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Veterinary Immunology and Immunopathology, 18 (1988) 109-117
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

Activation of Natural Killer Cells in Newborn Piglets by Interferon Induction

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(Accepted 8 September 1987)

ABSTRACT

Lesnick, C.E. and Derbyshire, J.B., 1988. Activation of natural killer cells in newborn piglets by interferon induction. *Vet. Immunol. Immunopathol.*, 18: 109-117.

Natural killer (NK) cell activity in the peripheral blood lymphocytes (PBL) of newborn piglets, normally negligible, was stimulated by in vitro treatment with porcine type I interferon (IFN), and the NK activity of PBL from weaned piglets was augmented by the same treatment. Binding of the PBL to the PK-15 targets used in the single cell cytotoxicity assay for NK activity was not affected by age or by IFN treatment. When newborn piglets were treated with a single intravenous dose at 2 days of age of 0.5 mg/kg of polyinosinic: polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose (poly ICLC), a synthetic IFN inducer, their IFN levels peaked at 6 h post-induction, and NK activity in their PBL peaked at 24 h post-induction at the level normally found in weaned piglets. The NK activity then declined until 7 days post-induction, when it increased again in a similar manner to that in untreated control piglets. Target-binding of the PBL was not affected by poly ICLC treatment of the piglets. Newborn piglets treated with poly ICLC and subsequently exposed to infection with transmissible gastroenteritis (TGE) virus showed a delay in onset of clinical signs of TGE compared with untreated control piglets. It was concluded that NK cells in newborn piglets can be activated by treatment of the piglets with poly ICLC, and that the presence of active NK cells is associated with some increase in resistance to challenge with TGE virus.

INTRODUCTION

In previous studies in this laboratory, Cepica and Derbyshire (1984a) showed that natural killer (NK) activity against cells infected with transmissible gastroenteritis (TGE) virus was lacking in the peripheral blood lymphocytes (PBL) of newborn piglets. Subsequently it was found that the adoptive transfer of PBL from an adult pig to newborn piglets established NK activity in the latter, and increased the resistance of the newborn piglets to challenge with TGE virus (Cepica and Derbyshire, 1984b). These findings suggested that it might be useful to attempt to stimulate NK activity in newborn piglets shortly

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after birth, in the hope of increasing their resistance to neonatal infections such as TGE.

The capacity of interferon (IFN) to enhance the cytotoxicity of human and rodent NK cells is well established (Kimber, 1985), and augmentation of the NK activity of adult pigs by in vitro treatment of their PBL with porcine interferon (IFN) was demonstrated by Chung et al. (1982) and Charley et al. (1983). Furthermore, the in vitro treatment of PBL from newborn piglets with porcine IFN, prepared by cocultivation of PBL with cells infected with TGE virus, rendered the PBL cytolytic against both uninfected and TGE virus-infected PK-15 target cells (Cepica and Derbyshire, 1986).

An alternative approach to the stimulation of NK cells is the use of IFN inducers. Polyinosinic:polycytidylic acid (poly IC), a synthetic double-stranded RNA which is a potent inducer of IFN, was shown to enhance the NK activity of adult porcine PBL in vitro by Chung et al. (1982) and Pinto (1985), but this compound has not been used in vivo in pigs to stimulate NK cells. In the only report of attempted reconstitution of neonatal porcine NK activity in vivo, Kim (1985) injected newborn piglets experimentally with OK-432, a streptococcal immune-modulating preparation, and found that this treatment led to accelerated development of NK activity, although Kim believed that this effect was not mediated solely by IFN induction.

Poly IC was used as an IFN inducer in swine by Vengris and Maré (1972) and Gainer and Guarnieri (1985), and in newborn piglets by Loewen and Derbyshire (1986). Newborn piglets were found to be more resistant than older animals to poly IC, and in subsequent studies poly IC complexed with poly-L-lysine and carboxymethylcellulose (poly ICLC) was shown to be a more effective IFN inducer than poly IC alone in newborn piglets (K.G. Loewen, personal communication, 1987). The main objective of the present study was to attempt to activate the NK cells of newborn piglets in vivo by IFN induction with poly ICLC. This was successfully accomplished, and a secondary objective was to determine whether piglets treated with poly ICLC were more resistant to challenge with TGE virus than untreated piglets. The in vitro activation of neonatal porcine NK cells by treatment with serum containing IFN is also recorded.

