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Glycosylation is Required for Coronavirus TGEV to Induce an Efficient Production of IFNα by Blood Mononuclear Cells

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Porcine peripheral blood mononuclear cells (PBMC) are induced to produce interferon alpha (IFNα) following in vitro exposure to coronavirus TGEV (transmissible gastroenteritis virus)-infected glutaraldehyde-fixed cell monolayers or to TGEV virions. In the present report, we examined the possibility that glycosylation of viral proteins could play a major role in interactions with PBMC leading to the production of IFNα. Con A pretreatment of TGEV-infected cell monolayers before fixation with glutaraldehyde and exposure to PBMC caused a dose-dependent inhibition of IFNα induction, implying that masking of carbohydrates at the surface of infected cells lowered IFN-α-induction. Similarly, inhibition of N-linked glycosylation by tunicamycin during viral infection of cell monolayers altered their ability to induce IFNα. In addition, complete cleavage of 'complex type' oligosaccharides by peptide-N-glycohydrolase F lowered the capacity of TGEV virions to induce IFNα. Thus, these findings strongly suggest that glycosylation of the viral proteins, and more precisely the presence of complex-type oligosaccharides, is an important requirement for a completely efficient interaction with PBMC leading to the production of IFN-α.

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Various stimuli such as viruses, bacterial products or tumour cells can induce leucocytes to produce interferon alpha (IFNα) [7]. The demonstration that inactivated viral particles or glutaraldehyde-fixed virus-infected cells could induce IFNα [2, 4, 10, 12, 13, 17] led to the suggestion that the IFN-inducing capacity was not related to virus replication but could rather reflect direct membrane interactions between leucocytes and viral proteins present at the surface of viral particles or virus-infected cells [7, 17].

Studies conducted to characterize the nature of IFNα-producer cells (IPC) in response to such stimuli, indicated that IPC were infrequent but highly efficient mononuclear cells [3, 11, 23]. IPC could be characterized in several mammalian species as non-adherent, non-T, non-B cells, expressing MHC class II and CD4 molecules [5, 9, 18, 21, 22, 24].

In contrast, few reports are available about the nature of the viral component involved in membrane interactions with lymphocytes leading to the activation of IFN-α-coding genes. Thus, in the course of studies on IFNα induction by herpes simplex virus (HSV), Lebon [19] showed that monoclonal antibodies (McAbs) directed at the major viral glycoprotein D could inhibit HSV-induced IFNα production. These data suggested that a defined glycoprotein could play a crucial role in IFNα-induction. In the case of transmissible gastroenteritis virus (TGEV), a coronavirus which induces acute diarrhoea and intense IFNα production in newborn piglets [14] we conducted experiments in which McAbs directed at the three different viral structural proteins were added during *in vitro* exposure of leucocytes to infected cells. It appeared that IFNα-induction could be blocked by two of four McAbs directed at the transmembrane glycoprotein M (formerly named E1) whereas McAbs specific for the other struc-

tural proteins (N and S) had no effects [4]. More recently, a series of epitope virus mutants were selected on the basis of their resistance to the IFN-induction blocking McAbs. All the relevant amino acid substitutions identified by sequencing the mutant M genes were localized within the first 22 N-terminal residues of the molecule. Furthermore, two mutations were shown to markedly lower the IFNα-inducing ability of the corresponding viral mutants (Laude *et al.*, submitted for pulication). All these findings strongly implied that IFNα-induction by TGEV could result from interactions between leucocytes and a short N-terminal domain of the viral glycoprotein M. Interestingly, one of the mutations altering IFNα-induction affected the unique functional N-glycosylation site of the glycoprotein M (Laude *et al.*, submitted for publication). It was therefore tempting to postulate that carbohydrate moieties on the viral protein M could play a crucial role for inducing IFNα. This question was addressed by using three different approaches. (1) Because of its high capacity to bind to carbohydrates [1] Concanavalin A (Con A) was added to infected cell monolayers before addition of leucocytes. (2) TGEV-infected cell monolayers used to induce IFNα were pretreated with tunicamycin, an antibiotic which inhibits N-glycosylation [8]. (3) Purified viral particles were treated with glycosidases which cleaved N-linked oligosaccharides from the viral protein M, before addition to leucocytes for inducing IFNα. This report shows that the presence of 'complex type' carbohydrates on TGEV polypeptides is required for efficiently inducing leucocytes to secrete IFNα.

