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**MYXOVIRUS- AND CORONAVIRUS-INDUCED  
IN VITRO STIMULATION  
OF SPONTANEOUS CELL-MEDIATED CYTOTOXICITY  
BY PORCINE BLOOD LEUKOCYTES**

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**SUMMARY**

Spontaneous cell-mediated cytotoxicity (SCMC) by porcine blood leukocytes toward human myeloid tumour target cells (K<sub>562</sub>) was shown by Koren and coworkers in 1978, using a 4-h <sup>51</sup>Cr-release assay. In the present paper, SCMC was found to be enhanced following a 24-h incubation of leukocytes with swine influenza virus (SIV) of the H<sub>3</sub>N<sub>2</sub> type, and with the transmissible gastroenteritis virus (TGEV), a porcine coronavirus.

In most of our experiments, both SIV and TGEV induced interferon (IFN) production by leukocytes (titres ranging from 30-7,500 units/ml). When porcine leukocytes were incubated with TGEV in the presence of anti-IFN antiserum, no SCMC stimulation occurred, suggesting that the virus-induced SCMC stimulation was mainly due to endogenously produced IFN. On the other hand, when either human  $\alpha$ IFN or porcine virus-induced IFN were added to porcine leukocytes, a rapid and marked SCMC increase was observed.

Both the SIV-induced SCMC increase and IFN production were suppressed when SIV was UV-inactivated. On the contrary, UV-inactivated TGEV was still able to enhance SCMC and to induce IFN production by porcine leukocytes.

On the whole, these results suggest that myxovirus and coronavirus are able to activate porcine leukocyte SCMC via endogenously produced IFN.

**KEY-WORDS:** Cytotoxicity, Myxovirus, Coronavirus, Interferon; Leukocytes, K<sub>562</sub>, Pig.

## INTRODUCTION

In the last few years, increasing interest has been devoted to viruses causing neonatal diarrhoea in man and animals, and to the defence mechanisms of the newborn against these infections. The pig appears to be a convenient animal model for studies on the pathogenesis of viral enteritis, as well as on the nature of early non-specific defence mechanisms against neonatal diarrhoea; indeed, the transmissible gastroenteritis virus (TGEV), a porcine coronavirus, produces an acute and fatal disease and induces high amounts of intestinal interferon (IFN) [7]. In addition to IFN, the presence of spontaneous cell-mediated cytotoxicity (SCMC) was investigated as a potential non-specific defence against enteropathogenic viruses. SCMC, or natural killing (NK), has been described in various mammalian and avian species [5, 4, 9], and there is increasing interest in its possible role in host defence against disease [5]. The treatment of rodent or human lymphocytes with IFN, or the infection of rodents with different viruses greatly increases SCMC [2, 3, 4, 13] but to our knowledge no similar data are available for other animal species.

In the present report, we provide results concerning the *in vitro* effects of TGEV on the spontaneous cytotoxicity of porcine leukocytes, using a short-term <sup>51</sup>chromium-release assay against human myeloid tumour target cells (K<sub>562</sub>) as described by Koren *et al.* [6]. We compare these data to the *in vitro* effects of a myxovirus which was previously shown to induce NK in mice [10]. Furthermore, we present results on the influence of porcine as well as human IFN on SCMC via porcine leukocytes.

## MATERIALS AND METHODS

*Animals.*

Conventionally reared pigs, 2-8 months old, were used.

*Interferon (IFN) preparations and assay.*

As a source of virus-induced porcine IFN, a serum obtained from a TGEV-infected neonate (IFN titre = 7,500 U/ml) was used.

Human  $\alpha$ IFN, with a titre of  $4 \times 10^6$  U/ml, was kindly provided by Institut Pasteur Production (Garches, France).

IFN was assayed on bovine MDBK cells using vesicular stomatitis virus as a challenge [7]. IFN titres were expressed with reference to an internal bovine standard IFN, yielding a mean titre of 2,500 U/ml by the 50 % plaque-reduction method.

