

**INHIBITION OF TRANSMISSIBLE  
GASTROENTERITIS CORONAVIRUS (TGEV)  
MULTIPLICATION *IN VITRO* BY NON-IMMUNE LYMPHOCYTES**

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**SUMMARY**

*In vitro* studies were undertaken to examine the effects of non-immune porcine peripheral blood leukocytes (PBL) on a coronavirus infection due to transmissible gastroenteritis virus (TGEV). The assay consisted of TGEV-infected epithelial cells expressing viral antigens on the cell surface and producing low amounts of interferon (IFN). Non-immune PBL were found to limit virus replication at an effector-to-target ratio of 100/1 even when effector cells were depleted of phagocytic cells. Neutralizing anti-IFN antibodies did not abrogate the effect. PBL from newborn animals were as effective as adult cells, whereas fibroepithelial cells, human and mouse lymphoid cells did not exert antiviral effects. Under similar conditions, PBL from adult animals could lyse TGEV-infected cells even in the presence of anti-IFN antibodies. However, newborn PBL were not cytotoxic. Moreover, depletion of NK cells by monoclonal antibodies plus complement did not alter the inhibitory effect. These latter observations suggest that virus multiplication-inhibition effects and cytotoxic (or NK) activities are unrelated.

**KEY-WORDS:** Coronavirus, TGEV, Cytotoxicity, Lymphocyte; Interferon, Pig.

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## INTRODUCTION

Non-specific defence mechanisms against viral infections include interferon (IFN) production and cell-mediated reactions. Non-immune mononuclear cells have been shown to counteract virus spreading by at least three distinct mechanisms: IFN production, spontaneous lysis of virus-infected cells (or natural killing, NK [14, 33]) and inhibition of virus replication [26]. In some cases, such as MHV3 or HSV1 infections, the great susceptibility of young animals was correlated with their immature cell-mediated immune reactions [16, 32].

In the present report, we have studied transmissible gastroenteritis virus (TGEV), a coronavirus which induces fatal diarrhoeas in up to 100 % of infected newborn piglets [12]. We show that non-immune porcine blood leukocytes (PBL) can inhibit TGEV multiplication *in vitro*. This inhibitory effect is an early mechanism which seems unrelated to the cytotoxic functions of PBL. In addition, both activities (inhibition of replication and cell-mediated cytotoxicity) appeared to be independent of IFN $\alpha$  production.

## MATERIALS AND METHODS

### Animals.

We used conventionally reared pigs obtained from a herd with no history of TGE and lacking neutralizing anti-TGEV serum antibodies.

### Virus.

As a source of TGEV, we used the cell-adapted Purdue 115 strain. Methods for propagation and titration (plaque-forming units, or PFU) were as already described [20].

### Cells.

Peripheral blood leukocytes (PBL) were obtained from heparinized blood by Ficoll density centrifugation [8, 29]. In some experiments, phagocytic cells were depleted

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ADCC = antibody-dependent cell-mediated  
cytotoxicity.  
E/T = effector-to-target.  
FCS = foetal calf serum.  
IFN = interferon.  
LGL = large granular lymphocyte.  
MEM = minimal essential medium.

MoAb = monoclonal antibody.  
m.o.i. = multiplicity of infection.  
NCS = normal calf serum.  
NK = natural killing.  
PBL = peripheral blood leukocyte.  
pfu = plaque-forming unit.  
TGEV = transmissible gastroenteritis virus.

by iron carbonyl ingestion [29] followed by centrifugation on lymphoprep (Nyegaard, Oslo, Norway). The epithelial pig kidney cell line RPTG was cultured as previously described [20].

Mouse thymocytes were obtained from BALB/c mice raised in our own facilities. SP<sub>2</sub>O mouse myeloma cells were provided by J. Grosclaude (INRA, Thiverval-Grignon). Human rectum carcinoma cells (HRT-18) and simian kidney cells (MA-104) were obtained from J.F. Vautherot (INRA, Thiverval-Grignon). Human erythro-leukaemia K562 cells were grown in suspension in RPMI supplemented with 10 % foetal calf serum (FCS).

