# Reconstituted coronavirus TGEV virosomes lose the virus ability to induce porcine interferon-alpha production

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*Vet Res* (1996) 27, 77-86




**Original article**

# Reconstituted coronavirus TGEV virosomes lose the virus ability to induce porcine interferon-alpha production

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(Received 3 June 1996; accepted 15 July 1996)

**Summary** — The transmissible gastroenteritis virus (TGEV) is a coronavirus which induces a strong interferon-alpha (IFN-α) production in vivo and in vitro. Previous studies have shown that the TGEV external protein M plays a major role in IFN-α induction by a non-infectious virus, whereas protein S is not involved. The present study extended these results by showing that monoclonal antibodies (MAbs) directed at the external viral protein sM could not block IFN-α induction, which argues against a direct role for this protein. In the same type of blocking experiment, MAbs to the TGEV receptor aminopeptidase N did not inhibit IFN-α induction, which strongly indicates that viral replication or entry through the receptor is not needed for TGEV induction of IFN-α in leukocytes. In an attempt to isolate functional envelope proteins, TGEV virions were detergent-solubilized and reconstituted in virosomes. Although BIAcore antigenic analysis revealed that the three external viral proteins were present on the virosomes, these proteins were unable to induce IFN-α in porcine leukocytes, and seemed to compete with the native virus for IFN-α induction. These data indicated that IFN-α inducing interactions between TGEV external proteins and leukocytes required a complex native envelope protein structure which has been lost in the virosomes.

**interferon-alpha / transmissible gastroenteritis virus / coronavirus / virosome / BIAcore**

**Résumé — Les virosomes reconstitués à partir du coronavirus de la gastroentérite transmissible ont perdu la propriété du virus à induire la production d'interféron alpha porcin.** Le virus de la gastroentérite transmissible (VGET) est un coronavirus induisant in vivo et in vitro une forte production d'interféron alpha (IFN-α). Des travaux antérieurs ont montré que la protéine externe M du VGET joue un rôle majeur dans l'induction d'IFN-α par le virus non infectieux, alors que la protéine externe S n'est pas impliquée. L'étude présentée ici élargit ces résultats en montrant que des anticorps monoclonaux (AcMo) dirigés contre la protéine externe sM ne bloquent pas

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l'induction d'IFN-α, ce qui réduit la possibilité d'un rôle direct de cette protéine. De la même manière, des AcMo dirigés contre le récepteur du VGET, l'aminopeptidase N, n'inhibent pas l'induction d'IFN-α, ce qui indique clairement que la réplication du virus ou son entrée dans les leucocytes via le récepteur n'est pas nécessaire à l'induction d'IFN-α. Afin d'isoler des protéines d'enveloppe fonctionnelles, les virions de VGET ont été solubilisés par des détergents et reconstitués en virosomes. Bien que l'analyse antigénique par BIAcore montre la présence des trois protéines externes du virus dans les virosomes, ces derniers n'induisent pas la production d'IFN-α par les leucocytes porcins mais seraient plutôt des compétiteurs du virus natif lors de l'induction d'IFN-α. L'ensemble de ces données montre que, pour être inductrices d'IFN-α, les interactions entre les protéines externes du virus et la membrane des leucocytes nécessitent l'organisation complexe de l'enveloppe protéique native du virus qui a été perdu dans les virosomes.

**interféron alpha / virus de la gastroentérite transmissible / coronavirus / virosome / BIAcore**

## INTRODUCTION

Interferons alpha and beta (IFN-α and -β) constitute critical elements of the early host response to viruses, serving both as antiviral and immunoregulatory agents. Although several different cell types have been shown to secrete IFN-α/β, the most efficient producers belong to the lymphohematopoietic lineage.

Monocytes/macrophages produce IFN-α/β when infected by viruses such as the Sendai virus, whereas an infrequent leukocyte population, referred to as natural interferon-producing cells (NIPC), was shown to secrete high amounts of IFN-α following a short contact with non-infectious viral structures (reviewed by Fitzgerald-Bocarsly, 1993). Studies performed on human or porcine cells have shown that blood NIPC are low density cells, expressing MHC class II and CD4 surface antigens but they are negative for CD2, CD3, B-cells, monocytes and NK cells markers (Fitzgerald-Bocarsly, 1993; Nowacki and Charley, 1993). Porcine NIPC are present during the early stages of gestation in fetal lymphoid tissues (Splichal et al, 1994), and have been shown to originate from bone-marrow precursors (Charley et al, 1995).