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EID<sub>50</sub> = egg infective dose 50 %.  
 FCS = foetal calf serum.  
 IFN = interferon.  
 MOI = multiplicity of infection.  
 MR = maximal release.  
 NK = natural killing.

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PFU = plaque-forming unit.  
 SCMC = spontaneous cell-mediated cytotoxicity.  
 SIV = swine influenza virus.  
 SR = spontaneous release.  
 TGEV = transmissible gastroenteritis virus.

Anti-human  $\alpha$ IFN antiserum was kindly provided by Dr Chany (Paris); after absorption on porcine leukocytes, it was used at a final dilution of 1/200, which was able to neutralize  $\approx$  750 units of porcine IFN.

*Preparation of mononuclear cells.*

Mononuclear cells were separated from heparinized blood by a Ficoll density centrifugation method: 20 ml of diluted blood (1/4 in 9 % NaCl) were layered onto 7.5 ml of a mixture containing 14 % Ficoll (Pharmacia, Uppsala, Sweden) and 32.8 % Telebrix (Guerbet, Aulnay-sous-Bois, France) [12]. After centrifugation for 15 min at 2,000 *g* at room temperature, the leukocytes were pipetted out, washed twice and resuspended in RPMI medium supplemented with 10 % foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (penicillin and streptomycin).

*Viruses.*

A strain of influenza virus type A ( $H_3N_2$ ) isolated from pig lung (Swine influenza virus: SIV), provided by Dr Hannoun (Paris), was passaged in embryonated eggs. Infectivity was expressed as the 50 % egg infective doses (EID<sub>50</sub>) per ml.

As a source of the TGEV cell-adapted Purdue 115 strain was used. Methods for propagation and titration (plaque-forming units: PFU) of this virus have previously been described [8].

*Chromium labelling of target cells.*

Human myeloid leukaemia cells ( $K_{562}$ , kindly provided by Dr Gutner, Villejuif, France) were labelled with  $Na_2^{51}CrO_4$  (Amersham, UK, 40  $\mu$ Ci/ $2 \times 10^6$  cells in 250  $\mu$ l RPMI) for 1 h. The cells were then washed three times with RPMI-10 % FCS and adjusted to a final concentration of  $10^5$ /ml in RPMI-10 % FCS.

*Assay for cytotoxicity.*

Porcine leukocytes were incubated in round-bottomed microtitre plates (Nunc, Roskild, Denmark) for 24 h at 37° C in a humidified atmosphere containing 7 % CO<sub>2</sub>. Triplicate cultures were prepared with  $10^6$  leukocytes per well in RPMI-10 % FCS. Then,  $10^4$  labelled target cells were added in a total volume of 0.2 ml (leukocyte: target ratio of 100), and plates were centrifuged for 5 min at 70 *g* as described by Koren *et al.* [6]. After 4 h of incubation, the assay was terminated by spinning the plates for 10 min at 300 *g*, and samples (0.1 ml) of the supernatants were collected for radioactivity measurement. The % cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{cpm experimental} - \text{cpm SR}}{\text{cpm MR} - \text{cpm SR}} \times 100$$

where SR (spontaneous release) is defined as the radioactivity released from target cells incubated in medium alone (RPMI-10 % FCS) and MR (maximal release) as cpm in the supernatants of targets lysed with Triton  $\times$  100.

## RESULTS

*SIV stimulation of SCMC in vitro.*

It was checked that SIV ( $2.5 \times 10^6$  EID<sub>50</sub>/ml) was unable to increase <sup>51</sup>Chromium SR by labelled target cells within 4 h.

When porcine blood leukocytes were incubated with SIV 24 h before the addition of labelled  $K_{562}$  cells, SCMC was found to increase: this stimulation was significant ( $p < 0.001$ , paired *t* test) in results from 17 different

leukocyte preparations. An IFN production was detectable in the supernatants of infected leukocytes, with IFN titres ranging from 275 to 7,500 Ug/ml (table I: exp. 1). Furthermore, when an aliquot fraction of the virus preparation was completely inactivated by UV irradiation, it no longer enhanced SCMC, and IFN production was markedly reduced (table I: exp. 2)

TABLE I. — SIV-induced SCMC enhancement and IFN production by live and UV-inactivated viruses.

