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DIFFERENTIAL EFFECTS OF HUMAN AND PORCINE INTERLEUKIN 2 ON NATURAL KILLING (NK) ACTIVITY OF NEWBORN PIGLETS AND ADULT PIGS LYMPHOCYTES

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RÉSUMÉ

EFFETS DIFFÉRENTS DES INTERLEUKINES 2 HUMAINE ET PORCINE SUR L’ACTIVITÉ NK DES LYMPHOCYTES DE PORCELETS NOUVEAU-NÉS ET DE PORCS ADULTES. — Nous avons étudié in vitro, à partir de lymphocytes sanguins de porcelets et de porcs adultes, les effets de l’Interleukine 2 (IL2) sur leur activité NK. Pour cela, de grandes quantités d’IL2 porcine ont été préparées par stimulation à la PHA de cellules ganglionnaires mésentériques irradiées. La purification par chromatographie en filtration sur gel a montré que la masse moléculaire apparente de l’IL2 était de 15 000 Da. Les IL2 naturelles purifiées humaine et porcine ainsi que l’IL2 humaine recombinante sont capables d’accroître très nettement l’activité NK des lymphocytes de porc adulte. Par contre, bien que les lymphocytes de porcelets soient activables par des surnageants bruts contenant l’IL2 porcine (ou par l’Interféron a comme nous l’avions montré précédemment) leur faible activité NK n’est pas accrue par l’IL2 purifiée. Ces résultats suggèrent donc l’existence de différences dans le développement ontogénique de l’aptitude des cellules NK porcines à répondre à diverses lymphokines.

Natural killing (NK) activity can be defined as the ability of normal unsensitized lymphoid cells to lyse tumor cells and virus-infected cells. There is some evidence, at least in the mouse, that NK activity might be involved in the rejection process of transplanted tumors and in the natural resistance to virus infections (Herberman and Ortaldo 1981, Bancroft et al 1981, Bukowski et al 1983, 1985). NK activity can be activated in vitro or in vivo by a variety of substances which include Interferons (IFN) and Interleukin 2 (IL2) (Rager-Zisman and Bloom 1985, Hefeneider et al 1983, Ortaldo et al 1984). In porcine species, the NK activity, which is very low or even undetectable in neonates (Huh et al 1981, Cepica and Derbyshire 1984, Charley et al 1985a), can be stimulated in vitro by natural or recombinant human or porcine IFNα (Charley et al 1983, Martin and Wardle 1984, Kim and Chung 1985, Charley et al 1985a, Cepica and Derbyshire 1986). In the present report, we have investigated in vitro the role of IL2 in the modulation of NK activities of newborn piglets or adult pigs. For that purpose, porcine IL2 was prepared from mesenteric lymph node cells and purified by gel filtration. We found that purified porcine or human IL2 could markedly increase adult pigs NK but were without significant effects on piglets NK.

Materials and Methods

Animals

Naturally-farrowed, colostrum-fed Large-White pigs were used for these experiments.

Production of IL2

Natural human IL2 was produced by PHA stimulation of blood lymphocytes as previously described (Chouaib et al 1984). Recombinant human IL2 was kindly provided by Biogen (Switzerland). Porcine IL2 was produced by PHA stimulation of either peripheral blood lymphocytes (PBL) or mesenteric lymph node (MLN) cells. Production of IL2 from PBL was performed as previously described (Charley et al 1985 b). Briefly, PBL were cultured in RPMI 1640 (Flow, Irvine, Scotland) supplemented with 5 % foetal calf serum (FCS) for 24 hours before addition of PHA (HA-16 from Wellcome, Dartford, UK) at 2.5 μg/ml. Supernatants were collected 48 hours later and used as crude porcine IL2. In order to prepare large volumes of supernatants for partial purification by gel-filtration, IL2 was produced by MLN cells: lymph nodes were aseptically removed from swine immediately after euthanatization. Single-cell suspensions were obtained by mincing and teasing the organ. Cell debris and clumps were removed by gravity sedimentation for 10 minutes and the cells present in the resulting supernates were washed three times. MLN cells were irradiated at 1 000 rads (Cobalt 60 gamma irradiator), adjusted to 106 cells per ml in
RPMI 1640 supplemented with 5 % normal pig serum, 5 x 10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 μg/ml gentamycin sulfate (Sigma, St Louis, MO, USA) and 1 % PHA-P (Difco, Detroit, MI, USA).

