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Original article

**Specific cytotoxic lymphocyte response in swine
against structural proteins of transmissible
gastro-enteritis virus: a study using lymphoblastoid
cell line and recombinant vaccinia virus**

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Abstract – To determine the specificity, if any, of cellular cytotoxicity against transmissible gastro-enteritis virus (TGEV) infected cells, we developed a test using B lymphoblasts from a MHC histocompatible (d/d haplotype) cell line (L14), as stimulating and target cells. These cells were previously infected with recombinant vaccinia virus including different TGEV structural genes, either the spike (vS), membrane (vM) or nucleoprotein gene (vN). Lymphocytes from a TGEV immunized (d/d) swine developed a cytotoxic activity after secondary in vitro stimulation in the presence of vS, vM or vN infected L14 cells. The cytotoxic activity was induced and directed against the homologous vS and vM infected cells but no cytotoxic activity occurred at all against vN infected cells. While vM infected cells induced a cytotoxic activity against vM infected cells only, vS infected cells stimulated a cross-reactive cytotoxic activity against vM and vN infected cells in addition to that against vS infected cells. This latter cytotoxicity may be due to an increase in a non-specific background of Natural Killer or lymphocyte activated killer activity, which is seen also after coculture with wild type vaccinia virus (vW) infected cells. Thus these results are of practical importance in two respects. First, lymphoid B cell lines represent an excellent tool for determining which viral antigens are recognized by cytotoxic lymphocytes and second, they indicate the need to incorporate the M and S genes into a TGEV vaccine to induce cellular immunity against TGEV. © Inra/Elsevier, Paris

transmissible gastro-enteritis virus / cellular cytotoxicity / recombinant vaccinia virus / structural protein / pig

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Résumé – Activité cytotoxique des lymphocytes de porc contre les protéines structurales du virus de la gastroentérite transmissible : mise en évidence par utilisation de cellules lymphoblastoïdes de lignées infectées par différentes constructions recombinantes du virus de la vaccine. Afin de déterminer l'existence et la spécificité de la réaction lymphocytaire cellulaire-cytotoxique contre les cellules infectées par le virus de la gastro-entérite transmissible (TGEV) nous avons utilisé les cellules lymphoblastoïdes de la lignée B (L14, histocompatible d'haplotype d/d) et différentes constructions recombinantes du virus de la vaccine comprenant les gènes des protéines structurales tels que celui de la protéine des spicules (vS), de la membrane (vM) ou de la nucléoprotéine (vN) du virus TGE. Les cellules L14 ont tout d'abord été infectées avec l'une de ces trois constructions puis irradiées et mises en culture avec les lymphocytes de porc (d/d) préalablement immunisés par voie orale avec du TGEV. Après 5 j de coculture, les lymphocytes immuns ont développé une activité cytotoxique contre les cellules stimulantes infectées par les constructions vS et vM, mais pas contre celles infectées par la construction vN. Alors que la cytotoxicité engendrée par les cellules stimulantes vM+ était strictement restreinte aux seules cellules cibles comportant cette même construction vM, la cytotoxicité engendrée par les cellules stimulantes vS+ incluait une cytotoxicité supplémentaire vis-à-vis des deux autres cibles infectées par les constructions cibles vM+ et vN+. Cette cytotoxicité pourrait procéder d'une cytotoxicité non spécifique de type *Natural Killer* ou *Lymphocyte Activated Killer* que nous avons également observée après coculture des lymphocytes avec les cellules L14 infectées par la souche sauvage du virus de la vaccine. Ainsi ces résultats préliminaires illustrent l'intérêt des lymphoblastes B en lignée pour l'étude de la cytotoxicité cellulaire : ils montrent de plus l'existence d'une réponse cellulaire cytotoxique spécifique vis-à-vis des protéines M et S, qui doivent donc être incluses dans un vaccin contre la gastro-entérite transmissible. © Inra/Elsevier, Paris

virus de la gastro-entérite transmissible / cytotoxicité cellulaire / virus de la vaccine recombinant / porc

1. INTRODUCTION

Recombinant vaccinia viruses (rVV) have been gradually recognized as a general expression vector for stimulation of humoral and cell-mediated immunity to specified antigens. They show promise for the determination of target antigens of cytotoxic T lymphocytes (CTL) [4] and as potential vaccines against viral and protozoan diseases in medical and veterinary field [13]. In pigs, however, few fundamental studies have been carried out so far that elucidate the effect of rVV application except for a limited number of preliminary field trials [3, 9].

