Interferon-induction in mouse spleen cells by the Newcastle disease virus (NDV) HN protein

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Summary — Newcastle disease virus (NDV) envelope glycoproteins that are expressed at the surface of fixed NDV (Ploufragan strain)-infected chick fibroblasts induce interferon (IFN) in mouse spleen cells. HN protein appears to be involved, since an anti-HN monoclonal antibody (Mab 3115) reduces the IFN production to 6% at most. However, the precise site of the molecule responsible for IFN induction is probably not exactly superimposed on the Mab 3115-epitope, since the NDV (83309 strain)-HN protein, which exhibits a modified Mab 3115-epitope, is also able to induce IFN. These preliminary results require further investigation in order to characterize the IFN herein demonstrated, to establish whether this induction mechanism exists in chicken lymphoid cells and to more accurately define the part of the HN molecule involved.

interferon / glycoprotein / Newcastle disease virus

Résumé — Induction d'interféron par la protéine HN du virus de la maladie de Newcastle dans les splénocytes de souris. *Les glycoprotéines d'enveloppe du virus de la maladie de Newcastle (NDV) qui sont exprimées à la surface de fibroblastes d'embryon de poulet infectés par le virus NDV (souche Ploufragan) puis fixés, induisent de l'interféron (IFN) dans les splénocytes de souris. La protéine HN apparaît impliquée, étant donné qu'un anticorps monoclonal anti-HN (Ac Mo 3115) réduit la production d'interféron à moins de 6%. Cependant le site précis, au niveau moléculaire, concerné dans l'induction d'interféron, n'est vraisemblablement pas superposé à l'épitope reconnu par l'Ac Mo 3115. En effet la protéine HN de la souche NDV 83309 qui présente un épitope correspondant modifié, est également capable d'induire de l'IFN. Ces résultats préliminaires requièrent des investigations complémentaires en vue de caractériser l'IFN présentement mis en évidence, d'établir si ce mécanisme d'induction existe chez les cellules lymphoïdes de poulet et de définir plus précisément la partie de la molécule HN impliquée.*

interféron / glycoprotéine / virus de la maladie de Newcastle

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INTRODUCTION

Interferons (IFNs) are part of the primary defense mechanism against viruses (Billiau, 1985) and it has been shown that human IFN-α administered in conjunction with the administration of infectious bovine rhinotracheitis (IBR), parainfluenza 3 (PI3), bovine viral diarrhea (BVD) vaccine in cattle, significantly stimulates specific antibody titres and enhances vaccine efficiency (Cummins, 1985).

On the other hand, myxoviruses and paramyxoviruses have been found to be inducers of IFN in murine and human lymphocytes, in the absence of genetic material and virus penetration (Ito *et al,* 1974, 1978, 1982; Dianzani *et al,* 1980). Moreover, the leucocyte IFN is now referred to as IFN-α (Steward *et al,* 1980; Interferon Nomenclature Committee, 1983) and it is now established that IFN-α is produced by murine, human and porcine lymphocytes following their interactions, in the absence of genetic material, with cell membrane-bearing viral antigens, such as influenza proteins (Reiss *et al,* 1984), herpes simplex virus (HSV) proteins (Capobianchi *et al,* 1985; Lebon, 1985), Dengue virus proteins (Kurane *et al,* 1986), and transmissible gastroenteritis coronavirus proteins (Charley and Laude, 1988).

Newcastle disease virus (an avian paramyxovirus) contains 2 envelope glycoproteins, F and HN. The former is involved in cell fusion, hemolysis and virus penetration, whereas the latter exhibits hemagglutinin and neuraminidase activity (Scheid *et al,* 1972; Scheid and Choppin, 1973, 1974). Further evidence indicates that the HN protein might be involved in interferon induction in mouse lymphoid cells. In fact, Youngner *et al* (1966) suggested a relation between the hemagglutinating activity of NDV and its ability to induce IFN *in vivo* in mouse plasma. Then Ito *et al* (1978) suggested that hemagglutinating activity might be closely related to interferon induction in mouse spleen cells by separated Sendai virus (hemagglutinating virus of Japan: HVJ) glycoproteins, whereas hemolytic and neuraminidase activities did not appear to be essential. Finally, Ito *et al* (1982) demonstrated that NDV strains with uncleaved Fo, characterized by a lack of haemolytic and cell fusion activity, were also able to induce IFN in mouse spleen cells.