#### **IFN assay.**

IFN was assayed on bovine MDBK cells using vesicular stomatitis virus as a challenge [18]. In this assay system, our laboratory unit (U) is equal to 0.25 international units of human IFN $\alpha$ . Anti-human IFN $\alpha$  antiserum was kindly provided by C. Chany (INSERM, Paris); it was absorbed on porcine PBL and used at a final dilution of 1/300, which neutralized at least 98 % of porcine IFN present in these experiments (table I). Control experiments showed that this antiserum was devoid of anti-TGEV antibodies and did not inhibit TGEV replication in RPTG cells.

#### **Cell-mediated inhibition assay of virus multiplication.**

RPTG cells were plated for 24 h in 25 cm<sup>2</sup> tissue culture flasks ( $2 \times 10^6$  cells per flask). Cells were then washed and infected with TGEV for 1 h in serum-free minimal essential medium (MEM) at a multiplicity of infection (m.o.i.) of 2. Cultures were then incubated at 38°C overnight in MEM supplemented with 10 % normal calf serum (NCS), trypsinized and washed. RPTG cells ( $10^4$ ) were incubated with  $10^6$  PBL (effector-to-target (E/T) ratio of 100/1, unless otherwise stated) in a final volume of 200  $\mu$ l (RPMI + 10 % FCS) in wells of round-bottomed microtitre plates (Nunc, Roskild, Denmark) for 22 h. Plates were then frozen and thawed in order to titrate together free and cell-associated TGEV. Controls included infected RPTG cells in the absence of PBL.

#### **Cell-mediated cytotoxicity against virus-infected cells: <sup>75</sup>Se-release assay.**

TGEV-infected RPTG cells were obtained as stated above except that, during overnight incubation, cells were labelled with <sup>75</sup>selenomethionine [24]; 1  $\mu$ Ci (4 Ci/mmol; Amersham, UK) for  $10^5$  cells in methionine-free RPMI medium (Eurobio, Paris) supplemented with 10 % NCS. Trypsinized cells were extensively washed and added to PBL at different E/T ratios, in a final volume of 200  $\mu$ l (RPMI + 10 % FCS) for 22 h (unless otherwise stated). Control experiments were performed with non-infected labelled RPTG cells. At the end of the assay, plates were centrifuged (300 g for 10 min) and 100  $\mu$ l samples of supernatant were collected for radioactivity measurement. Percent cytotoxicity was calculated as:

$$100 \times (\text{cpm exp} - \text{cpm SR}) / (\text{cpm MR} - \text{cpm SR})$$

where SR (spontaneous release) relates to labelled cells in the absence of PBL, and MR (maximal release) to cells with 10 % Triton-X100.

#### **Cell-mediated cytotoxicity against K562 cells: <sup>51</sup>Cr-release assay.**

A 4-h <sup>51</sup>Cr-release assay was performed as previously described [6] in round-bottomed culture plates with  $10^4$  K562 cells added to various effector cell dilutions. The % cytotoxicity was calculated as stated above.

**Complement-dependent cytotoxicity.**

Non-toxic one-month old rabbit sera served as a source of complement. One volume of PBL ( $3-10 \times 10^6$  cells per ml) was mixed with one volume of complement (1/15 final dilution) and three volumes of antibodies for 30 min at 37°C [28]. Then, the residual cell viability was measured by Trypan blue dye exclusion and the cells were assayed after readjustment of the viable cell concentration. Anti-porcine PBL hybridomas 76-2-11, 76-7-4 [27] and MSA4 [13] were kindly provided by Dr M. Pescovitz (NIH), and the corresponding monoclonal antibodies (MoAb) were used as ascitic fluids at a final dilution of 1/200.

**RESULTS****Characterization of TGEV-infected RPTG cells.**

Several experiments indicated that viral antigens were expressed on the surface of TGEV-infected cells: by performing antibody-dependent cell-mediated cytotoxicity (ADCC) assays, a higher cytotoxicity was obtained when infected target cells were incubated with porcine PBL in the presence of anti-TGEV antiserum [7]. Surface immunofluorescence assays were also performed on RPTG cells trypsinized 16 h after infection using monoclonal antibodies directed against viral glycoprotein E<sub>2</sub>. Most cells expressed surface antigens (data not shown) as also shown on cell monolayers [22].