Exp. n <sup>o</sup>	UV inactivation	MOI	% cytotoxicity	IFN (Ug/ml)
1	—	0	5 ± 0 (*)	< 10
	—	2.5 × 10 <sup>-1</sup>	12.6 ± 1.1 (**)	2,500
	—	2.5 × 10 <sup>-2</sup>	11.6 ± 0.3 (**)	7,500
2	—	0	8 ± 1.5	< 10
	—	2.5 × 10 <sup>-1</sup>	20.6 ± 0.6 (**)	ND
	+	2.5 × 10 <sup>-6</sup>	8.6 ± 0.6	30

MOI = multiplicity of infection (EID<sub>50</sub> per cell).

ND = not done.

Results of exp. 2 are representative of 4 different cell suspensions.

(\*) Mean of triplicates ± SEM.

(\*\*) Significantly different from controls (p < 0.01, Student's *t* test).

#### *TGEV-induced SCMC enhancement.*

Controls showed that TGEV (1.2 × 10<sup>7</sup> PFU/ml) and absorbed anti-IFN antiserum did not increase SR by labelled K<sub>562</sub> within 4 h.

SCMC increased significantly following 24 h incubation of porcine leukocytes with TGEV (p < 0.001, paired *t* test, with data from 12 different cell suspensions). IFN was detected, with titres ranging from 30 to 2,500 µg/ml. Since virus-induced porcine IFN was shown to be neutralized by anti-human αIFN antiserum (La Bonnardière and Laude, manuscript in preparation), we were able to show that anti-αIFN antiserum, when added to porcine leukocytes with TGEV, suppressed virus-induced SCMC enhancement and IFN production (table II). Control experiments showed that ultracentrifuged virus suspensions were no longer able to stimulate SCMC, indicating that the enhancing effect was related to the virus particle; however, contrary to findings with SIV, when TGEV was UV-inactivated, it was still able to induce SCMC enhancement and IFN production (table III).

#### *Enhancement of SCMC in vitro by exogenous virus-induced porcine IFN or human αIFN.*

Since the above results suggested that SCMC enhancement by SIV and TGEV was related to IFN production, we tried to determine whether the addition of IFN to porcine leukocytes would affect their cytotoxicity: table IV shows that 10<sup>3</sup> units of porcine IFN per ml increased the SCMC of two cell suspensions but was inactive on a third preparation. Human

TABLE II. — TGEV-induced SCMC enhancement and IFN production with or without anti- $\alpha$ IFN anti-serum.

Animal nb	Anti-IFN	% cytotoxicity		Virus-induced IFN (U/ml)
		without TGEV	with TGEV	
48	—	3.9 $\pm$ 0.6	10.5 $\pm$ 1.5 (p < 0.02)	475
	+	4.8 $\pm$ 0.4	7.5 $\pm$ 1.4 (NS)	< 10
23	—	30.9 $\pm$ 0.5	34.4 $\pm$ 0.7 (p < 0.02)	160
	+	33.0 $\pm$ 1.6	34.3 $\pm$ 2.0 (NS)	< 10

Final dilution of anti-IFN = 1/200.  
 Multiplicity of infection = 1.2 PFU/cell.  
 NS = not significantly different from uninfected cells (Student's *t* test).  
 Mean of triplicates  $\pm$  SEM.

TABLE III. — Influence of UV inactivation of virus on TGEV-induced SCMC enhancement and IFN production.