MATERIALS AND METHODS

Preparation and administration of poly ICLC

Poly ICLC was prepared by the method of Levy et al. (1975) with minor modifications. Briefly, poly IC (Miles Scientific, Naperville, IL), reannealed by heating at 71°C for 1 h and allowed to cool slowly, was added to a mixture of equal volumes of 3 mg/ml of poly-L-lysine (Sigma Chemical Co., St. Louis, MO) in normal saline and 1% carboxymethylcellulose (Sigma Chemical Co.,

St. Louis, MO) to give a final concentration of poly IC of 1 mg/ml. The resulting complex (poly ICLC) was stored at 4°C. Piglets were inoculated intravenously with poly ICLC at a dosage of 0.5 mg/kg of poly IC within 2 days of birth. Four litters of crossbred Yorkshire piglets were used, from a specific pathogen-free herd which lacked virus neutralizing antibodies to TGE virus. The sows farrowed in isolation, and suckled their piglets for the duration of each experiment. Three litters were used to determine the effect of poly ICLC treatment on NK activity. Approximately half the piglets in each litter were randomly selected for treatment, with the remainder as untreated controls. The fourth litter was used to determine the effect of poly ICLC treatment on the response to challenge with TGE virus, as described below.

Assay of NK activity

At appropriate intervals the piglets were anaesthetized with halothane (Somnothane: Hoechst Canada Inc., Montreal, Que.) and 30 ml of blood were collected by cardiac puncture, after which each piglet was killed. PBL were isolated and purified by centrifugation over Ficoll–Hypaque, with removal of monocytes by plastic adherence as described by Cepica and Derbyshire (1984a). NK activity of the PBL was determined by the single cell cytotoxicity assay (SCCA) previously described by Cepica and Derbyshire (1986), based on the procedure of Grimm and Bonavida (1979), utilizing PK-15 cells as targets. The percentage of target-binding lymphocytes was recorded, based on at least 200 cells, and NK activity was determined by recording the percentage of 500 lymphocyte-target cell conjugates with non-viable target cells (% cytotoxicity). The significance of differences between groups of treated and control piglets was determined by the *t*-test. For determination of the in vitro effect of IFN on NK activity, PBL from six newborn and six weaned piglets were incubated for 18 h at 37°C with pooled serum collected 6 h after inoculation of newborn piglets with poly ICLC as described above, diluted to contain 320 antiviral units of IFN/ml, or with a similar dilution of fetal bovine serum as a control. After incubation the PBL were washed twice and then used in the SCCA. The significance of differences between the mean % cytotoxicity of IFN-treated and control PBL was determined by the *t*-test.

Assay and characterization of interferon

Sera were collected for IFN assay from all piglets 6 h after treatment with poly ICLC, and at the same times that PBL were collected for the SCCA. The IFN titres in the sera were determined by a plaque inhibition assay in Madin–Darby bovine kidney cells challenged with vesicular stomatitis virus as described by Loewen and Derbyshire (1986), and recorded as the reciprocal of the highest dilution of serum which inhibited at least 50% of 30–40 plaque-

forming units of virus. The antiviral activity of selected representative sera was characterized as IFN according to standard criteria (Lockhart, 1973), and found to be abolished by trypsin treatment, partially susceptible to heat, and resistant to pH 2.0 and ultracentrifugation, consistent with the properties of type 1 (alpha/beta) IFN.

Challenge with TGE virus

Five piglets were inoculated intravenously with 0.5 mg/kg of poly ICLC at 2 days of age, and seven litter-mates were left untreated. Six hours later, two of the untreated piglets were dosed orally with 1.5 ml of a 20% suspension of small intestinal mucosa and contents, prepared as described by Ristic et al. (1965), from a specific pathogen-free piglet which had been infected 48 h previously with the Purdue (Doyle and Hutchings, 1946) strain of TGE virus. The purpose of these two piglets was to serve as a source of virus for contact transmission to the remainder of the litter. They showed clinical signs of TGE on the following day, and they were killed on day 4, when TGE had appeared in the other piglets. All the piglets were examined clinically twice daily, and weighed once or twice daily, since rapid weight loss is a sign of TGE (Saif and Bohl, 1986), for the duration of the experiment, which was terminated when the piglets were 10 days old. Fresh water was provided for the piglets twice daily from the third day of life, when diarrhoea began.

RESULTS

In vitro stimulation of NK activity

As shown in Table 1, there were no significant differences in conjugate formation in the SCCA between neonatal and weaned piglets, and treatment of the PBL with IFN-containing serum had no significant effect on conjugate formation. The control PBL from the newborn piglets showed virtually no NK activity, but the neonatal porcine PBL were activated by treatment with IFN

TABLE 1

The effect of in vitro treatment with IFN on conjugate formation and NK activity of PBL from six neonatal and six weaned piglets

Piglets	% Conjugation[a]		% Cytotoxicity[a]	
	Control	Treated	Control	Treated
Neonatal	16.9 ± 1.7	16.7 ± 1.6	0.4 ± 0.1	5.5 ± 0.4
Weaned	16.7 ± 0.8	17.1 ± 0.5	6.2 ± 0.3	9.9 ± 0.3

[a]Values shown are means ± standard deviations.