Supernatants were collected following 4 to 6 days of incubation, centrifuged and used for partial purification by gel-filtration.

Purification of IL2

Concentrated supernatants were applied on an AcA 54 gel-filtration column as described previously (Wakasugi et al 1984). Fractions (8 ml) were collected, filter-sterilized, supplemented with 2 % normal pig serum and kept at 4 °C.

The column was calibrated with ovalbumin (OVA, mw 45,000 Da) and ribonuclease (RNase, mw 13,700 Da). For thymidine incorporation assays, fractions were diluted 1/10 in RPMI 1640.

Generation of porcine lymphoblastoid cells

We have prepared two distinct long-term cultures of porcine IL2 dependent lymphoblastoid cells, derived from mixed lymphocyte cultures (Baker and Knoblock 1982, Gasbarre personal communication). Stimulator cells were porcine PBL which were irradiated (3 000 rads), aliquoted and kept frozen in liquid nitrogen. Responder cells were PBL obtained from other pigs, cultured at 2-4 x 10^6 cells per ml of RPMI 1640 supplemented with 10 % FCS, 2-ME, glutamin, gentamycin, as stated above, and 1 mM sodium pyruvate (Gibco, Puisley, Scotland) in the presence of an equal number of stimulator cells. After 7 days of culture, dead cells were depleted by centrifugation over warm Ficoll-Telebrix, d :1.09, solution (Salmon 1979, Mishell and Shiigi 1980) and viable cells were re-incubated with freshly thawed stimulator cells for 7 days. This procedure was performed three times. Cultures were then maintained in 20 % crude porcine IL2 and readjusted twice a week to a cell concentration of 2 x 10^5 cells per ml. By this method, two different long-term cultures were obtained and were named 4041 and 4042.

PHA-stimulated porcine lymphoblasts were also prepared as previously described (Charley et al 1985b) following 3-5 days of culture.

Quantitative microassays for IL2 activity

Supernatants were tested for their ability to support the growth of IL2 dependent murine CTL-L2 cells and of porcine lymphoblastoid cells. Briefly, murine cells (4 x 10^6 per ml) or porcine cells (10^6 cells per ml) were incubated in microtiter plates with dilutions of supernatants, respectively for 48 hours or 72 hours. Cultures were then pulsed overnight with 0.5 μCi (18.5 kBq) tritiated thymidine and cells were collected with a multiple sample harvester. The IL2 activity was calculated as defined by the Biological Response Modifiers Program (NCI, Frederick, MD, USA), using the BRMP Reference.

Table 1. – In vitro effects of crude supernatants containing porcine IL2, on the porcine NK activity

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Age of the Animals</th>
<th>Number of Animals</th>
<th>Mean (%) cytotoxicity (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control PBL</td>
</tr>
<tr>
<td>1</td>
<td>3-4 months</td>
<td>9</td>
<td>22.6</td>
</tr>
<tr>
<td>2</td>
<td>4 days</td>
<td>10</td>
<td>3.7</td>
</tr>
<tr>
<td>3</td>
<td>3 days</td>
<td>8</td>
<td>9.6</td>
</tr>
</tbody>
</table>

a : 4 hours Cr release assay against K562 cells   
b : PBL were pretreated overnight with a crude porcine IL2 preparation at a final concentration of 60 BRMP U /ml   
c : Statistical analysis by a paired « t » test

Fig 1. – IL2 activity after AcA 54 fractionation of concentrated porcine supernatants: 3H-thymidine incorporation in porcine PBL (O), porcine lymphoblastoid 4041 cells (△) and murine CTL-L2 cells (●).
Reagent and results were expressed in BRMP Units (BRMP U) per ml.

In order to characterize the presence of PHA in the fraction of gel-filtration, porcine PBL (5 x 10^5 per ml) were incubated with 1/10 diluted fractions for 72 hours before addition of tritiated thymidine.

**Chromium release assay for NK**

Porcine lymphocytes were prepared from peripheral blood by sedimentation of red blood cells in Dextran as described previously (Charley et al 1985a) followed by centrifugation over Lymphoprep (Nyegaard, Oslo, Norway). A 4 hours ^51^Cr-release assay was performed as previously described (Koren et al 1978, Charley et al 1983): porcine lymphocytes were preincubated overnight with IL2 or with control medium and incubated with labeled human K562 target cells. Results were expressed as percent cytotoxicity (Koren et al 1978).