The mechanisms by which non-infectious viruses induce NIPC to produce IFN-α are not yet fully understood. Because several studies have shown that inactivated viruses or virus-infected glutaraldehyde-fixed cells could induce IFN-α production, it was postulated that IFN-α induction in NIPC did not require the actual replication of the virus in the NIPC, but might result from membrane interactions between NIPC and external viral proteins (Lebon et al, 1982). Thus, in several viral systems, antibodies directed at some viral proteins could block IFN-α induction (Lebon, 1985; Charley and Laude, 1988; Capobianchi et al, 1992). More recently, recombinant viral glycoproteins gp120 from the human immunodeficiency virus (HIV) (Capobianchi et al, 1992) and HN from parainfluenza virus (Ito et al, 1994) were found to be able to induce IFN-α in vitro.

In the porcine species, the coronavirus transmissible gastroenteritis virus (TGEV) was shown to induce high levels of IFN-α in infected newborn piglets (La Bonnardière and Laude, 1981). TGEV is an RNA-enveloped virus, containing three external glycoproteins designated S, M (Laude et al, 1986) and the more recently described sM (Godet et al, 1992). Its major receptor on the enterocytes is aminopeptidase N (APN) (Delmas et al, 1992). The crucial role played by the external glycoprotein M in IFN-α induction was suggested by in vitro studies in which antibodies to, and mutations in, the N-terminal domain of M altered viral induction of IFN-α (Charley and Laude, 1988; Laude et al, 1992). No studies however were undertaken to determine if the TGEV external proteins themselves were sufficient to induce IFN-α. In the present

study we attempted to clarify the role of the TGEV external proteins in IFN-α induction.

Several examples of the functional reconstitution of viral envelopes into virosomes have been described (Bron et al, 1993). The reconstitution procedure involves the solubilization of the viral envelope using nonionic detergents, removal of the viral nucleocapsid by ultracentrifugation, and the subsequent controlled removal of the detergent from the supernatant. In the present work, external TGEV proteins were detergent solubilized and reconstituted into virosomes for in vitro IFN-α induction assays on porcine blood leukocytes. Although the BIAcore analysis indicated that the three external proteins were antigenically present in the virosome, no IFN-α induction was obtained. In contrast, the virosomes seemed to compete with TGEV for IFN-α induction. These data suggest that more complex mechanisms than direct viral envelope proteins interactions with NIPC are needed for TGEV induction of IFN-α.

## MATERIALS AND METHODS

### Antibodies

The characteristics of murine monoclonal antibodies (MAbs) directed against external domains of TGEV viral proteins M and S in terms of polypeptide specificity, neutralizing ability and epitope mapping, were described in Delmas et al (1986) and Laude et al (1986). The characterization of murine MAbs directed against the C-terminal domain of TGEV viral proteins sM was described in Godet et al (1992). The characterization of murine MAbs directed against the TGEV binding site on its receptor aminopeptidase N (APN) was described in Delmas et al (1992). The specificity of the different MAbs is shown in table I and in figure 1.

### Virus

The high-passage Purdue-115 strain of TGEV was used as a virus source. The procedures for virus propagation in the pig kidney cell line PDH, and the titration of infectivity in the swine testis ST cell line have been reported previously (Laude et al, 1986).

The procedure for preparing TGEV-infected, fixed monolayers was previously described (Charley and Laude, 1988). Briefly, PDH cells were plated for 24 h in 96-wells plates at a density of 5 x $10^5$/mL (0.1 mL per well). TGEV infection was performed for 1 h in serum-free minimum essential medium (MEM) at a multiplicity of infection of 1. Cultures were then incubated at 38 °C overnight in medium supplemented with 5% new-born calf serum. The TGEV-infected and control monolayers were treated with 0.25% glutaraldehyde in PBS for 1 h at 4 °C and then with 3% glycine in PBS for at least 1 h at room temperature. The plates were then stored at 4 °C.