Under these conditions, infected RPTG cells produced very low IFN amounts (less than 10 U/ml) within 22 h. In contrast, supernatants of infected RPTG cells incubated for 22 h with porcine PBL contained IFN with titres ranging from 100 to 800 U/ml. The leukocytic origin of this IFN was confirmed by the fact (table I) that it was almost completely neutralized by an anti-human IFN $\alpha$  antiserum previously shown to cross-react with porcine IFN [19].

**Cell-mediated inhibition of viral multiplication.**

When non-immune PBL were incubated for 22 h with TGEV-infected cells, virus multiplication in RPTG cells was markedly reduced (85-99 % inhibition; table I, exp. n° 1 to 4). In kinetic experiments, the inhibitory effect was observed when PBL were incubated for at least 9 h with infected RPTG cells: about 45 to 55 % inhibition was observed after 9 h, as compared to 85-90 % following 22 h of incubation (2 experiments). The requirement of a minimal duration of incubation suggested that the decrease in virus titres was not due to adsorption of virus particles on PBL. In fact, control experiments showed that virus titres were identical when cell-free virus suspensions were incubated in medium alone or with PBL.

TABLE I. — Cell-mediated inhibition of TGEV replication *in vitro*.

Exp. n°	Effector cells	Presence of anti-IFN antiserum	Virus titre (pfu/ml)
1	0	—	$7 \times 10^3$
	Adult pig PBL	—	$< 5 \times 10^2$
2	0	—	$5.3 \times 10^4$
	Adult pig PBL	—	$2.5 \times 10^3$
		+	$1.7 \times 10^3$
3	0	—	$7.3 \times 10^3$
	Adult pig PBL	—	$< 50$
		+	50
4	0	—	$2 \times 10^4$
	Adult pig PBL	—	$2.4 \times 10^3$
	8-day old pig PBL	—	$1.1 \times 10^3$
		—	$5.9 \times 10^3$
5	0	—	$4 \times 10^4$
	Adult pig PBL	—	$1.3 \times 10^3$
	HRT-18	—	$7.1 \times 10^4$
	MA-104	—	$3 \times 10^4$
	Mouse thymocytes	—	$3.8 \times 10^4$
	SP2	—	$6.2 \times 10^4$
	K-562	—	$3 \times 10^4$

Average IFN $\alpha$  titre in co-cultures of PBL + TGEV-infected cells was 480 U/ml. In the presence of anti-IFN $\alpha$  antiserum, the IFN $\alpha$  titre was  $\leq 10$  U/ml.

RPTG cells were infected with TGEV (m.o.i. = 2) for 16 h, trypsinized and incubated for 20 h with various cells at an E/T ratio of 100.

Since IFN $\alpha$  is present in supernatants of infected cells incubated with PBL (table I and fig. 1), and since TGEV is susceptible to IFN [18], the viral multiplication inhibition could well be due to the IFN antiviral effect. In fact, in the presence of a dilution (1/300) of anti-IFN $\alpha$  antiserum which neutralized more than 98 % endogenous porcine IFN, PBL still inhibited virus multiplication (table I, exp. n° 2 and 3), which implies that this inhibition was mostly independent of extracellular IFN.

Inhibition of TGEV multiplication was observed when PBL were incubated, with E/T of 100/1 and 50/1 (table II). PBL from 8-day old piglets were as active as cells from adults (table I). However, mouse and human lymphoid cells (thymocytes, SP20, K562) or TGEV-non replicating cell lines (HRT-18, MA-104) could not significantly inhibit virus multiplication (table I). It was concluded that this viral multiplication-inhibition was selectively mediated by porcine leukocytes (including gut lymphocytes; Petit and Charley, unpublished results).

TABLE II. — Inhibition of TGEV replication *in vitro* by PBL and by phagocyte-depleted lymphocytes at different E/T ratios.