Duration of UV inactivation (min)	Residual infectivity titre (PFU/ml)	Multiplicity of infection (PFU/cell)	% cytotoxicity	IFN titre (U/ml)
0	10 <sup>7</sup>	0 (*)	9.6 $\pm$ 0.8 (**)	< 10
0.5	2.5 $\times$ 10 <sup>5</sup>	1	21.8 $\pm$ 0.6	825
1.0	9 $\times$ 10 <sup>4</sup>	2.5 $\times$ 10 <sup>-2</sup>	20.5 $\pm$ 0.3	825
2.0	0	9 $\times$ 10 <sup>-3</sup>	21.5 $\pm$ 1.4	825
20.0	0	0	15.9 $\pm$ 0.6	825
		0	19.8 $\pm$ 1.6	275

Results are representative of 4 different cell suspensions.  
 (\*) Control without TGEV.  
 (\*\*) Mean of triplicates  $\pm$  SEM.

TABLE IV. — Enhancement of SCMC by virus-induced porcine IFN.

Animal nb	Porcine IFN titre (Ug/ml)			
	0	12.5	125	1,250
21	3 $\pm$ 0.8 (*)	3.3 $\pm$ 0.6	7 $\pm$ 0.5	15 $\pm$ 1.1 (**)
22	20 $\pm$ 2.3	15 $\pm$ 4	19 $\pm$ 1.1	20 $\pm$ 1.7
24	6 $\pm$ 1.1	1 $\pm$ 0.5	6 $\pm$ 2.3	15 $\pm$ 0.8 (**)

(\*) Mean of triplicates  $\pm$  SEM.  
 (\*\*) Significantly different from control cells (p < 0.01 by Student's *t* test).

$\alpha$ IFN ( $2 \times 10^3$  Ug/ml) significantly increased SCMC ( $p < 0.01$  in paired  $t$  test with data from nine different cell preparations). This enhancement was dose-dependent and had already been observed with  $2 \times 10^2$  Ug/ml (fig. 1). Furthermore, the IFN « boosting » effect was also observed against two other human tumour cell lines (CEM and PDe-B1 [9]) and was inhibited by anti  $\alpha$ IFN antiserum (data not shown).

Controls showed that human and porcine IFN did not increase SR by labelled target cells.

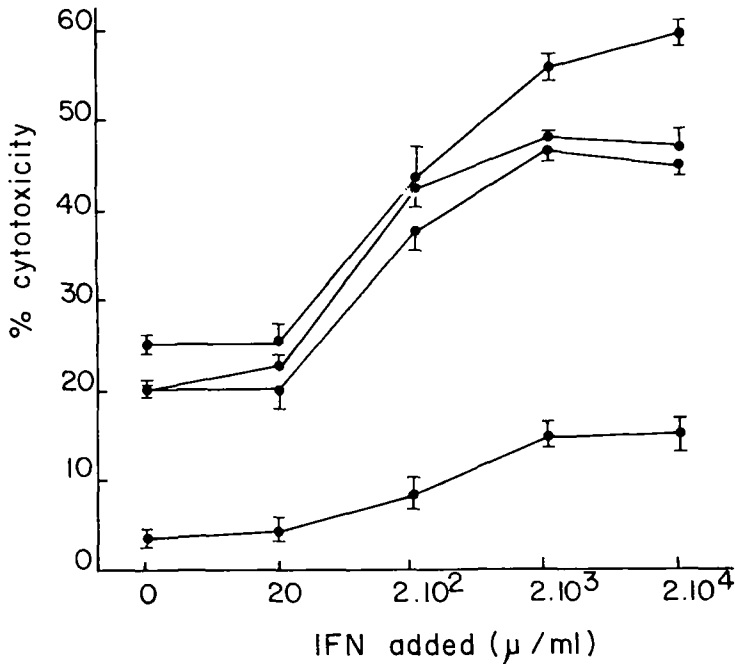


FIG. 1. — Enhancement of SCMC by human  $\alpha$ IFN. Each curve is related to one leukocyte preparation.

## DISCUSSION

This demonstrates that SIV and TGEV increase the SCMC of porcine leukocytes. Both viruses induced IFN production by leukocytes, and moreover, anti-human  $\alpha$ IFN antiserum, which has been shown to neutralize porcine IFN (La Bonnardière and Laude, in preparation) suppressed the TGEV-induced SCMC stimulation. Both porcine and human IFN were also shown to stimulate SCMC *in vitro*.