MATERIALS AND METHODS

Preparation of PBMC. Porcine peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood collected from 2–4-month old animals, by centrifugation on Ficoll (MSL, density 1.077, Eurobio, Paris, France). The PBMC were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and antibiotics.

Virus. As a source of TGEV, we used the cell-adapted Purdue 115 strain. Methods for propagation and titration were as described by Laude *et al.* [15].

IFN induction. PBMC were induced to produce IFNα by overnight incubation on TGEV-infected, glutaraldehyde-fixed cell monolayers as described previously [4]: briefly, pig kidney cells (PD5) were plated in 96-well microplates, infected by the coronavirus TGEV for 18 h, then fixed with 0.25% glutaraldehyde (1 h at 4°C) and stored with 3% glycine. Monolayers were washed before addition of PBMC (100 μl per well at 5×10^6/ml). Supernatants were collected after 18 h of incubation at 37°C and assayed for IFN activity.

Con A treatment of TGEV-infected cell monolayers. Pig kidney cell (PD5) monolayers, infected for 18 h, were washed once with PBS, then incubated for 1 h at 37°C with various amounts of Con A (Miles-Yeda, Israel) before being fixed with glutaraldehyde as described above.

Tunicamycin treatment of TGEV-infected cell monolayers. At 1 h post-infection, tunicamycin (Serva, Heidelberg, FRG) was added to the pig kidney cell monolayers at a dosage of 2 μg/ml for 18 h, until fixation by glutaraldehyde.

Glycosidase treatments. Endo-β-N-acetylglucosaminidase H (endo H), endo-β-N-acetylglucosaminidase F (endo F) and peptide-N-glycosidase F (PNGase F) were purchased from Boehringer-Mannheim-France (Meylan, France). Aliquots of 20 μl of purified TGEV suspensions (500 μg/ml) prepared as described by Laude *et al.* [15], with the addition of 3 μl of ^{35}S-labelled TGEV [6], were incubated with 20 μl of glycosidase and 20 μl of 20 mM sodium phosphate (pH 7.2), 50 mM EDTA solution, for 18 h at 37°C. The final concentrations of enzymes were 60 U/ml for glycosidase F, 15 U/ml for endo F and 0.3 U/ml for endo H. The resulting preparations were used to induce PBMC as follows: serial 10-fold dilutions of 10 μl aliquots were added to PBMC (5×10^6/ml) in a final volume of 0.2 ml for 18 h at 37°C. In order to ascertain the enzymatic cleavage, 6 μl aliquots of each preparation were subjected to electrophoresis on 10% polyacrylamide gels which were then processed for fluorography.

IFN bioassay. Log3 dilutions of PBMC supernatants were assayed for IFN on bovine MDBK cells using vesicular stomatitis virus as a challenge [14]. A standard porcine IFNα was included in each assay. This standard was calibrated on MDBK cells with the human international reference IFN B69/19 (NIH, Bethesda, MD, USA). In our results, 1 U is equivalent to 1 IU of human IFN.

RESULTS

Effects of Con A treatment of TGEV-infected cells on IFNα induction

PBMC exposed to TGEV-infected glutaraldehyde-fixed cell monolayers are induced to secrete IFNα: in the results shown in Table I, 5×10^6 PBMC per ml produced 1000 U/ml IFN following 18 h incubation at 37°C. In order to evaluate the role played by viral glycoproteins present at the surface of infected cells for the induction of IFNα, Con A was added to TGEV-infected cell monolayers before fixation with glutaraldehyde. Table I shows that Con A treatment causes a dose-dependent inhibition of IFNα-induction. Control experiments showed that uninfected cell monolayers, with or without Con A treatment,

TABLE I. Effect of Con A treatment of TGEV-infected cell monolayers on their ability to induce PBMC to secrete IFNα[1]

Con A concentration[2] (μg/ml)	0	0.5	5	50	500
IFN titres (U/ml)[3]	1000	300	30	>3	>3

1. Data representative of two experiments.
2. Various amounts of Con A were added to TGEV-infected pig kidney cell monolayers 1 h before glutaraldehyde fixation.
3. IFN produced by PBMC at 5×10^6/ml exposed to cell monolayers for 18 h.

did not induce PBMC to produce IFNα (not shown).