Exp. n°	Nature of effector cells	E/T ratio	Virus titre (pfu/ml)	IFN titre (U/ml)
1	0	0	$10^5$	< 10
	Adult pig leukocytes	100/1	$5.7 \times 10^3$	825
	Phagocytic cell-depleted lymphocytes	100/1	$6 \times 10^3$	2,500
2	0	0	$6 \times 10^5$	< 10
	Adult pig leukocytes	100/1	$1.8 \times 10^5$	100
	»	50/1	$1.8 \times 10^5$	160
	»	25/1	$4.1 \times 10^5$	50
	Phagocytic cell-depleted lymphocytes	100/1	$2.7 \times 10^5$	8,250

RPTG cells were treated as in table I.

PBL were depleted of phagocytic cells by carbonyl iron ingestion followed by centrifugation over lymphoprep (see «Materials and Methods»).

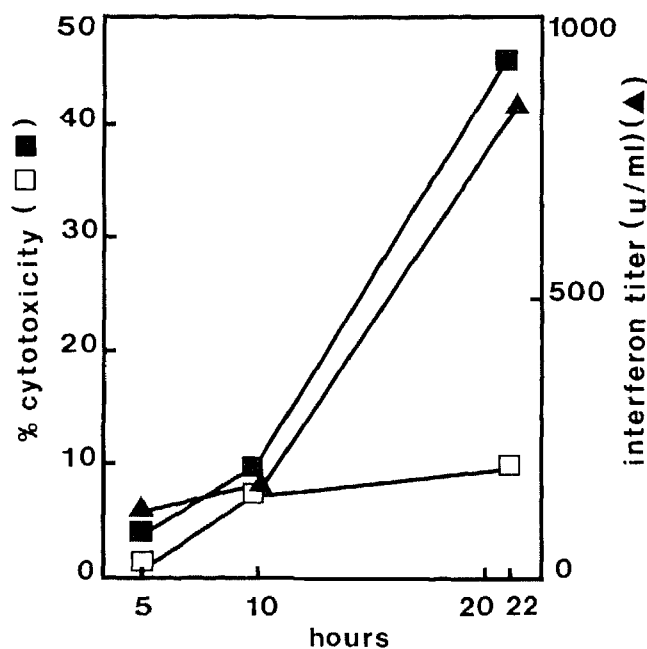


FIG. 1. — Lysis of infected (black symbols) and uninfected cells (open symbols) and IFN production by non-immune PBL: kinetic experiments (E/T ratio of 100/1).

Mean values of spontaneous release by control and infected cells after 22 h were 994 and 1,680 counts per min, respectively. Maximal release values were 5,063 and 5,370, respectively.

When porcine PBL were depleted of phagocytic cells, they could still inhibit TGEV multiplication. Complement-mediated lysis assays were used to characterize the effector cells involved in the inhibition of virus multiplication (table III). MoAb 76-2-11, which recognizes porcine cytotoxic T cells (PT8) [27] and which abolishes porcine NK activity against K562 cells (table III and Pescovitz, personal communication) did not alter the ability of PBL to limit TGEV multiplication (table III). Similarly, MoAb MSA4, a pan-T-cell reagent [13] and MoAb 76-7-4 which reacts with porcine B cells [27], had no effects on the effector functions studied (table III).

TABLE III. — Effects of complement-mediated lysis of PBL upon their ability to lyse K562 cells and to inhibit TGEV replication.

	Complement alone	MoAb 76-2-11 (PT8) + complement	MoAb MSA4 (PanT) + complement	MoAb 76-7-4 (B) + complement
Percentage of dead cells	2-6 (1)	10-40	43-58	10-23
NK (K562) % cytotoxicity	E/T = 50 16 (2) E/T = 100 36	0	3	33
Inhibition of TGEV replication (percentage)	71 ± 2 (3)	73 ± 14	66 ± 19	66 ± 7

(1) Range of data in 4 experiments.

(2) % cytotoxicity in a 4-h <sup>51</sup>Cr-release assay, with K562 cells as target cells, at E/T ratio of 50 and 100. Data are representative of two experiments.

(3) Mean ± SEM of 4 experiments. PBL were incubated with virus-infected cells at an E/T ratio of 100.

Percentage of inhibition was calculated as: 100 × (virus titre without PBL – virus titre with PBL)/virus titre without PBL.

