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Interferon induction in porcine leukocytes with transmissible gastroenteritis virus

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ABSTRACT

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Leukocytes were harvested from the peripheral blood, mesenteric lymph node and small intestinal lamina propria from groups of three piglets before, and 1,2 and 3 weeks after infection with virulent transmissible gastroenteritis virus (TGEV) at 2 weeks of age. The donor piglets developed clinical signs of transmissible gastroenteritis which persisted for up to 3 days, and they developed peak serum titres of TGEV-neutralizing antibodies 2 weeks post-infection. The leukocytes were cultured in the presence of pokeweed mitogen (PWM), various dilutions of purified TGEV, or control media for 3 or 5 days, and the culture supernatants were tested for antiviral activity in MDBK cells challenged with vesicular stomatitis virus. The antiviral activity was characterized as porcine interferon (IFN)-α or porcine IFN-τ on the basis of its stability at pH 2.0 and neutralization by anti-human IFN-α antibodies. Viability of the leukocytes in culture, determined by trypan blue exclusion, was highest for the peripheral blood leukocytes and lowest for the mesenteric lymph node leukocytes. There were no consistent differences in antiviral activity between cultures incubated for 3 or 5 days. Porcine IFN-α was found in the supernatants of the leukocyte cultures stimulated with TGEV antigen, harvested before or after infection of the donor piglets with TGEV. Porcine IFN-τ was demonstrated in the supernatants of the leukocyte cultures stimulated with PWM, more frequently when the leukocytes were harvested post-infection. This was the first demonstration of IFN induction in vitro in leukocytes from porcine gut-associated lymphoid tissue.

INTRODUCTION

Transmissible gastroenteritis virus (TGEV) is a porcine enteric coronavirus which causes severe diarrhoea, associated with villous atrophy in the small intestine, and high mortality in newborn piglets (Bohl, 1989). La Bonnardière and Laude (1981, 1983) demonstrated high levels of interferon (IFN) early in experimental TGE in the blood and intestine of newborn piglets, and they suggested that the IFN might have arisen from the enterocytes in the small intestine. In subsequent studies however, La Bonnardière et al. (1986) identified the IFN as leukocytic IFN or porcine IFN-α, but the tissue source

of the IFN was not determined. It is well established that porcine IFN-α can be induced in peripheral blood leukocytes (PBL) by a variety of viruses (reviewed by Weingartl and Derbyshire, 1990) and in the case of TGEV it was shown by Charley and Laude (1988) that IFN induction was associated with an interaction of the viral transmembrane E1 or M glycoprotein with the PBL cell membrane. In a recent study, Charley and Lavenant (1990) characterized the blood mononuclear cells which produced porcine IFN-α in response to induction with TGEV as non-phagocytic, non-adherent, non-T, non-B, $CD4^+$ MHC-class-II-positive cells.

Few studies on the induction of porcine IFN-α in leukocytes from sources other than peripheral blood have been described, although Salmon et al. (1989) induced IFN in splenic lymphocytes with influenza virus. In an earlier paper, La Bonnardière and Laude (1983) described the production of IFN in porcine alveolar macrophage cultures in response to infection with TGEV. The in vitro induction of porcine IFN-α in leukocytes from the gut-associated lymphoid tissue (GALT) has not been described, but immunoreactive IFN-α was found to be widely distributed in normal human tissues, including spindle-shaped cells in the jejunal lamina propria (Khan et al., 1989).

Porcine IFN-τ has been less extensively studied than IFN-α. As in other species, porcine IFN-τ is a product of activated T-lymphocytes, and it was first induced by phytohaemagglutinin (PHA) in PBL by Yilma (1983). Subsequently, Piasecki (1988) showed that porcine IFN-τ from the same source exerted a synergistic antiviral action with porcine IFN-α. Charley et al. (1988) studied the antiviral properties of recombinant porcine IFN-τ, which was shown to have a protective effect on porcine cells challenged with TGEV, but the recombinant IFN was less protective than natural porcine IFN-α.