Since in the future, recombinant F, HN or F + HN NDV vaccines will be available (Wemers *et al,* 1987; Boursnell *et al,* 1990; Edbauer *et al,* 1990; Nagy *et al,* 1990; Ogawa *et al,* 1990), it is of interest to know whether these recombinant proteins are able to induce IFN-α, and if so to what extent; or if not, the consequences in view of other vaccines' efficiency and protection against viral diseases.

The purpose of the present preliminary experiment was to demonstrate by the means of monoclonal antibodies that the HN protein of NDV was able to induce IFN in mouse spleen non-adherent cells and to partially localize the region of the protein involved.

MATERIALS AND METHODS

Viruses

The following NDV strains were used: the velogenic French challenge Ploufragan strain and a mesogenic pigeon isolate referenced as 83309, the characteristics of which have been detailed previously (Jestin *et al,* 1989). This strain does not bind to Mab 3115 because its corresponding epitope 3115 is modified. Both 2 strains belong to our laboratory collection; all of them were grown and titrated in the allantoic fluid of 9–10-day-old specific pathogen-free (SPF) eggs.

Cell cultures

Primary chick embryo cell monolayers (fibroblasts: CF) were prepared from 9–10 day-old SPF embryos (Hitchner *et al,* 1975). The cells were seeded with 9 x 10^5 cells/ml. They were then grown in a BHK_{21} medium supplemented with 2% calf serum, and used 24 h later.

Spleen cells were prepared from C57/Bl SPF 6–15-week-old mice (Iffa Credo-Lyon) as previously described (Ito *et al,* 1973, 1974). Several concentrations of spleen cell suspensions were tested, so that the fibroblast/spleen cell ratio varied from 0.1–0.025. The separation of spleen cells into glass-adherent (macrophages) and non-adherent cells was adapted from the technique of Ito *et al* (1973). The spleen cells were distributed into glass tissue-culture dishes, and then incubated for 2 h at 37 °C. The non adherent cells were subsequently counted, then ready for use.

L-929 cells were grown in DMEM medium, supplemented with 20 mM HEPES and 10% foetal calf serum. They were seeded with 4 x 10^4 cells per well in 96-well plates for IFN assay.

Infection of CF cells with NDV and fixation with glutaraldehyde

Infection was performed by adsorbing NDV strains for 30 min at 37 °C. Several multiplicities of infection (moi) expressed as egg infective doses (EID_{50})/cell and incubation times having been tested, in order to obtain maximal haemadsorption, incubation time was defined as 16 h and moi as 3.2 using the Ploufragan strain; with the 83309 strain, the time of infection was 27 h with a moi of 0.3.

NDV-infected and control CF cells were fixed using glutaraldehyde as previously reported (Lebon, 1985; Charley and Laude, 1988).

Haemadsorption assay with NDV-infected glutaraldehyde-fixed CF cells

The method of Alexander *et al* (1973) was applied.

Interferon induction in vitro

Spleen cells were added to triplicate glutaraldehyde-fixed CF cells in such a way that several ratios of CF/non adherent spleen cells were evaluated. They were incubated for 19 h at 35 °C. Then the cell culture supernatant was collected. During the first experiments, the supernatant was dialyzed against 0.01 M HCl pH 2 at 4 °C for 48 h then brought to pH 7.2 by dialyzing against PBS at 4 °C for a further 24 h. They were clarified by centrifugation at 2 000 *g* for 10 min and ultracentrifuged for 1 h at 4 °C at 100 000 *g*. Later, the steps of dialyzing and ultracentrifugation were omitted. When specified, the samples were concentrated before IFN assay using centricon 10 (Amicon) according to the manufacturer's instructions.

Finally, a CF/non adherent spleen cells ratio of 0.05 was selected and IFN induction assays were duplicated.

IFN assay

The assay involved protection of L cells against infection with vesicular stomatitis virus (VSV) as assessed by inhibition of cytopathic effect. The method described by Su *et al* (1990) was applied, except that the L cells were inoculated with 35 tissue cell infective dose 50 ($TCID_{50}$), and the titrations were directly scored microscopically. The highest dilution of the titrated sample producing 50% protection of the cells was taken as the endpoint. A laboratory standard murine IFN was included in each assay. This standard was calibrated with a murine international reference (National Institute of Health) α/β IFN obtained from the Pasteur Institute (Paris). Our results were expressed as IU/ml and corresponded to the mean of 2 repetitions.