**Results**

**Production of porcine IL2**

When porcine PBL were preincubated 24 hours without mitogens and cultured for two days with PHA, supernatants were obtained, with IL2 titers ranging from 60 to 150 BRMP U/ml (Charley et al 1985b). However, this procedure required the isolation of PBL by centrifugation over Ficoll and yielded relatively small volumes of supernatants. In order to prepare larger volumes of IL2 for gel filtration, we adopted an alternative way of IL2 production. Since we found that spleen cells yielded low IL2 titers (less than 9 BRMP U/ml), we used mesenteric lymph nodes (MLN) as a source of lymphocytes. From an adult animal it is easy to obtain 3-7 x 10^9 MNL cells without any purification step and containing a very low percentage of red blood cells; from such a cell suspension, about 5 liters of supernatants are prepared. When MLN cells were irradiated and treated as described in Materials and Methods, IL2 titers ranged from 90 to 240 BRMP U/ml.

Concentrated supernatants were submitted to AcA 54 gel filtration and fractions were assayed by thymidine incorporation in pig lymphocytes, in pig lymphoblastoid 4041 cells and in murine CTL-L2 cells (fig 1). With pig lymphocytes, a major peak of incorporation was obtained corresponding to the elution profile of PHA. With murine CTL-L2 cells and with porcine 4041 cells, a single peak of IL2 was obtained, with an apparent molecular weight of 15,000 Da (fig 1). This dialysed material was thereafter referred to as «semipurified IL2».

**Effects of porcine IL2 on NK activity**

PBL prepared from young piglets or from adults were preincubated overnight with crude porcine IL2 and then tested for NK activity by a 4 hours ^51^Cr-release assay: a significant increase of NK activity was obtained (table 1). Under similar conditions, when 4 days-old-piglets PBL were pretreated with an optimal amount of human IFN (5 x 10^3 IU/ml; Charley et al 1983), the NK activity was less markedly enhanced (Charley et al 1985a) than with crude porcine IL2. In order to determine if the

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**Table 2. — Effect of semipurified porcine IL2 on NK Activity of blood lymphocytes derived from young piglets.**

<table>
<thead>
<tr>
<th>Dosage of porcine IL2 (BRMP U/ml)</th>
<th>% cytotoxicity (E:T = 50:1)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Pig n° 9</td>
</tr>
<tr>
<td>0</td>
<td>6.3</td>
</tr>
<tr>
<td>7</td>
<td>4.8</td>
</tr>
<tr>
<td>14</td>
<td>6.3</td>
</tr>
<tr>
<td>28</td>
<td>5.9</td>
</tr>
</tbody>
</table>

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**Fig. 2. — Dose-response effects of semipurified porcine (- - - - and human I I - I) IL2 on NK activity of blood lymphocytes derived from 2 adult pigs (●●). The NK assay was performed at an effector to target ratio of 50:1.**
enhancing effect was due to IL2 or to other factors present in crude supernatants, semipurified porcine IL2 was tested. Several experiments performed with adult PBL indicated that, at various effector to target (E:T) ratios, a marked NK enhancement was obtained with semi-purified porcine IL2 (data not shown). Clear-cut dose-responses of adult PBL to porcine and human IL2 were observed (fig 2) with IL2 doses of 3 BRMP U/ml or more. However, when PBL obtained from newborn animals were incubated with semipurified porcine IL2 (IL2 titers ranging from 7 to 28 BRMP U/ml), no NK enhancement was observed (table 2).

**Effects of human IL2 on porcine NK activity**

Results in figure 2 show similar dose-response curves when adult PBL were pretreated with human or with porcine semipurified IL2. This enhancement was observed at various effector to target ratios with the most pronounced effect at E:T = 50.

Recombinant human IL2 was also found to increase markedly cytotoxic activities of cells obtained from adults but had only marginal effects on NK activity of piglets-derived PBL (table 3).

**Proliferative response of porcine lymphoblastoid cells to IL2**

To determine if activated porcine mononuclear cells were sensitive to human IL2, we examined proliferation of PHA blasts and of two lymphoblastoid cell-lines in the presence of serial dilutions of IL2. Table 4 indicates that porcine PHA blasts and 4042 cells were sensitive to homologous and not to heterologous IL2 whereas the 4041 lymphoblastoid cell-line could proliferate in the presence of human or porcine IL2. PHA blasts and 4042 cells were not sensitive to PHA whereas 4041 cells retained a low level of responsiveness to PHA.