The induction of porcine IFN-τ in lymphocytes from the GALT has not been described, but there is evidence of cell-mediated immune (CMI) responses to TGEV in these tissues. The earliest study was by Frederick and Bohl (1976), who demonstrated the production of macrophage migration inhibition factor by leukocytes from the lamina propria (LP) of the small intestine of pigs infected with TGEV. Blastogenic responses to TGEV antigen were demonstrated in lymphocytes from Peyer's patches and mesenteric lymph nodes (MLN) by Shimizu and Shimizu (1979a), and by Welch et al. (1988), while Shimizu and Shimizu (1979b) showed that cytotoxic lymphocytes were generated in the Peyer's patches and MLN of pigs infected orally with TGEV.

The objective of the present study was to determine the ability of leukocytes from the porcine GALT to produce IFN-α or IFN-τ in response to stimulation with TGEV. Leukocytes were collected from the MLN and LP, as well as from the peripheral blood, from groups of piglets before and at intervals after infection with virulent TGEV. The leukocytes were cultured in the presence of TGEV antigen, and tested for the production of porcine IFN-α or

IFN-τ. Pokeweed mitogen (PWM) was also used as a non-specific inducer of porcine IFN-τ. The antibody response to the infection in the donor piglets was measured by a virus neutralization (VN) test.

MATERIALS AND METHODS

Antigen preparation

A stock of the Diamond Laboratories vaccine strain of TGEV was prepared by cultivation on monolayers of stable swine testis (SST) cells (McClurkin and Norman, 1966), which were frozen at $-70°C$ after incubation for 24 h. After thawing, the medium and cells were clarified by centrifugation at 5000 *g* for 20 min, and the supernatant, containing 10^7 median cell culture infectious doses per ml as determined by a microtitre infectivity assay on SST cells, was concentrated 15 times using the Minitan ultrafiltration system (Millipore Corporation, Bedford, MA 01730, USA) with 100 000 mw cut-off filter plates. The concentrated virus was purified as described by Welch and Saif (1988) on a discontinuous sucrose gradient, and the purified virus was used as antigen for the in vitro stimulation of leukocytes. A control antigen was prepared in a similar way from uninfected SST cell cultures.

Leukocyte separation

Leukocytes were isolated from heparinized blood by centrifugation on Ficoll-paque as described by Cepica and Derbyshire (1984a), except for the omission of monocyte removal. For the isolation of MLN leukocytes, the MLN was minced and the tissue forced through a 0.19 mm pore size stainless steel screen. The cells were pelleted and washed three times in Hanks' balanced salt solution (HBSS), and erythrocytes removed by hypotonic lysis. Leukocytes were separated from the LP of the jejunum as described by Ernst et al. (1987), by treatment with ethylenediamine tetraacetate followed by dispase, erthryocyte removal by hypotonic lysis, and centrifugation through 30% Percoll. Leukocytes from each of the above sources were finally suspended in RPMI-1640 (Gibco BRL Life Technologies Inc., Grand Island, NY 14072-0068, USA) supplemented with 15% foetal bovine serum (FBS).

Experimental piglets

Twelve 2 week-old piglets born to a specific pathogen-free (SPF) Yorkshire sow were used. They were weaned at 10 days of age. They were fed reconstituted evaporated cows' milk (Carnation milk) twice daily, and had continual access to water and to a commercial creep fed for piglets. Three piglets were killed for leukocyte collection at 2 weeks by an intravenous overdose of sodium pentobarbitone, at the same time the remaining 9 piglets were infected orally with 2 ml of a 20% suspension of small intestinal mucosa and contents. This was prepared as described by Ristic et al. (1965), from a SPF newborn

piglet which had been infected 48 h previously with virulent Purdue strain TGEV. Three piglets were killed at 1,2 and 3 weeks post-infection (pi). Food was withheld for 18 h before euthanasia to facilitate the collection of leukocytes from the lamina propria.