Effects of tunicamycin treatment of TGEV-infected cells on IFNα-induction

Pig kidney cell monolayers were treated by tunicamycin for 18 h following initiation of viral infection, then fixed with glutaraldehyde. Tunicamyin-treated cell monolayers induced PBMC to produce 10–100 times less IFN than mock-treated cell monolayers (Table II; four independent experiments). It was shown previously that tunicamycin (at 2 μg/ml) inhibited N-linked glycosylation with slight effects on the overall cell protein synthesis (Delmas and Laude, submitted for publication). Our present data indicate that tunicamycin markedly affects the ability of TGEV-infected cells to induce IFNα production.

TABLE II. Effect of tunicamycin treatment of TGEV-infected cell monolayers on their capacity to induce PBMC to secrete IFNα[1]

Tunicamycin (2 μg/ml) treatment[2]	PBMC concentration (10^6/ml)		
	2.5	5	10
−	1000[3]	9000	9000
+	100	300	1000

1. Data representative of four experiments.
2. TGEV-infected cell monolayers were treated or not with 2 μg/ml tunicamycin during 18 h, then fixed with glutaraldehyde.
3. IFN (U/ml) produced by PBMC exposed to cell monolayers for 18 h.

Effects of virus digestion by glycosidases on IFNα induction

In order to further analyse the role played by oligosaccharide moieties of TGEV proteins for IFNα induction, we examined the effects of pretreating virus particles by glycosidases before incubation with PBMC. Three endoglycosidases able to cleave N-linked oligosaccharides were used: endo H and endo F cleave predominantly high mannose oligosaccharide chains, whereas PNGase F cleaves both high mannose and complex-type saccharides [27]. Virus particles pretreated with endo H or endo F induced the same amounts of IFNα as control virions (Fig. 1). In contrast, PNGase F markedly decreased the virions' ability to induce PBMC production of IFNα: Fig. 1 shows that the same amount of PNGase F-treated virus could induce 10–100 times less IFNα than other viral preparations. SDS-polyacrylamide gel electrophoresis control analysis, performed on the mixture of cold and 35S labelled TGEV, showed that endo H and endo F caused a Mr shift of the major 29 kDa M protein species to 26 kDa, while 30–36 kDa species remained unaffected (Fig. 2). In contrast, PNGase F caused a complete carbohydrate cleavage leading to the presence of only one 26 kDa molecular species. The nucleoprotein N was not affected by digestion which indicates that proteolytic cleavages of viral polypeptides were unlikely to occur during glycosidase treatments. When

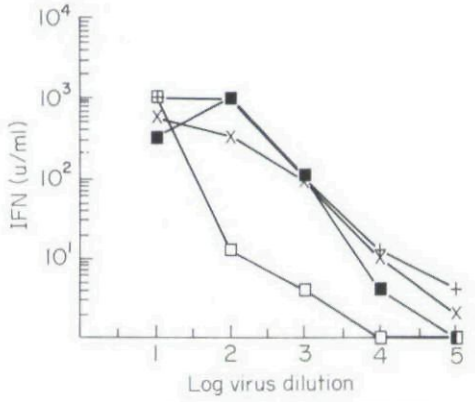

FIG. 1. Effects of glycosidase treatment of TGE virions on IFNα induction. Aliquots of virus particles were pretreated with PNGase F (□), endo F (+), endo H (×) or control medium (■) for 18 h. PBMC (2.5×10^6/ml) were induced to produce IFNα by incubation (18 h) with 10-fold dilutions of each virus preparation. (Data representative of four experiments.)