### Virosomes

The clarified supernatant culture of TGEV infected cells was concentrated 200 times by ultracentrifugation at 100 000 *g* in a 45Ti rotor (Beckman) for 2 h at 4 °C. The virions were resuspended in sterile water and stored at –80 °C.

Detergent-extraction was performed either with octyl glucoside (OG), or with sodium deoxycholate/Nonidet P40 1/1 (NaDOC/NP40). Concentrated virions (0.5 mL) were solubilized in 0.5 mL OG 4% or NaDOC/NP40 0.5% in 200 mM NaCl, 20 mM Hepes, pH 7.2 (NaCl/Hepes buffer). The mixture was sonicated 30 s and then incubated 30 min on ice. Non-solubilized material was removed by centrifugation (100 000 *g* in the TL100.2 rotor for 30 min at 4 °C). The supernatant fraction obtained after extraction with OG was dialysed at room temperature against two changes of 250 mL NaCl/Hepes buffer for 1 h each, followed by dialysis against 500 mL buffer for 2 h. Dialysis was continued at 4 °C against 2 L of NaCl/Hepes buffer for 48 h with three buffer changes (Harmsen et al, 1985). The supernatant fraction obtained

**Table I.** Effect of MAbs directed against TGEV external proteins and TGEV receptor APN on IFN-α induction.

| *MAbs* | *Specificity* | *IFN yield (% of control)* | | |
|---|---|---|---|---|
| | | *MAb dilution* | | |
| | | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| C29 + Q3 | sM | 45 | > 50 | > 50 |
| R16 + V17 | sM | > 50 | > 50 | > 50 |
| S2 | sM | > 50 | > 50 | > 50 |
| 49.22 | M | 12 | 27.5 | > 50 |
| 25.22 | M | 8.5 | 14.4 | > 50 |
| 9.34 | M | 39 | > 50 | > 50 |
| 51.13 | S | > 50 | > 50 | > 50 |
| 25b.21 | S | > 50 | > 50 | > 50 |
| 10.4 + 40.1 | S | > 50 | > 50 | > 50 |
| G43 | APN | > 50 | > 50 | > 50 |
| W26 + G18 | APN | > 50 | > 50 | > 50 |

TGEV infected, fixed monolayers were preincubated for 1 h at 37 °C with antibodies before PBMC addition ($4 \times 10^5$ cells per well) for overnight incubation. poIFN-α production was assayed by a specific ELISA. The data presented here are representative of three independent experiments including one with infectious TGEV particles.