Interferon induction

Leukocyte cultures were prepared in 96-well flat bottom Falcon microtitre plates (Becton Dickinson Labware, Lincoln Park, NJ 07035, U.S.A.), with 100 μl of 10^6 cells ml^{-1} in RPMI with 15% FBS in each well. Each of three wells received 100 μl of one of four tenfold dilutions of TGEV or control antigen, from 10^{-1} to 10^{-4}. Four wells received 100 μl of a solution of PWM (Sigma Chemical Company, St. Louis, MO 63178-9916) containing 10 μg ml^{-1}. This concentration of PWM had been shown in a preliminary study to be optimal for the stimulation of a lymphocyte proliferative response in porcine leukocytes. A further four control wells were cultured only in RPMI plus 15% FBS. Two identical plates were set up for each source of leukocytes, and the supernatants were collected from one plate after incubation for 3 days at 37°C in 5% CO_2, and from the second plate after incubation for 5 days. Cell viability was determined by trypan blue exclusion when the cultures were initiated, and when the supernatants were harvested after 3 or 5 days.

Interferon assays

Leukocyte culture supernatants were tested for antiviral activity by the cytopathic effect (CPE) inhibition assay of Familletti et al. (1981), in MDBK cells challenged with vesicular stomatitis virus. The supernatants were tested undiluted, and recorded as positive if they inhibited CPE. For the differentiation of antiviral activity due to porcine IFN-α or IFN-τ, the supernatants of positive cultures of leukocytes from the blood, MLN and LP were pooled as follows. Pool number 1 included the leukocyte culture supernatants from the piglets killed at 2 weeks of age and stimulated with PWM, while pools, 2,3 and 4 contained the supernatants from the PWM-stimulated leukocytes from the piglets killed 1,2 and 3 weeks after infection respectively. Pool numbers 5 to 8 consisted of the supernatants from the leukocyte cultures stimulated with TGEV antigen 0,1,2 and 3 weeks after infection respectively. The pH of a portion of each pool was adjusted to 2.0 for 24 h at 4°C, and then readjusted to pH 7.0. Each treated and untreated sample was then titrated in the CPE inhibition assay, and the titre was recorded as the reciprocal of the highest dilution which inhibited the CPE. The same supernatant pools were also tested for neutralization of antiviral activity by anti-human IFN-α, which cross reacts with porcine IFN-α (La Bonnardière et al., 1986). Each supernatant pool was mixed with an equal volume of an equine polyclonal anti-human IFN-α antibody (Boehringer Mannheim Canada Ltd., Laval, Québec H7V 3Z9) containing 100 units of antibody, or with normal horse serum, and each mix-

ture, together with an untreated control sample, was titrated for antiviral activity in the CPE-inhibition assay as above. Neutralization of the antiviral activity of the porcine IFN was indicated by the presence of CPE.

Virus neutralization tests

Sera were collected from the piglets before infection, and when their leukocytes were harvested for IFN induction, and tested for TGEV neutralizing antibodies by a microtitre procedure in SST cells with 100 median cell culture infectious doses of the Diamond strain of virus.

RESULTS

Clinical findings

All the piglets were clinically normal before infection. The nine infected piglets showed clinical signs of TGE within 48 h of inoculation. Vomiting was followed by diarrhoea, which persisted for 2 to 3 days. The piglets remained alert, and since they continued to drink they showed little evidence of dehydration.

TABLE 1

Viability of leukocytes collected from piglets[1] before and after infection with TGEV, and cultured for 0,3 or 5 days

Weeks after infection	Source of leukocytes[2]	% viable leukocytes ($\bar{x} \pm SD$)		
		Day 0	Day 3	Day 5
0	Blood	89± 6	85± 8	79± 2
	MLN	89± 1	63± 6	47± 2
	LP	93± 2	75± 5	64± 5
1	Blood	89± 2	79± 1	79± 1
	MLN	89± 8	63± 3	55± 2
	LP	97± 4	75± 4	67± 9
2	Blood	85± 5	77± 3	72± 2
	MLN	89± 5	56±18	38± 7
	LP	96± 1	73±11	67±10
3	Blood	94± 1	73± 6	69± 9
	MLN	75±15	63±13	47± 3
	LP	94± 1	76± 8	69± 5

[1]Three piglets per group

[2]MLN, mesenteric lymph node; LP, lamina propria.