#### Cell-mediated cytotoxicity against TGEV-infected RPTG cells.

As a possible mechanism explaining the viral multiplication inhibition by PBL, we analysed, in the same experimental conditions as stated above, the ability of non-immune PBL to lyse virus-infected cells *in vitro*. Infected RPTG cells were labelled with <sup>75</sup>Se-methionine instead of <sup>51</sup>Cr in order to reduce spontaneous isotope release in long-term assays [24]. In <sup>75</sup>Se-release assays, non-immune PBL could, within 22 h, lyse infected target cells much more efficiently than control cells (fig. 1). In the same period, IFN $\alpha$  gradually

appeared in supernatants (fig. 2). We also performed  $^{75}\text{Se}$ -release assays in the presence of neutralizing anti-IFN $\alpha$  antiserum: under these conditions (fig. 2), infected cells were still more extensively lysed than control cells. In contrast to adults, PBL obtained from young animals could not lyse TGEV-infected cells (table IV).

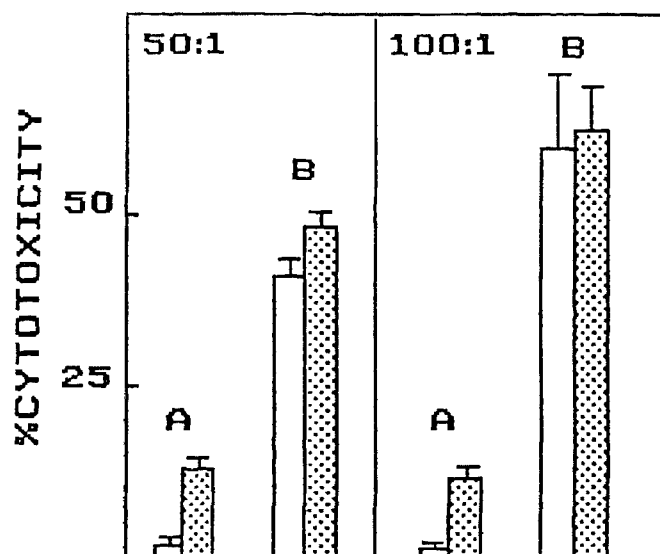


FIG. 2. — Effect of anti-IFN antibodies on cell-mediated cytotoxicity of non-immune PBL against virus-infected cells.

Open bars = presence of anti-IFN antiserum (1/300); dotted bars = absence of antiserum. A = uninfected cells; B = infected cells.

TABLE IV. — Comparison of young and adult animal PBL in their ability to mediate lysis of TGEV-infected cells.

PBL origin	% cytotoxicity at E/T ratios of		
	25/1	50/1	100/1
8-day old piglets	0 0	0 0	0 0
Adults	0 9.3 ± 1.5	9.4 ± 1.4 23.8 ± 2.8	18.1 ± 2.2 36 ± 4.7

RPTG were treated as in table I.  
 $^{75}\text{Se}$ -release assay performed in 22 h.



## DISCUSSION

Our findings indicate that non-immune PBL can limit TGEV multiplication *in vitro*: several mechanisms could account for that observation. Firstly, NK cells could lyse infected cells before virus progeny was produced and therefore limit viral replication: this mechanism was proposed for herpesviruses [11, 34]. In the present report as well as in previous studies [5], non-immune porcine PBL are shown to be cytotoxic to TGEV-infected target cells. This cytotoxicity differs from ADCC in that it requires a longer period of incubation [5, 7]. However, PBL obtained from newborn piglets have no spontaneous cytotoxicity, either against tumour target cells [8, 15] or against TGEV-infected cells ([5]; our present results). Since PBL from young piglets do inhibit virus multiplication *in vitro*, it is unlikely that TGEV multiplication inhibition could be due to NK lysis of infected cells. This assumption is further strengthened by the fact that complement-mediated lysis of PBL by MoAb 76-2-11, which abolished the porcine NK activity against K562 cells, did not alter the inhibitory effect. However, with the MoAb used, we could not characterize the precise nature of the effector cells involved in the inhibition phenomenon.