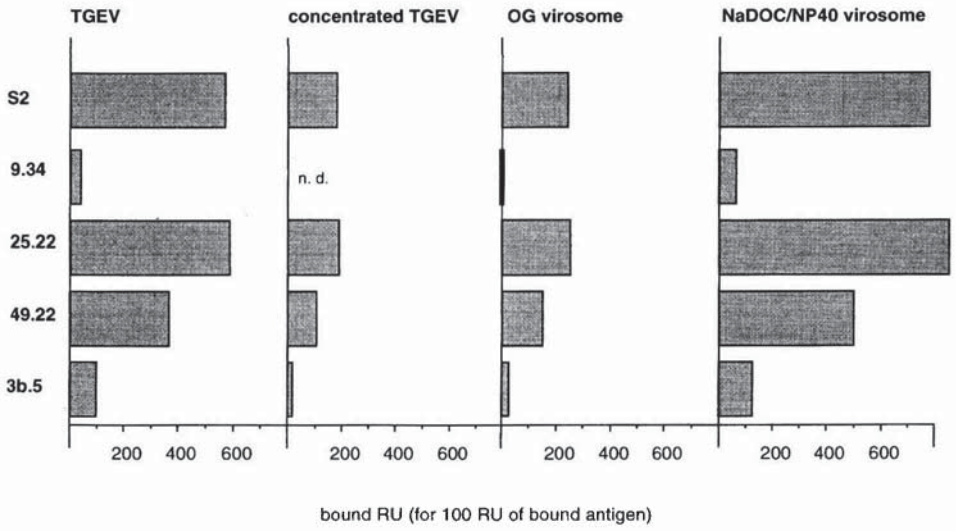


**Fig 1.** Preservation of antigenic pattern between TGEV virions and reconstituted virosomes as analysed with BIAcore. Anti TGEV S (3b.5) MAb was used for capturing native virions or reconstituted virosomes. The MAbs anti-sM (S2), -M (9.34, 25.22, 49.22) or -S (3b.5) were then added in independent runs. The amounts of bound MAb are expressed as resonance units (RU) for 100 RU of bound viral material. These data are representative of three experiments.

after extraction with NaDOC/NP40 was incubated for 3 h (1 h at room temperature and 2 h at 4 °C) with 300 mg of biobeads SM-2 (Bio-Rad), previously washed in methanol and NaCl/Hepes buffer. Another 300 mg beads were added for overnight incubation at 4 °C. The biobeads were removed by centrifugation for 4 min at 250 *g* through siliconized glass wool as described in Horigome and Sugano (1983). After the detergent (OG or NaDOC/NP40) was removed, a turbid suspension formed. The newly formed particules, called 'virosomes', were washed by repeated ultracentrifugations (100 000 *g* in the TL100.2 rotor for 1 h min at 4 °C) and resuspended in 0.5 mL of MEM. The virosomes (OG and NaDOC/NP40) were stored at –80 °C.

### BIAcore experiments

BIAcore (Pharmacia Biosensor, Uppsala, Sweden) allows the analysis, in real time, of the kinetics and stoichiometry of the interactions between molecules trapped sequentially on a miniaturized biocaptor (Johnsson and Löfas, 1991). The biocaptor consists of a hydrogel of dextran polymer. The amounts of interacting ligands are monitored by surface plasmon resonance; the signal is expressed in resonance units (RU): a variation of 1 000 RU corresponds to a variation of immobilized or trapped material of 1 ng/mm$^2$.

Purified rabbit anti-mouse (RAM) IgG Fc immunoglobulins (RAMFc; Pharmacia) were first covalently linked to the dextran chips. For all experiments, a flow rate of 5 µL/min was used in EDTA-free 150 mM NaCl, 10 mM HEPES buffer, pH 7.4. The 3b.5 anti S capture MAb (ascite fluid diluted 1:50 or 1:100) was injected at a volume of 25 µL. Then, TGEV-viral particles, TGEV reconstituted virosomes or control virosomes (generated from new-born calf serum) were injected, followed by 50 µL of mouse IgG (80 µg/mL) to block the unoccupied sites of the RAMFc. Then different MAbs directed against the three viral membrane proteins (as shown in figure 1) were injected independently as undiluted hybridoma supernatant to a volume of 30 µL. RAM was regenerated by 15 µL of 1 M formic acid.

### Leukocytes

Non-adherent porcine peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Ficoll density centrifugation on MSL® (density 1.077, Eurobio, Paris) followed by adherent cell depletion on tissue culture flasks as already described (Nowacki and Charley, 1993). PBMC were suspended in the RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) and antibiotics for IFN-α induction assays.

### IFN-α induction

PBMC were induced to produce IFN-α by overnight incubation at 37 °C with TGEV in 96-well microplates. Non-adherent cells were incubated at a final concentration of 4 x 10$^6$ cells per millilitre in a total volume of 200 µL with TGEV, virosomes, or both, at various dilutions as shown in *Results*.

In some experiments, PBMC (4 x 10$^6$/mL) were cultured with TGEV-infected cell monolayers overnight at 37 °C in RPMI plus 10% FCS. MAbs against TGEV S, M, sM as well as against TGEV receptor aminopeptidase N (APN) were added at various dilutions (see *Results*) in order to block the IFN-α induction by TGEV.

Cell supernatants were harvested and IFN-α levels were tested by titration using immunoassay.