Pulford and Britton [17, 18] have developed rVVs expressing porcine transmissible gastro-enteritis virus (TGEV) antigens, of which the spike (S) protein is known to elicit neutralizing antibodies in the sow [10]. The role of

this protein in the induction of local cellular responses remains unclear but it seems likely that the protein can activate a cytotoxic T-lymphocyte response which occurs in gut-associated lymphoid tissues in animals naturally or experimentally infected with TGEV [21].

The purpose of this study was to examine the specificity of the cytotoxic activity, if any, of immune lymphocytes originating from the mesenteric lymph nodes of swine immunized by the TGEV.

2. MATERIALS AND METHODS

2.1. Isolation of lymphocytes from pigs

Two 2-year-old inbred MHC histocompatible (SLA, d/d haplotype) miniature sows were employed as lymphocyte donors. One pig #83116 was orally immunized twice, with 10^9

PFU of an attenuated TGEV Nouzilly strain [2], at an interval of 3 weeks. The other pig #83405 was kept uninfected (in a different location) as a control. The animals were killed and their lymphocytes were immediately purified from mesenteric lymph-nodes as previously described [19]. They were then cryopreserved in liquid nitrogen until being used for the cytotoxicity test.

2.2. Preparation of stimulating and effector cells

The cytotoxicity test was performed by using a lymphoblastoid B cell line (L14), established from a histocompatible d/d miniature pig [11] and infected cells were used as both stimulating and target cells. It has been shown previously that 100 % of L14 cells exhibit MHC class I and 10 % MHC class II [11], synthesize and express TGEV antigens in the cytoplasm and on the membrane following infection with rVV [22]. Three vaccinia recombinants were used in this study, vS, vM and vN expressing the spike (S), integral membrane protein (M) and nucleoprotein (N), respectively [17, 18]. The wild vaccinia virus strain (vW), used for producing the rVVs expressing the TGEV antigens, was used as a negative control of the recombinant strains. L14 cells were infected with each viral strain at 10 PFU/cell for 2 days. They were then gamma-ray irradiated at 3,000 rads to block their cell division, if they were to be used as a stimulating cell.

The lymphocytes recovered from cryopreservation were not spontaneously cytotoxic against the infected L14 cells (data not shown). In contrast, after a cell culture period in the presence of L14 infected cells at a lymphocyte/L14 cell ratio of 20:1 for 5 days at 37 °C, they exhibited cytotoxic activity.

Thus after this secondary in vitro stimulation with L14 cells, the activated lymphocytes were mixed with target cells. In one assay of cytotoxicity inhibition the lymphocytes were incubated with anti-CD18a, (H20A) kindly provided by Dr W. Davis (Washington State University, Pullman, WA, USA) at 1:100 final dilution of ascites.

2.3. Cytotoxicity assay

The target L14 cells were prepared in the same manner as for the stimulation but were not irradiated. Each target was labelled with $\text{Na}_2 \text{}^{51}\text{CrO}_4$ (NEN, specific activity 490.35 mCi/mg Cr) at a radioactivity of 10 microCi/ 10^6 cells for 1 h. The activated lymphocytes were mixed with ^{51}Cr -labelled L14 cells at different effector/target (E/T) ratios from 10:1 to 100:1, each in triplicate, for 4 h at 37 °C. Then, the supernatants harvested were counted for ^{51}Cr γ -emission in a scintillation counter (PACKARD 1600TR). Results were expressed as % cytotoxicity calculated by the formula:

$$\% \text{ cytotoxicity} = \{ [\text{mean cpm (experimental)} - \text{mean cpm (SR)}] / [\text{mean cpm (MR)} - \text{mean cpm (SR)}] \} \times 100$$

where spontaneous release (SR) is defined as the radioactivity released from target cells incubated in medium alone (no CTL) and maximal release (MR) as the cpm in the supernatants of target cells lysed with 5 % of triton X-100. In this study, SR levels ranged from 21 to 45 % of MR values.