TABLE 2

Detection of antiviral activity in supernatants of leukocytes harvested from piglets[1] before and after infection with TGEV, and cultured with PWM or TGEV antigen

Weeks after infection	Source of leukocytes[2]	Days of incubation	Number of piglets responding to stimulation				
			PWM	Log_{10} dilutions of TGEV antigen			
				1	2	3	4
	Blood	3	0	3	3	2	1
		5	0	3	3	2	1
0	MLN	3	3	3	3	1	1
		5	2	3	3	1	0
	LP	3	0	2	2	1	0
		5	0	2	2	1	0
	Blood	3	1	3	3	3	1
		5	2	3	3	2	0
1	MLN	3	3	3	3	0	0
		5	2	3	3	1	1
	LP	3	1	3	3	2	0
		5	0	3	2	2	0
	Blood	3	2	3	3	1	0
		5	2	3	3	3	2
2	MLN	3	3	3	2	0	0
		5	3	3	3	0	0
	LP	3	3	3	3	0	0
		5	0	3	2	0	1
	Blood	3	2	3	3	3	0
		5	2	3	3	3	1
3	MLN	3	2	2	1	1	0
		5	2	2	1	0	0
	LP	3	1	2	1	0	1
		5	1	2	1	0	0

[1]Three piglets per group
[2]MLN, mesenteric lymph node; LP, lamina propria.

Serum antibody titres

The sera were all negative for TGEV neutralizing antibodies before infection. The mean log_2 VN antibody titres ($\pm$SD) 1,2 and 3 weeks after infection were 9.0 ± 1.7, 10.6 ± 0.6 and 9.3 ± 0.6 respectively.

Viability of cultured leukocytes

Leukocyte viabilities, determined by trypan blue exclusion, are given in Table 1. The initial viabilities were generally close to 90%. During cultivation, viability was maintained at the highest level by the PBL, and at the low-

TABLE 3

The effects of treatment at pH 2.0 on the antiviral activities of pooled supernatants from leukocytes collected before or after infection of piglets with TGEV and cultured in the presence of PWM or TGEV antigen

Pool[1] number	Weeks after infection	Stimulus	Antiviral titre	
			Pre-treatment	Post-treatment
1	0	PWM	400	0
2	1	PWM	100	0
3	2	PWM	200	0
4	3	PWM	200	0
5	0	TGEV	1600	800
6	1	TGEV	1600	1600
7	2	TGEV	800	800
8	3	TGEV	800	400

[1]The composition of each pool is described in Materials and Methods.

est level by the MLN leukocytes. The MLN leukocytes also showed the greatest variability in viability. There were no differences in the viability of leukocytes collected from the piglets before or after infection with TGEV.

Interferon induction

Antiviral activity was not found in the supernatants of leukocytes cultured in RPMI alone or in the presence of the control antigen. The detection of antiviral activity in the supernatants of leukocyte culture stimulated with PWM or TGEV antigen is summarized in Table 2. There were no consistent differences in antiviral activity between cultures incubated for 3 or 5 days. Before infection, PWM induced antiviral activity only in MLN leukocytes, but after infection of the donor piglets, leukocytes from all three sources produced antiviral activity in response to PWM, with the peak number of responses in leukocytes collected 2 weeks post-infection. Stimulation of the leukocytes with 10^{-1} or 10^{-2} dilutions of TGEV antigen resulted in a higher number of responses than when 10^{-3} or 10^{-4} dilutions of the antigen were used. The frequency of responses to the viral antigen was similar before and after infection of the donors with TGEV.

The effects of pH 2.0 treatment on the antiviral activity of the pools of supernatants from the leukocyte cultures are shown in Table 3. The antiviral titres of the supernatants from the cultures stimulated with TGEV were consistently higher than the titres of the PWM-stimulated culture supernatants, and the pH 2.0 treatment had no significant effect on these titres. Conversely, the antiviral activity produced in response to stimulation with PWM was abrogated by treatment of these pooled supernatants at pH 2.0.