In several experimental models, IFN $\alpha$  was produced by mononuclear cells during the antiviral NK assay, and it was therefore suggested that the preferential lysis of virus-infected over uninfected cells could be due to a non-specific augmentation of cytolysis by IFN $\alpha$  [30]. However, by using anti-IFN $\alpha$  antibodies, it was found that lysis of cells infected with HSV1 [10], measles [4] and dengue viruses [17] was independent of IFN $\alpha$  generation. Similarly, the present report indicates that porcine effector cells were capable of lysing TGEV-infected target cells independently of extracellular IFN $\alpha$  production. Whether the glycoprotein E2 expressed on the membrane of TGEV-infected cells [22] could directly activate NK, as previously shown for Sendai, influenza and measles virus glycoproteins [1, 2, 3] needs to be investigated.

A second possible explanation for the inhibition of viral replication could be that PBL alters target cell metabolism, thereby interfering with virus-driven protein synthesis [26]. However, in our experiments, the antiviral effect was mediated only by porcine PBL and not by other lymphoid or epithelial cells tested.

Thirdly, IFN could account for the inhibitory effect: porcine PBL, although unable to replicate TGEV, produce IFN $\alpha$  after exposure to infectious or UV-inactivated TGEV [21] or to TGEV-infected cells (this report). When the large majority of endogenously produced IFN $\alpha$  was neutralized by anti-IFN $\alpha$  antibodies, virus replication was still inhibited by PBL; however, one cannot rule out a possible antiviral effect of residual IFN. Previous studies on inhibition of herpesvirus multiplication have also shown that activated macrophages [26], Leu11<sup>+</sup> Leu4<sup>-</sup> human large granular lymphocytes (LGL) [11] and bovine leukocytes [31] could inhibit virus multiplication independently of IFN $\alpha$  production.

In order to propose a mechanism which could explain the inhibitory effect described here, it would also be necessary to examine the presence, in

porcine PBL, of natural antiviral compounds such as the neutralizing peptides MCP1 and MCP2 described in rabbit leukocytes [23].

Finally, what roles could be played by the activities described in this report in the resistance of adult animals to TGE infection? Cell-mediated inhibition of virus replication, which could act early in the course of the infection (within 9 h of incubation) might serve to limit TGEV replication in adult animals. In newborn piglets, although mononuclear cells are active *in vitro*, the reduced number of circulating mononuclear cells [25] and the very low number of intestinal intraepithelial lymphocytes [9] observed during the first 3 weeks of life, along with the inability of piglet-derived PBL to lyse virus-infected cells, could account for the great susceptibility of young animals to TGEV infection.

### RÉSUMÉ

INHIBITION DE LA MULTIPLICATION *IN VITRO* DU CORONAVIRUS DE LA GASTROENTÉRITE TRANSMISSIBLE (GET) PAR DES LYMPHOCYTES NON IMMUNS

Nous avons entrepris d'étudier *in vitro* les effets de leucocytes sanguins de porcs non immuns sur l'infection par le coronavirus de la gastroentérite transmissible (GET). En l'absence de lymphocytes, le virus GET se réplique dans des cellules épithéliales, induisant l'expression d'antigènes viraux de surface et la production de faibles quantités d'interféron (IFN). Des lymphocytes non immuns, mis en présence de cellules infectées, sont en mesure de limiter la multiplication virale quand ils sont utilisés à un rapport effecteur sur cibles de 100 et cela, même quand les cellules effectrices sont appauvries en cellules phagocytaires. Des anticorps anti-IFN neutralisants n'empêchent pas cet effet. Les lymphocytes d'animaux nouveaux-nés sont aussi actifs que ceux des adultes, alors que des cellules fibroépithéliales et des cellules lymphoïdes humaines ou murines n'ont aucun effet antiviral. Dans les mêmes conditions, des lymphocytes provenant d'animaux adultes détruisent des cellules infectées par le virus GET, même en présence d'anticorps anti-IFN. Cependant, puisque les lymphocytes de nouveaux-nés ne sont pas cytotoxiques et que la destruction des cellules NK par anticorps monoclonal plus complètement ne modifie pas l'effet inhibiteur, il apparaît que cet effet antiviral n'est pas dû aux fonctions cytotoxiques (NK) des lymphocytes. La nature du mécanisme responsable de l'effet antiviral décrit dans cet article reste donc à élucider.

MOTS-CLÉS: Coronavirus, TGEV, Cytotoxicité, Lymphocyte; Porc, Interféron.

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