($P<0.05$). The NK activity of the PBL from the weaned piglets was also significantly ($P<0.05$) augmented by treatment with IFN-containing serum.

In vivo stimulation of NK activity in newborn piglets

IFN assays were done on the sera of all piglets 6 h after treatment with poly ICLC. All the treated piglets had IFN titres, which ranged from 80 to 640 units/0.4 ml (mean 246 units/0.4 ml). IFN was not detected in any of the control piglets. Three poly ICLC-treated and three untreated control piglets were tested for IFN and NK activity at each of the times shown in Fig. 1, except at 12 and 16 days of age when single treated and control animals were tested. The mean titres of IFN at 12 h and 24 h after treatment were 160 units/0.4 ml and 53 units/0.4 ml, respectively. No IFN was found in the other sera tested except at low titres (10–20 units/0.4 ml) in control piglets at 10 and 14 days of age. NK activity (Fig. 1) was demonstrated in the PBL of the treated piglets 12 h post-induction, and peaked at 1 day post-induction. The level of NK activity in the treated piglets then declined until 7 days post-induction to a level similar to that found in the untreated controls at 9 days of age. NK activity was significantly ($P<0.05$) higher in the treated piglets than in the untreated controls from 12 h to 5 days post-induction. From 7 days post-induction (9

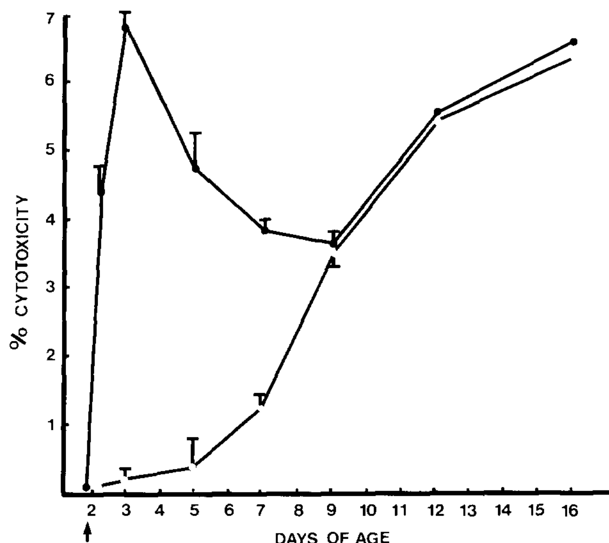

Fig. 1. Natural killer cell activity in the single cell cytotoxicity assay (% cytotoxicity) in piglets treated with 0.5 mg/kg poly ICLC (●) at 2 days of age (arrow), and in untreated control piglets (○). The points indicate mean % cytotoxicity, and standard deviations are shown by vertical bars. Three poly ICLC-treated and three untreated control piglets were tested at each time, except at 12 and 16 days of age, when single treated and control animals were tested.

days of age) to the end of the experiment, NK activity in the treated and control piglets increased at a similar rate, and at 16 days of age the levels of NK activity were similar to those described above for weaned piglets. Conjugate formation remained at a similar level to that found above in neonatal piglets throughout the experiment, and there were no significant differences in conjugate formation between the treated and control piglets.

Response to challenge with TGE virus

The mean body weights of the piglets in this experiment are given in Fig. 2. The poly ICLC-treated piglets lost weight during the 6-h period following treatment, when they were dull and listless, and did not feed, but they had recovered by 8 h post-induction, and gained weight during the following 24 h. Diarrhoea began in all the untreated control piglets on day 3, within 24 h of contact exposure to the two piglets which were dosed orally with TGE virus. During this day the untreated piglets showed considerable weight loss (Fig. 2) and all of them had rather watery diarrhoea. At this time the treated piglets remained clinically normal and continued to gain weight. Signs of TGE were not detected in any of the treated piglets until 18 h later, on day 4, when they

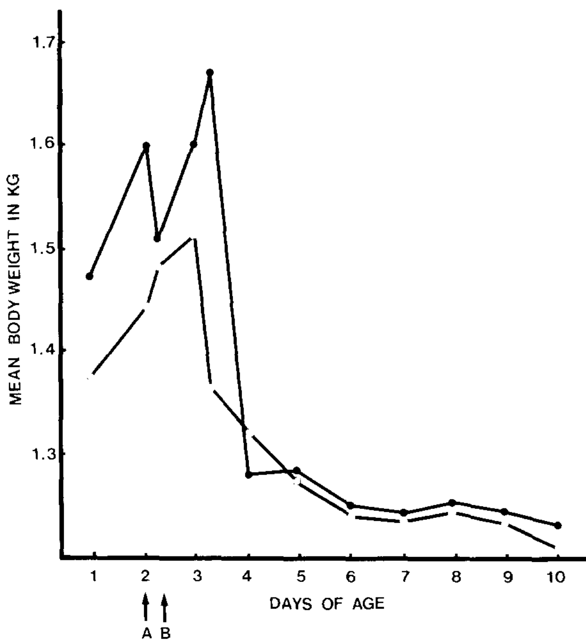

Fig. 2. Mean body weights of five piglets treated with poly ICLC (●) at 2 days of age (arrow A), and five untreated control piglets (○), exposed to infection with transmissible gastroenteritis virus 6 h later (arrow B).