### IFN-α immunoassay

A specific ELISA for porcine IFN-α was performed as previously described (De Arce et al, 1992; Splichal et al, 1994), using MAb K9 for coating, and peroxidase-conjugated F17 MAb as a second Ab. In each assay, our internal standard of recombinant porcine IFN-α was included.

## RESULTS

### Effect of anti-TGEV external protein-MAbs, and anti-TGEV receptor-MAbs on IFN-α induction

In order to evaluate the role of the recently described TGEV sM protein and TGEV receptor APN in IFN-α induction, blocking experiments with antibodies were performed. The IFN-α-inducing capacity of the infected fixed monolayers was not modified by addition to the cocultures with porcine PBMC of the anti-sM, anti-S or anti-APN MAbs (table I), but markedly blocked by addition of 49-22 and 25-22 anti-M MAb.

### Antigenic analysis of reconstituted TGEV virosomes

In order to determine the role of the TGEV envelope proteins in IFN-α induction, the viral membrane proteins were extracted with detergents and then envelope-like structures, referred to as virosomes, were reconstituted following detergent elimination. Such virosomes appeared as a slightly turbid suspension after detergent elimination and were pelletable by ultracentrifugation. The molecular composition and antigenic pattern of the virosomes were studied by surface plasmon resonance analysis using a BIAcore instrument. Preliminary studies done with MEGA10 as solubilizing detergent showed that the viral epitopes could be lost by detergent treatment (results not shown). This underlined the importance of using BIAcore analysis to select the proper detergent.

When both OG and NaDOC/NP40 virosomes were captured by the anti-S MAb 3b.5, epitopes of S, M and sM were detected on both virosome preparations as shown in figure 1. These results demonstrated that the virosomes were multiproteic structures (capture by anti-S and addition of anti-M, -S or -sM MAbs) including the three viral membrane proteins. In particular the two epitopes (25.22 and 49.22) described as being involved in IFN-α induction were detected on both virosome preparations. Furthermore the antigenic pattern of the reconstituted virosomes appeared identical to the one of the native or concentrated virions (fig 1).

### TGEV virosomes and IFN-α induction

Preliminary experiments in which detergent solubilized TGEV envelopes were reconstituted in the presence of exogenous lipids, such as azolectin (L-α-phosphatidylcholine, Sigma) showed the marked toxic in vitro effects of additional lipids on PBMC (data not shown). In the subsequent trials, the solubilized envelopes were therefore reconstituted without the addition of exogenous lipids.

In four independent experiments, OG- and NaDOC/NP40-prepared TGEV virosomes were assayed on porcine PBMC for IFN-α induction. No IFN-α induction was observed when using the virosomes in a range of dilutions from 1:3 to 1:81 (fig 2). Under the same experimental conditions, positive controls using equivalent amounts of native concentrated virus induced a strong IFN-α production (fig 2).

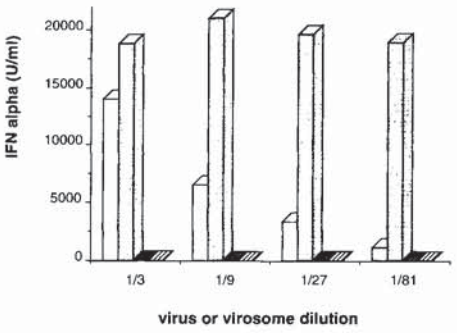


**Fig 2.** Induction of IFN-α by TGEV (□), concentrated TGEV (■) and virosomes (OG (▣) and NaDOC/NP40 (▨)). PBMCwere induced to produce IFN-α in an overnight incubation with the different viral preparations. Concentrated TGEV and virosomes were tested using roughly equivalent amounts of viral material. IFN-α levels in the supernatant culture was assayed by the specific immunoassay.

As shown in figure 3, the virosomes inhibited the IFN-α induction by native TGEV. In order to estimate the potential toxic effect for PBMC of any residual detergents left in the virosomes, PBMC were incubated with TGEV for 1 h before adding the virosomes at various dilutions, and the IFN-α levels were assayed in the supernatants after overnight induction. The results in figure 3a showed that the NaDOC/NP40 virosomes at a concentration of 2:10 had only marginal toxic effects on IFN-α induction, whereas the OG virosomes at the same concentration produced a 50% inhibition of IFN-α induction.