Assay for virus isolation from NDV-infected glutaraldehyde-fixed CF cells

In order to control the absence of live virus following CF cell fixation with glutaraldehyde, the cell culture supernatant collected after incubation of spleen cells with NDV-infected glutaralde-

hyde-fixed CF cells was inoculated into the allantoic cavity of 9–10 day old specific pathogen-free (SPF) hen eggs. Three passages were performed every 3–4 days, the eggs were candled daily, and the hemagglutination activity (HA test) of the amnio-allantoic fluids collected every 3–4 days was tested (Alexander, 1988).

Effects of antibodies on IFN induction

The preparation and characteristics of the Mabs listed in table II have been previously described (Jestin *et al*, 1989). Briefly, Mabs 3115, 1063, 1062, 3048 are anti-HN specific, whereas the protein specificity of Mabs 1035 and 2114 is doubtful: the former reacts in Western blot with a 150–200 kDa protein; the latter is thought to recognize an internal protein (Jestin *et al*, 1991). The NDV reference serum was prepared in 7-week-old SPF chickens vaccinated and then challenged with the Ploufragan strain. Its titre as assessed by the hemagglutination inhibition (HI) test (Alexander, 1988) was 320. The negative control serum was obtained from SPF chickens. Its HI titre was < 5. NDV-infected CF cells were incubated for 30 min at 37 °C, with the Mabs and sera mentioned above, having been previously heated to 56 °C and diluted 1/20 and 1/10 in PBS respectively. They were then fixed with glutaraldehyde and used as previously described.

In addition, the effect of Mab 3115 on IFN induction was also investigated by performing Mab 3115 incubation after glutaraldehyde fixation; apart from this, conditions were the same.

Effect of Mab 3115 on IFN titre

In order to verify that Mab 3115 did not exert any anti-IFN activity, titre of mouse (internal reference) IFN was measured following incubation with Mab 3115, diluted 1/20, and compared with that of reference IFN, similarly incubated with negative ascitic fluid on the one hand, and PBS on the other.

RESULTS

NDV-infected fibroblast cells were fixed when haemadsorption was maximal and the IFN titres induced by these cells were measured (table I). The level of IFN produced was equivalent to the IFN titre shown by mouse spleen cells stimulated by live NDV (63 UI.ml^{-1} in unconcentrated spleen cell supernatant, using spleen cells seeded at 5 x 10^6 cells/ml and a moi of 20). The IFN obtained was stable at pH 2 and was not pelleted following centrifugation of 100 000 *g* for 1 h at 4 °C. The IFN titres were the same (data not shown) when the dialyzing and ultracentrifugation steps were omitted. Moreover, it was also checked that no infectious NDV particle could be demonstrated from fixed NDV

Table I. IFN induced in mouse non adherent spleen cells by fixed NDV (Ploufragan strain)-infected chicken fibroblasts displaying maximal haemadsorption.

moi	*IFN titre (IU/ml)*	
	Assay a [a]	*Assay b* [a]
32	ND	35 [c]
3.2	18 [b]	40 [c]
0.6	35 [b]	ND

Controls < 4 and < 10 for assays a and b respectively : mouse non adherent spleen cells incubated with fixed uninfected chicken fibroblasts, and with fixed NDV-infected chicken fibroblasts incubated with medium. [a] Spleen cells were collected from 6- and 8-week-old mice for assays a and b respectively. [b] Cell supernatants having been concentrated 12 times as specified in *Materials and methods* before IFN titration, present results are thus divided by 12. [c] Unconcentrated.

(Ploufragan)-infected fibroblasts. Following 3 successive series of propagations in allantoic fluids of SPF eggs, all the embryos were normal and allantoic fluids gave negative HA tests.

The effect of anti-NDV Mabs was then investigated. As shown in table II, Mab 3115 and NDV specific antiserum appeared to drastically reduce IFN induction. This reduction of IFN induction was observed irrespective of the moment when Mab was incubated with fibroblasts, *ie* before or after glutaraldehyde fixation. Mab 3115 did not exhibit any anti-IFN activity (data not shown).

In order to evaluate the site of the HN protein involved in IFN induction, the same assays were carried out using fixed fibroblasts which had been infected by a NDV strain (83309) previously shown to lack the "3115" epitope. We found that the 83309 strain induced IFN at the same level as compared to the Ploufragan strain-infected fibroblasts (270 and 340 IU/ml respectively) in 7-times concentrated supernatant of stimulated spleen cell culture (mean of 2 repetitions).