all showed watery diarrhoea and a dramatic loss in body weight (Fig. 2). For the remainder of the experiment the treated and control piglets were clinically similar. All had persistent, watery diarrhoea, and they failed to gain weight. One treated piglet died on day 10, when the remaining piglets were killed. At post-mortem examination the carcasses of the control and treated piglets were indistinguishable. All showed dehydration and cachexia, with fluid contents throughout the intestinal tract.

DISCUSSION

The in vitro activation of PBL from newborn piglets by incubation with serum from piglets treated 6 h previously with poly ICLC was probably due to the presence of IFN in the serum. Any residual poly ICLC in the serum would be well below the concentration found by Chung et al. (1982) to be required for direct activation of the PBL by poly IC. This finding extended the earlier observation of neonatal NK activation by the treatment of PBL with IFN prepared by cocultivation of PBL with TGE virus-infected cells (Cepica and Derbyshire, 1986). The demonstration of in vitro neonatal porcine NK cell activation was also in agreement with the activation of PBL from newborn piglets with human IFN by Charley et al. (1985), although it contrasted with the failure of Kim and Chung (1985) to reconstitute NK activity in neonatal porcine PBL with human IFN. The augmentation by IFN of the NK activity of PBL from weaned piglets which we observed was in agreement with the reported in vitro augmentation of NK activity in PBL from adult pigs by treatment with porcine IFN (Chung et al., 1982; Charley et al., 1983), and with previous studies in this laboratory (Cepica and Derbyshire, 1986).

Poly ICLC consistently induced an IFN response, the kinetics of which were similar to those reported for poly IC in newborn piglets (Loewen and Derbyshire, 1986). Poly ICLC treatment caused rapid activation of NK cells in vivo, but had no effect on conjugate formation by the PBL. By 24 h after inoculation with poly ICLC the level of NK activity in the PBL of the treated piglets was as high as that found in normal weaned piglets, but the activity subsequently declined. It was particularly noteworthy that the rise in serum IFN was immediately followed by NK cell activation, and that the decline of NK activity was preceded by falling IFN titres. This relationship is consistent with the hypothesis that the IFN induced by the poly ICLC treatment resulted in transient activation of the NK cells. The kinetics of NK activation were similar to those described in mice treated with poly IC in an early paper on this topic (Djeu et al., 1979). At 9 days of age the treated piglets showed evidence of natural activation of their NK cells, similar to that observed in the untreated controls, indicating tht they were not refractory to subsequent activation.

The level of lymphocyte-target cell conjugate formation in our studies was similar for newborn and weaned piglets, and was unaffected by IFN treatment

in vitro or in vivo. This indicates that the lack of NK activity in neonatal porcine PBL is not due to a lack of target cell recognition and binding, but is associated with a subsequent cytolytic defect. In contrast, it has been found in humans that the target-binding of neonatal PBL is lower than that of adult PBL (Baley and Schacter, 1985; Nair et al., 1985), but as in our studies, the target-binding capacity of human NK cells was not increased by IFN treatment (Silva et al., 1980; Marumo et al., 1984).

When poly ICLC-treated piglets were exposed to infection with TGE virus they showed a delay in onset of clinical signs in comparison with their untreated litter mates. This corresponded with the period of peak NK activity in the treated piglets, but as IFN levels and NK activity declined the resistance of the piglets was lost, and the subsequent course of the disease was similar to that in the untreated controls. It is noteworthy that in an earlier study (Cepica and Derbyshire, 1984b), in which NK activity was established in newborn piglets by the adoptive transfer of adult porcine PBL, there was also an increase in incubation period when the piglets were challenged with TGE virus. However, in the present study, direct antiviral effects of the induced IFN, as well as the activated NK cells, may have contributed to the delayed onset of TGE in the poly ICLC-treated piglets.

ACKNOWLEDGEMENTS

This work was supported by the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Agriculture and Food. We acknowledge the technical assistance of Mrs. Nancy Kemp and Mr. H. Hanlon. The study formed part of a thesis submitted by the senior author to the Faculty of Graduate Studies at the University of Guelph in partial fulfilment of the requirements for the Master of Science degree.

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