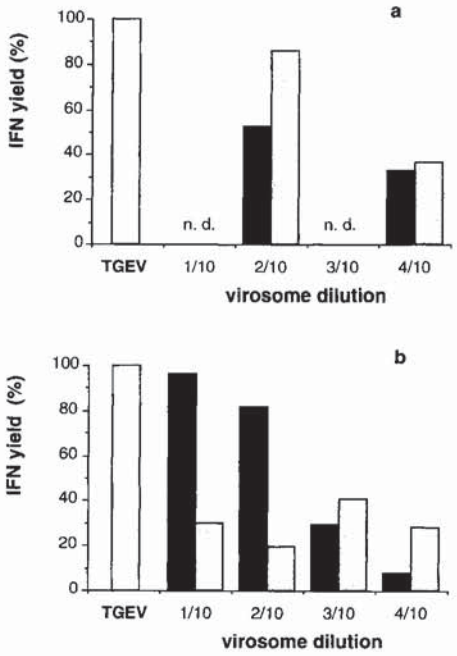


**Fig 3.** Inhibitory effects of the OG ( ■ ) and NaDoc/NP40 ( ■ ) virosomes on TGEV-induced IFN-α production. PBMC were induced to produce IFN-α in an overnight incubation with TGEV (fixed concentration) and increasing amounts of virosomes. IFN-α levels in the supernatant culture was assayed by the specific immunoassay. **a.** PBMC were pretreated with TGEV for 1 h at 37 °C before adding the virosome (OG or NaDoc/NP40). **b.** PBMC were pretreated with virosome (OG or NaDOC/NP40) for 1 h at 37 °C before adding the TGEV.

To determine whether the inhibitory effects of virosomes on IFN-α induction by native TGEV were due to a competition between virions and virosomes at the PBMC surface, the latter were incubated with the virosomes before adding the TGEV virions. The results in figure 3b showed that NaDOC/NP40 virosomes produced a strong inhibition of IFN-α induction even at the lower concentration tested (1:10). In the same experiment the OG virosomes showed a strong inhibitory effect on IFN-α induction but only at the higher concentrations (3:10 and 4:10).

## DISCUSSION

The present study was undertaken to determine the precise role played by the external viral glycoproteins in TGEV-induced IFN-α production following contact with porcine PBMC.

In our initial study on MAb-mediated inhibition of IFN-α induction, we demonstrated the inhibitory effects of two anti-M MAb (25.22 and 49.22) whereas anti-S MAb had no effect on IFN-α induction (Charley and Laude, 1988). More recently, additional MAbs were produced to a third TGEV external protein called sM (Godet et al, 1992), and to the TGEV receptor APN (Delmas et al, 1992). These MAbs were tested in IFN-α induction experiments with TGEV-infected fixed monolayers, and were shown in the present study to have no blocking effects. Our present data indicated therefore that the induction of IFN-α by non-infectious TGEV could be blocked by MAb to viral M protein, but not by MAb to the other external proteins S and sM. This argues against the TGEV external protein sM having a direct role in IFN-α induction. Even so, the only MAbs available were all directed against the C-terminal part of the protein whose orientation in the viral envelope remains still unclear. Considering the fact that APN is known to be expressed by murine and human leukocytes from the myeloid lineage (CD13), the finding that IFN-α induction could not be blocked by MAb to the TGEV receptor APN, supports the idea that the possible entry of

TGEV into porcine leukocytes through APN is not needed for inducing IFN-α. The mechanism by which non-infectious TGEV induced IFN-α in porcine PBMC seems therefore to differ from IFN-α induction by HIV for which the MAbs directed at the receptor (ie, the CD4 molecule) are shown to block the IFN-α induction (Capobianchi et al, 1992). Furthermore, in the HIV models the CD4 binding viral protein gp120 is shown to induce IFN-α (Capobianchi et al, 1992), whereas the TGEV receptor binding protein S does not seem to be involved in IFN-α induction (Charley and Laude, 1988).