Treatment of the pooled supernatants with anti-human IFN-α antibodies had no effect on the antiviral titres of pools 1–4, from the leukocyte cultures stimulated with PWM. However, the antiviral activities of pools 5–8, from the cultures stimulated with TGEV, were completely neutralized by the anti-human IFN-α antibodies.

DISCUSSION

The effects of pH 2.0 treatment and exposure to anti-human IFN-α antibodies on the antiviral activities of the leukocyte culture supernatants provided strong evidence that stimulation with TGEV induced porcine IFN-α, while stimulation with PWM induced porcine IFN-τ. It has already been shown that the transmembrane E1 or M glycoprotein of TGEV is a potent inducer of porcine IFN-α (Charley and Laude, 1988), and that porcine IFN-τ can be induced by T-cell mitogens (Yilma, 1983).

The finding that TGEV was able to induce porcine IFN-α in leukocytes from the LP was of interest since it suggests that these cells may be the source of intestinal IFN induced by TGEV in vivo (La Bonnardière and Laude, 1981; 1983). Porcine IFN-α induced in the LP would be highly accessible to the cryptal enterocytes, in which it might induce an antiviral state and hence contribute to recovery from the infection. Weingartl and Derbyshire (1991) demonstrated the antiviral activity of porcine IFN-α against TGEV in porcine intestinal explant and intestinal epithelial cell cultures. An indirect antiviral activity of porcine IFN-α induced in the LP might be mediated by activation of NK cells among the intraepithelial lymphocytes, which may contribute to resistance against TGE (Cepica and Derbyshire, 1984b). Conversely, porcine IFN-α induced in LP leukocytes could contribute to the pathogenesis of TGE by reducing the replacement rate of enterocytes, since it is well recognized that IFN may have an antiproliferative effect (Tamm et al., 1987), and Laude and La Bonnadière (1984) described a cytocidal effect of porcine IFN-α on porcine renal epithelial cells.

There was no evidence that in vitro stimulation with TGEV antigen induced porcine IFN-τ in this experiment, since no greater antiviral activity was detected in the culture supernatants of leukocytes harvested after infection than before infection of the donor piglets, and the antiviral activity was fully resistant to pH 2.0 treatment and completely neutralized by anti-human IFN-α antibodies. However, the donor piglets mounted a strong immune response as indicated by the appearance of high levels of circulating TGEV antibodies, and studies elsewhere have demonstrated sensitized T-lymphocytes in the LP (Frederick and Bohl, 1976) and MLN (Shimizu and Shimizu, 1979a; Welch et al., 1988) of pigs infected with TGEV. It is possible that small amounts of porcine IFN-τ in these culture supernatants may have been undetectable in the presence of an excess of porcine IFN-α, or that the latter

may have inhibited the proliferative response of sensitized T-lymphocytes upon exposure to antigen which is necessary for IFN-τ production. For the induction of porcine IFN-τ with TGEV antigen it would be prudent to exclude the highly interferonogenic M glycoprotein of the virus, and use purified S glycoprotein for in vitro stimulation,

Our findings demonstrated the ability of GALT lymphocytes to produce porcine IFN-τ in response to stimulation with PWM. The titres produced were relatively low, and higher levels might have been induced with phytohaemagglutinin, which is more frequently used for IFN-τ induction since it is a more potent T-cell mitogen (Kristensen et al., 1982). We used PWM because we were also attempting to stimulate antibody production in the leukocyte cultures (Naidoo, 1990), and PWM is mitogenic for B-cells as well as T-cells (Kristensen et al., 1982). It was of interest that more cultures responded to porcine IFN-τ induction with PWM when the leukocytes were harvested from piglets which had been infected with TGEV. The peak responses to PWM were in cultures containing leukocytes harvested 2 weeks post-infection, which corresponded to the peak antibody response to the infection. Welch et al. (1988) also found peak blastogenic responses to TGEV antigen with lymphocytes from the blood and MLN harvested between 6 and 14 days after exposure of the donors to virulent virus. In the present study, the increased responsiveness of the leukocytes to PWM after infection may have been due to a relative increase in the number of T-lymphocytes in response to the infection.

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