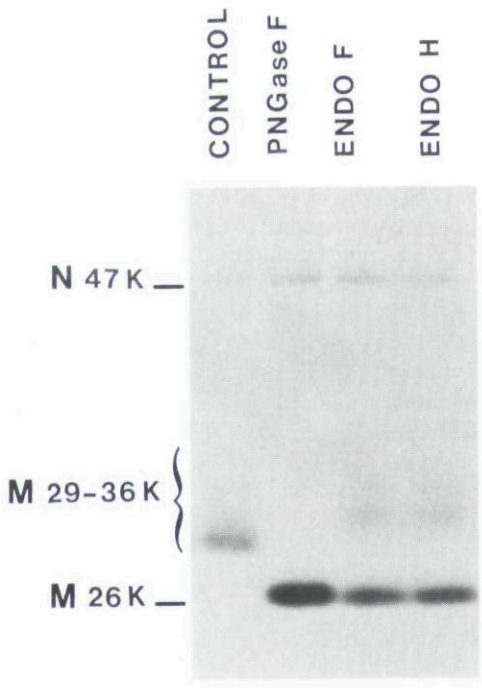

FIG. 2. SDS-polyacrylamide gel electrophoresis of control or glycosidase-treated virus particles.

aliquots of virus preparations were incubated with PBMC, a residual PNGase F activity might have directly acted on PBMC, thereby altering their ability to secrete IFNα. This did not appear to be the case since the induction of IFNα remained unchanged in a control experiment in which PNGase F was added to the virus just before incubation with PBMC (Fig. 3).

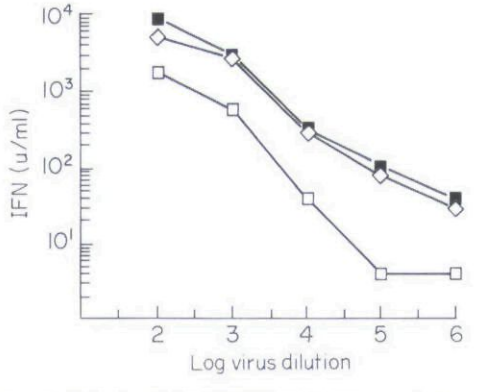

FIG. 3. Direct effect of PNGase F on virus-induced PBMC. PNGase F was incubated with virus particles for 18 hr at 37°C (□) or just before addition to PBMC (◇). Controls consisted of untreated virus particles (■).

DISCUSSION

The results of the present investigation indicate that glycosylation of coronavirus TGEV proteins is of major importance for induction of IFNα production by blood mononuclear cells.

The fact that potent IFNα induction is achieved by fixed cells, as already described [2, 4, 13, 17], strongly suggested that IFNα induction is independent of virus penetration into PBMC but could rather reflect direct interaction between membrane-associated viral antigens and PBMC membranes [7, 19]. In a first set of experiments, TGEV-infected cells were treated with Con A before fixation with glutaraldehyde: such treatments caused a dose-dependent inhibition in the ability of cell monolayers to induce PBMC to produce IFNα (Table I). Because Con A has a high capacity to bind to complex-type carbohydrates [26], these data imply that masking of carbohydrates present at the surface of infected cells will impede adequate interaction with PBMC leading to IFNα-production. However, the possibility that the Con A inhibitory effect might be due to steric hindrance of the relevant structure could not be excluded. In addition, Con A could bind to cell membrane glycoproteins as well as viral glycoproteins.

A more precise experimental approach consisted, therefore, in treating TGEV-infected cells with tunicamycin, an antibiotic which blocks N-linked glycosylation [8]. Such a treatment was previously shown to have slight effects on the overall cell protein synthesis and M and S polypeptides were produced with Mr corresponding to those predicted for the apoproteins (Delmas and Laude, submitted for publication). In our experiments, tunicamycin reduced the ability of infected cell monolayers to induce IFNα (Table II), which implies that inhibition of glycosylation in cell monolayers has negative effects on the expression of an 'IFNα-inducing-signal' at the cell surface. However, since tunicamycin can affect glycosylation of cell membrane proteins in addition to virus-coded proteins, it is not possible to determine from these data whether reduction of IFN-induction is related to the absence of carbohydrate moieties on viral or on cellular proteins. Moreover, tunicamycin could also block the transport of the viral apoproteins to the cell surface (see for example, Ref. 20).