3. RESULTS

3.1. Induction of vS cytotoxic activity

The results presented in *table 1* show that, when immune lymphocytes were cocultured with non-infected stimulated cells, there was only a background of non-specific cytotoxic activity (6–16 %). After their culture in the presence of the rvS infected cells, the immune lymphocytes exerted higher cytotoxicity (19–31 %) against the L14 target cells conveying vS than against the vW infected cells (10–12 %) or non-infected cells (13–17 %). In contrast, the MLN cells from the non-infected pig did not show any specific cytotoxic activity (data not shown).

3.2. Specificity of cytotoxic activity

To examine the specificity of the cytotoxic activity against the various structural proteins of TGEV, the lymphocytes of the same immunized pig were stimulated with L14 cells infected by three different rVV strains (vS, vM and vN) and the cytotoxic activity was tested against the same target cells (*table II*). When cultivated with vS infected cells, the lymphocytes developed higher cytotoxic activity (23–39 %) against L14 cells infected by the same recombinant virus (vS-infected) than that developed by the other recombinant viruses vM or vN infected target cells (14–26 % and 18–19 %, respectively). The net levels of the specific cytotoxicity to the TGEV S-protein, i.e. the difference between the cytotoxicity levels against vS and those against other strains, ranged from 5 to 13 % even though the background levels were relatively high. Thus, while higher activity was found against the homologous S antigen, there was some cross reactivity against the vM and vN infected cells. In contrast, a restricted cell cytotoxicity against vM infected cells was seen

after vM stimulation. No detectable cytotoxic activity was encountered after stimulation by vN infected cells against the same target rvN infected cells or the others.

3.3. Non-specific cytotoxicity using wild vaccinia virus

The observation of a cross-cytotoxic activity after stimulation by vS infected cells may suggest cross-reactive recognition by CTL of M and N proteins expressed on the L14 cells; however, this explanation was excluded due to the absence of known aminoacid homology between the S, M and N proteins. Thus the hypothesis remains that the recombinant S protein stimulates a level of non-specific cytotoxic activity due to this particular recombinant construction. Such non-specific activity was also seen when the lymphocytes were incubated in presence of L14 cells infected by the vW strain of vaccinia virus as seen in *table III*. The results show that lymphocytes developed cytotoxic activity against various target cells whether they were

Table I. Induction of specific cytotoxic activity of immune MLN lymphocytes against the vS infected L14 cells after coculture with the same infected cells (secondary stimulation).

Infected L14 cells ^a		% cytotoxicity at different E/T ratios		
Stimulant	Target	10:1	50:1	100:1
NI	NI	10 ^b	14	14
NI	vW	8	18	16
NI	rvS	6	15	16
rvS	NI	13	13	17
rvS	vW	10	12	12
rvS	rvS	19	27	31

^a L14 were infected by recombinant vaccine Spike (rvS), wild vaccinia virus (vW) or non-infected (NI).

^b The values represent the means of cytotoxicity percentages of two experiments at lymphocyte/L14 ratios of 10, 50 and 100:1.

Table II. Specificity of cytotoxic activities of MLN lymphocytes from TGEV immunized pigs after secondary *in vitro* stimulation by rVV infected cells

Infected L14 cells ^a		% cytotoxicity at different E/T ratios		
Stimulant	Target	10:1	50:1	100:1
rvS	rvS	23 ^b	31	39
rvSd	rvM	14	21	26
rvS	rvN	18	23	19
rvM	rvS	2	2	3
rvMd	rvM	17	20	20
rvMd	rvN	4	1	0
rvN	rvS	3	2	3
rvN	rvM	2	1	5
rvN	rvN	3	0	4

^a L14 cells were infected by different recombinant rvS, rvM and rvN and used either as stimulants or targets for lymphocytes of a swine immunized by TGEV

^b Values represent the means of cytotoxicity percentage triplicates.

infected or non-infected. Thus vW infection enhanced the cytotoxicity of the lymphocytes towards L14 cells non-specifically up to a maximum 42 % (*table III*), whereas lysis level against non-infected L14 cells after stimulation by rvS remained at less than 17 % for any E/T ratio (*table I*). This increased cytotoxicity was suppressed to or below the background level when the lymphocytes were incubated in the presence of a monoclonal antibody (H20A, anti CD18a) against the porcine leucocyte function antigen, LFA-1 (*table III*).