The present data are in agreement with previous studies showing that different viruses (including lymphocytic choriomeningitis, mumps, measles and herpes simplex viruses) induced IFN production by leukocytes, and that NK cells in these cell preparations became activated (reviewed by Welsh [13]). Influenza virus has also been shown to induce IFN production

by infected human leukocytes [11] and to stimulate NK cells *in vitro* [13] and *in vivo* [10]. However, to our knowledge, this the first demonstration that a coronavirus is able to induce IFN production by blood leukocytes and to increase SCMC.

UV-inactivation experiments produced distinct results with the two viruses, since inactivated SIV was unable to stimulate SCMC or to induce IFN, whereas inactivated TGEV retained its inducing effect: this data further indicated that virus-induced SCMC enhancement is correlated with IFN production.

The discrepancy in the results of UV-inactivation experiments using the two viruses is probably due to differences in the doses of UV treatment: in order to obtain almost total SIV inactivation, it was necessary to irradiate the virus suspension up to 4 h, whereas the TGEV was fully inactivated following 2 min of UV treatment. It therefore appears that prolonged UV irradiation abolishes the ability of the virus to induce IFN and SCMC enhancement. Indeed, such a result was observed in preliminary experiments with TGEV. However, it is worth noting that some exceptions to this rule do exist, since Casali *et al.* [1] described increased SCMC by a measles virus glycoprotein, with no detectable IFN production.

Finally, porcine leukocytes were found to express activated SCMC when incubated with IFN, as was shown for rodents and man [2, 3, 4]. According to our results, porcine IFN seemed less active than human IFN. This seems to be consistent with the finding that porcine IFN has a more pronounced antiviral effect upon heterologous (bovine) cells than upon homologous cells [7].

The role of SCMC- and IFN-« boosted » SCMC *in vivo* in TGEV-induced enteritis is currently under investigation in our laboratory.

## RÉSUMÉ

STIMULATION « IN VITRO », PAR UN MYXOVIRUS OU UN CORONAVIRUS,  
DE LA CYTOTOXICITÉ SPONTANÉE À MÉDIATION CELLULAIRE  
PAR LES LEUCOCYTES SANGUINS DE PORC

Une cytotoxicité spontanée à médiation cellulaire par les leucocytes sanguins de porc est mise en évidence par un test de relargage de  $^{51}\text{Cr}$  en 4 h, vis-à-vis d'une cible tumorale humaine ( $\text{K}_{562}$ ). Cette cytotoxicité spontanée est accrue après 24 h d'incubation des leucocytes avec un virus grippal porcin (de type  $\text{H}_3\text{N}_2$ ) ou le virus de la gastroentérite transmissible (GET), un coronavirus du porc.

Ces deux virus ont induit une production d'interféron par les leucocytes. Quand les leucocytes ont été incubés avec le virus de la GET en présence de sérum anti-interféron, aucune stimulation de la cytotoxicité spontanée n'est apparue, ce qui suggère que cette stimulation était principalement due à de l'interféron produit par les leucocytes. Par ailleurs, quand de l'interféron  $\alpha$  humain ou de l'interféron viro-induit porcin est ajouté aux



leucocytes de porc, on observe une stimulation rapide et importante de la cytotoxicité spontanée.

La stimulation de cytotoxicité spontanée et la production d'interféron sont supprimées quand le virus grippal porcine est inactivé par les rayons ultraviolets, mais non quand le virus de la GET est inactivé.

L'ensemble de ces résultats suggère que le myxovirus ou le coronavirus peuvent stimuler l'activité cytotoxique spontanée des leucocytes de porc par production d'interféron endogène.

MOTS-CLÉS : Cytotoxicité, Myxovirus, Coronavirus, Interféron ; K<sub>562</sub>, Leucocytes, Porc.

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