Table II. Effect of antibodies against NDV proteins upon IFN induction of mouse non-adherent spleen cells, by fixed NDV (Ploufragan strain)-infected chicken fibroblasts.

Antibodies	*IFN yield (% of control)*
Negative ascitic fluid	100
3 115 [a]	≤ 6 [c]
1 062 [a]	50
3 048 [a]	62
1 063 [a]	62
1 035 [b]	62
2 114 [b]	100
Positive antiserum	≤ 6
Negative serum	88

[a] Anti-HN Mabs

[b] Mabs of doubtful specificity (see *Materials and methods*)

[c] ≤ 14% when Mab 3115 incubation was performed after glutaraldehyde fixation, otherwise antibody incubation was performed before glutaraldehyde fixation.

DISCUSSION

IFN was produced following contact of NDV infected and glutaraldehyde-fixed chicken fibroblasts with mouse spleen cells. However, the IFN level observed in this experiment appeared very weak. Ito *et al* (1978) found that low-titer IFN was produced in mouse spleen cells incubated with an appropriate concentration of Sendai spike samples, but these titers were not expressed in IU/ml. Using adenovirus fiber protein as inducer for IFN production in mouse spleen cells and the same conditions as the present study for measuring viral inhibitory activity, Tiensiwakul and Khoobyarian (1983) obtained IFN titres as high as 320 UI/ml (with a negative upper limit of 160 UI/ml). Moreover, mouse spleen cells are also regarded as poor IFN producers in comparison with human or pig blood lymphocytes (Charley, personal communication). Finally, under the conditions of the present study, live NDV did not induce higher titres; this is in agreement with the finding of Tiensiwakul and Khoobyarian (1983) comparing IFN production as a result of cell stimulation by live adenovirus or fiber protein.

The IFN in this study has not been characterized as IFN-α, despite filtration observations indicating that the present IFN likely had a molecular weight below 25 kDa, which is compatible with established data for murine IFN-α characterized as 18–20 kDa (Interferon Nomenclature Committee, 1983; Brehm and Kirchner, 1986).

Our results using monoclonal antibodies demonstrate that the HN protein is involved in IFN induction. The interferogenic site appears near the hemagglutinin because Mab 3115 strongly inhibits hemagglutin activity as well as IFN induction and does not inhibit neuraminidase activity, using *N*-acetyl neuramin lactose as substrate (Jestin *et al,* 1989). The negative results obtained with the other anti-HN Mabs used cannot be explained by poor binding with viral protein since they also have high antibody titres by ELISA. But it should be noted that the latter Mabs also exhibit poor ability to inhibit hemagglutinin activity (Jestin *et al,* 1989). To our knowledge, this observation has not previously been reported. Spatial localization of the 7 epitopes listed up to now on the HN protein (Iorio *et al,* 1989) are not available. Nevertheless, obtention of variants using Mabs exhibiting the same pattern of reactivity as described for MAB 3115 (Jestin *et al,* 1989) has been reported (Gotoh *et al,* 1988; Yusoff *et al,* 1988). Sequencing of these Mab-resistant mutants has shown amino acid substitution at positions 345 or 347 (Yusoff *et al,* 1988) or at position 495 (Gotoh *et al,* 1988). Thus, both 3115 epitope and "interferogenic" determinant might be located near the carboxy-terminus of the HN protein which is 571 to 616 aa long, depending on the strain (Sato *et al,* 1987; Gotoh *et al,* 1988). Previous papers have demonstrated that NDV HN protein, unlike most viral proteins, has its carboxy terminus exposed at the external surface of the viral envelope (Schuy *et al,* 1984) and is anchored to the envelope bilayer by its amino terminus (Mc Ginnes *et al,* 1987; Morrison, 1988).

If significant IFN production is demonstrated in the future following chicken lymphocyte stimulation by HN protein, it would be of interest to more accurately localize the "interferogenic" determinant. Although one NDV mutant lacking the 3115 epitope was shown, in the present paper, to induce as much IFN as the wild virus, it will be necessary to evaluate the IFN induction ability of several other Mab 3115-resistant mutants, in order to select and sequence the one(s) that would exhibit a defect in triggering IFN induction *via* their HN protein.

Finally, the role of IFN produced *in vivo*, after the exposure of chickens to recombinant HN protein, remains to be investigated. Whether it is of importance or not has to be considered in the future when employing NDV F or F/HN recombinant vaccine.

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