The MLN cells from the non-infected pig did not show any augmentation of non-specific lysis. Thus, coculture of immune lymphocytes with vW infected cells revealed the natural cytotoxicity of the immune MLN cells against rVV infected cells.

4. DISCUSSION

The present results clearly demonstrate that when used as a stimulating cells, vS infected L14 cells can activate an *in vitro* CTL response specific to TGEV S antigens in the porcine mesenteric lymph-node primed *in vivo* with TGEV. The activation of the specific cytotoxic response was evident at any E/T ratio, even though the net levels of specific cytotoxicity appeared low due to a relatively high non-specific background.

In addition to vS, the vM protein gene was also able to activate M protein specific CTLs, whereas the vN conveying N protein gene failed to develop CTLs specific to the N protein. Unlike the CTL response to the surface viral antigens (S and M proteins), the CTL specific to the intracellular antigen (N protein) might

Table III. Suppression of non-specific cytotoxicity of MLN lymphocytes by treatment with anti-Leucocyte function antigen-1 (LFA-1) antibody.

Infected L14 cells		% cytotoxicity at different E/T ratios		
Stimulant	Target	10 : 1	50 : 1	100 : 1
No treatment				
vW	NI	25	32	31
	vW	31	41	38
	rvS	33	35	34
	rvN	32	42	38
Antibody treatment ^a				
vW	NI	6	7	8
	vW	9	11	13
	rvS	5	4	14
	rvN	8	10	6

^a The lymphocytes were incubated with anti CD 18a (H20A, 1/100 final dilution of ascite) during vW-stimulation. Values represent the cytotoxicity means of cytotoxicity percentage triplicates.

have not been well primed in the MLN by TGEV immunization; however, mice immunized by hepatitis virus, another coronavirus, did not develop CTL against N protein but against S protein [5].

Non-specific lysis of non-infected cells as well as infected cells occurred after stimulation either with vW or vS infected cells, respectively; similar results have been described in pigs after stimulation by vW [15] or pseudorabies virus (PRV) infected cells [12, 23]; this non-specific lysis may be due either to non-specific activity of the effector cells (NK) or to lymphokine-activated killer cells [20], in conformity with our results of cytotoxicity inhibition by anti LFA-1, known to inhibit the NK activity [8]; spontaneous and non-MHC restricted cytotoxic activity, NK or LAK activity, is ascribed now to the activity of CD6-, CD2+ T cells [16]. Alternatively, since vS infected cells express vS protein at the membrane cell surface in contrast to the vM and vN proteins which remain in the

cell cytoplasm [17, 18]; there is potentially a greater number of T cell epitopes by the internal processing and by MHC class II association at the cell membrane; this suggestion is in agreement with recent results [1] showing a higher blastogenic response with S than M or N protein; thus it could be hypothesized that immune CD4 T cells are much more stimulated by this rVV construction and hence secreted much more interleukins, which in turn increases the background of LAK activity. Lastly, the S protein itself is known as an interferon inducer [6] which in turn increases NK activity [7].

Thus, our results fit with the view of specific killing with vM, but both specific and non-specific killing with rvS; however, further analysis of the cytotoxic effector cells, class I distribution on target cells after infection [12] and MHC restriction are needed to understand and define precisely the mechanisms of cytotoxicity.

This study did not consider whether these recombinants could prime the TGEV-specific CTL when inoculated into intact pigs. Bennink et al. [4] have reported that a rVV expressing the influenza virus haemagglutinin (HA) is not only able to stimulate HA-specific CTL responses in mice but also to prime them. If such an ability is also confirmed in these TGEV-recombinant strains, they could serve as a promising vaccine aimed at reinforcing mucosa-associated lymphocytes such as intraepithelial lymphocytes which exert cytotoxicity on virus-damaged infected epithelial cells [14].

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