To find out whether IFN-α induction was triggered by direct interaction between TGEV external proteins, presumably glycoprotein M, and a putative receptor on PBMC, the viral envelope proteins were reconstituted into virosomes following detergent extraction.

This methodology was previously described to reconstitute the fusogenic activity mediated by the viral spike protein of enveloped viruses such as the sendai virus (Harmsen et al, 1985), sindbis virus (Scheule, 1986), influenza virus (Nussbaum et al, 1987; Bron et al, 1993) and vesicular stomatitis virus (Metsikkö et al, 1986). Of crucial importance is the proper choice of the detergent which should not denature the membrane proteins. The use of non-ionic detergents such as octyl glucoside is therefore classically recommended (Harmsen et al, 1985). In the case of the mouse hepatitis coronavirus, however, the combination of NP40 and NaDOC is described to maintain specific interactions between S and M glycoproteins (Opstelten et al, 1995). Furthermore, the use of NP40 permits the reconstitution of the Sendai virus membrane particles which display IFN inducing capacity (Ito et al, 1978).

We chose to try to reconstitute TGEV virosomes using either octyl glucoside or the combination of NP40 with NaDOC.

The presence of the three TGEV external proteins, and in particular of the two epitopes characterized as being involved in IFN-α induction (25.22 and 49.22) was demonstrated in the reconstituted virosomes (OG and NaDOC/NP40) by the use of a BIAcore instrument. Despite the fact that the virosomes appeared to be multiproteic structures antigenically similar to native TGEV virions, they had lost their ability to induce IFN-α production following contact with PBMC. Furthermore such reconstituted virosomes could inhibit IFN-α induction by native TGEV. These inhibitory effects could be due to toxicity for PBMC of any residual detergent or to competition between the virosomes and virus for fixation on a putative receptor for IFN-α induction. In particular, at a low concentration (2:10), the NaDOC/NP40 virosomes exhibited only marginal inhibitory effects on IFN-α induction when they were added after the contact between TGEV virions and PBMC. In contrast, for a similar concentration, when they were put first into contact with PBMC they strongly inhibited the TGEV-induced IFN-α production. This discrepancy might indeed reflect the capacity of the NaDOC/NP40 virosomes to bind a putative receptor on PBMC. Such competition for a receptor on PBMC for porcine IFN-α induction was already suggested for the Aujesky Disease Virus (ADV) by Artursson (1993): when the ADV capacity to induce IFN-α was lost by UV treatment, these ADV preparations were still able to inhibit IFN-α induction by non-treated ADV.

The inability of the reconstituted TGEV virosome to induce IFN-α production despite its correct antigenic pattern could therefore indicate that the binding of a TGEV external protein, presumably the M protein, on NIP cells is not a sufficient triggering signal. Indeed IFN-α induction by HIV was shown to require at least two signals, one given by the CD4 binding site of gp120 and the other given by the V3 loop of gp120 binding to membrane-sulfatide-related glycolipids (Ankel et al, 1994, 1995).

After the envelope TGEV proteins were reconstituted into virosomes, they could compete with native virions for IFN-α induction via the first signal but they might have lost a specific superstructure (eg, association of two envelope proteins) during the detergent solubilization procedure. Recent studies have demonstrated the importance of the association between the sM

and M proteins in order to achieve the reconstitution of coronavirus-like particles by co-expression of viral envelope protein genes (Baudoux, 1996; Vennema et al, 1996). This association usually takes place during the budding of virions through cellular membranes to finally achieve the viral envelope (Vennema et al, 1996). A chemical reconstitution of a virosome by detergent removal might not therefore maintain such a specific association. In the case of TGEV the putative second signal might well be a superstructure formed by the association between the sM and M protein which may be lost in the virosomes.

From the data obtained in the present study and the recent data on coronavirus assembly, we propose that the induction of IFN-α by TGEV is mediated by interactions between viral envelope proteins, presumably M and sM, and putative receptors on NIP cells, which are clearly distinct from the TGEV receptor APN.

## ACKNOWLEDGMENTS

We thank C de Vaureix for providing us with the IFN-α reagents and A Ghazi (Orsay) for his helpful introduction to liposome methodology.

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