method, complex formation in the RV protocol resulted in a decrease of free secreted antibody titers as revealed by ELISA tests. For this reason, the normal viral stimulation (V) method, performed with only one addition of viral antigens at the beginning of the leukocyte cultures, was the method chosen for subsequent experiments.

The methodology described here, based on an *in vitro* antigenic boost following an initial *in vivo* immunization, can be extended to leukocytes from other organs of the pig's immune system and will permit the characterization of differences in the properties of humoral immune responses expressed by different lymphoid tissues. Moreover use of the ELISASPOT test provides additional data to those obtained from the ELISA test. The use of these enzyme-linked immunosorbent assays permit both a precise analysis of the antigen-specific antibody response and studies of the mechanisms of Ig-isotype regulation. Thus immunomodulating factors might act in regulating either the cell's rate of Ig secretion or the proportion of sensitized lymphocytes secreting antibody. These techniques using enteropathogenic TGE coronavirus as antigen may constitute a valuable *in vitro* model for basic studies of the regulation of antigen-specific IgA synthesis in the secretory immune system.

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