For these reasons a last set of experiments was conducted in which virus particles were treated

with glycosidases before being incubated with PBMC in order to induce IFNα. Three enzymes were used: two glycosidases (endo H and endo F) cleave predominantly high mannose oligosaccharide chains whereas peptide-N-glycohydrolase F (PNGase F) cleaves both high mannose and 'complex-type' oligosaccharides [27]. Our results show that PNGase F was the only enzyme which could reduce TGEV virions ability to induce IFNα (Fig. 1). A control experiment showed that this reduction was not due to a direct effect of PNGase F on PBMC (Fig. 3). When treated virion aliquots were tritrated on PBMC (Fig. 1) it appeared that a reduced IFNα-induction was obtained with diluted PNGase F-treated viral preparations whereas high concentrations of PNGase F-treated virus (about 1.5 μg virions for 5×10^5 PBMC) induced as much IFNα as control, endo H- or endo F-treated virus. We have already reported strong evidence for the role of glycoprotein M as the effector viral molecule for IFNα-induction ([4]; Laude *et al.*, submitted for publication). It is therefore interesting to notice that PNGase F was also the only glycosidase tested which could cleave completely oligosaccharides from the viral protein M, leading to the production of a 26 kDa molecule (Fig. 2), corresponding to the size predicted for the apoprotein M [16] and found after tunicamycin treatment (Delmas and Laude, submitted for publication). Moreover, since PNGase F is able to cleave 'complex-type' oligosaccharides in contrast to endo H or endo F [27], our results indicate that only a complete cleavage of 'complex-type' oligosaccharides from the viral protein M alters the virus' ability to induce PBMC to produce IFNα.

What is the role(s) of carbohydrates, and more precisely complex-type oligosaccharides, present at the surface of virus particles or virus-infected cells, in the induction of IFNα? It is unlikely that carbohydrates alone are the 'IFNα-inducing signal': in fact, we show here that deglycosylated virus particles do induce IFNα when exposed at high concentrations to PBMC (Fig. 1). One possible explanation is that glycosylation of viral protein M is an important structural characteristic creating the adequate conformation required for it to interact with a putative receptor on PBMC membrane, leading to activation of IFNα genes. The need for adequate conformation for IFNα-induction was also suggested by our previous observation that a mutation on the glycoprotein M, which should cause important conformational changes, greatly impaired IFNα-induction (Laude *et al.*, submitted for publication). The existence of a membrane receptor involved in IFNα-induction was suggested by the results from Lebon [18] that IFNα-induction was sensitive to lysosomotropic drugs.

Alternatively, carbohydrate moieties of viral polypeptides may influence IFNα-induction by increasing the stability of complexes formed between virus particles or virus-infected cells and PBMC. With that respect, adhesion receptors termed 'selectins' were recently demonstrated on lymphocytes [25]: these molecules contain an N-terminal lectin domain potentially able to interact with carbohydrates. Cell-surface molecules equivalent to such 'selectins' could bind virus-associated carbohydrates, which in turn would facilitate stable interactions between PBMC and the actual 'IFN-inducing domain' of the viral polypeptide, leading to IFNα synthesis. Obviously it would be interesting to know whether carbohydrates are important for induction of IFNα by viruses other than TGEV, and which of the mechanisms we suggested (i.e., adequate conformation or adhesion requirement) is involved in the IFNα-induction process. A consequence of our findings is that synthetic peptides corresponding to the M protein or *Escherichia coli*-produced unglycosylated recombinant M proteins by themselves will be unlikely to efficiently induce IFNα, unless used at high concentrations. In addition, carbohydrates such as 'complex-type' oligosaccharides may be useful tools to probe the infrequent leucocyte subpopulation involved in the production of IFNα.

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