**Discussion**

Production of porcine IL2 has already been described by several authors who used freshly prepa-
red (Gasbarre et al 1984, Ohlinger 1984, English et al 1985) or precultured PBL (Mertsching et al 1984, Charley et al 1985b) stimulated with lectins or by mixed lymphocyte reaction.

The procedure described in the report allows the production of large volumes of porcine supernatants rich in IL2, through the simple preparation of mesenteric lymph node cells which does not require any purification step and after gamma irradiation which presumably destroyed suppressor cells (Chouaib et al 1984). Fractionation of concentrated supernatants by gel-filtration yielded active fractions corresponding to an apparent molecular weight of 15 000 Da, a value consistent with the findings of Gasbarre et al (1984) and Ohlinger (1984) whereas English et al (1985), found a mw of 23 000 Da. Porcine IL2 was described as being unable to promote the growth of human lymphoblasts (Charley et al 1985b, English et al 1985), but contradictory results were published on the proliferative effects of human IL2 on pig cells. Thus, in two reports, lectin activation or allogeneic stimulation were found to render porcine cells sensitive to human IL2 (Charley et al 1985, Fong and Doyle 1986) whereas we found that human IL2 had only marginal effects on PHA blasts when compared to similar amounts of porcine IL2 (Charley et al 1985b). A possible explanation for these discrepancies is that the sensitivity of porcine blasts to human IL2 could greatly vary from one cell preparation to another; thus, in the present report (table 4), two porcine lymphoblastoid cell-lines generated by mixed lymphocyte culture differed markedly in their responsiveness to heterologous IL2.

The direct stimulatory effect of IL2 on human or mouse NK cells has been extensively studied in vitro as well as in vivo (Ortaldo et al 1984, Trinchieri et al 1984, Lanier et al 1985, Talmadge 1985). From the data presented in this report, lectin-free semipurified porcine and human IL2 appeared to activate efficiently NK activity of adult pigs. Moreover, recombinant human IL2 could stimulate porcine NK (table 3) to much higher level than previously described (Fong and Doyle 1986). These studies suggest therefore that human IL2 have a potential for use as in vivo immunomodulators of the NK system of adult pigs.

Regarding newborn piglets, several reports indicate that their lymphocytes have low or undetectable NK activity against virus-infected target cells (Cepica and Derbyshire 1984) or against K562 cells (Huh et al 1981, Charley et al 1985a). However, they were rendered cytotoxic by pre-treatment with purified human IFN (Charley et al 1985a) or with crude porcine IFN (Cepica and Derbyshire 1986). The data presented in this report indicate that purified IL2 is unable to activate NK activity of newborn animals-derived PBL. This suggest therefore differences in the ontogenic development of IFN- and IL2- responsiveness of porcine NK system. In human species also, differential effects of lymphokines on NK activity were observed in neonates: thus, IL2 and IFNa could potentiate immature NK cells whereas IFNy had no effects (Ueno et al 1985, Nair et al 1985, Seki et al 1985). Finally, since crude porcine supernatants could activate newborn NK activity (table 1) the nature of the lymphokine (such as IFNy) possibly involved in this phenomenon needs now to be investigated.

Acknowledgements

We wish to thank M Bonneau (INRA La Minière) for providing Large White piglets.

Abstract

The in vitro effects of human or porcine Interleukin 2 (IL2) on Natural Killing (NK) activity were studied with blood lymphocytes of newborn piglets and of adult pigs. Large volumes of porcine IL2 were prepared by PHA stimulation of irradiated mesenteric lymph node cells and, following purification by gel-filtration chromatography, the apparent molecular weight of IL2 was 15 000 Da. Purified human and porcine IL2 as well as recombinant human IL2 were found to increase markedly NK activity of lymphocytes derived from adult animals. However, although newborn piglets-derived lymphocytes are sensitive to crude porcine IL2 supernatants (and to Interferon α as previously shown) their low NK activity is unaffected by purified IL2. These data suggest therefore the existence of differences in the ontogenic development of the lymphokines responsiveness of porcine NK cells.

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