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Morphology and functional characteristics of isolated porcine intraepithelial lymphocytes

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SUMMARY

We have examined the morphology and functional characteristics of porcine intraepithelial lymphocytes (IEL). A subpopulation of IEL contains granules as seen in other species, and their ultrastructure was also similar. They were capable of producing T-cell growth factor and interferon on *in vitro* stimulation. IEL killed P815 cells in the presence of PHA, but did not kill K562 cells.

INTRODUCTION

In a previous paper we have described the isolation and growth *in vitro* of porcine intraepithelial lymphocytes (IEL). There are strong response of porcine IEL to T-cell mitogens in the pig (Wilson, Stokes & Bourne, 1986). In the species studied so far, guinea-pig IEL show a response to mitogens (Arnaud-Battandier & Nelson, 1982), human and rat IEL do not (Greenwood, Austin & Dobbins, 1983; Nauss *et al.*, 1984), and mouse IEL are intermediate (Dillon & MacDonald, 1984). IEL from other species are characterized by having cytoplasmic granules and T-cell surface markers (Guy-Grand, Griscilli & Vassali, 1978; Marsh, 1985; Lyscom & Brueton, 1982). In this paper we show that, morphologically, porcine IEL are similar to those in other species, and they show other functional characteristics consistent with the hypothesis that at least a proportion of the IEL population are T lymphocytes.

MATERIALS AND METHODS

Isolation

Porcine IEL were isolated as described previously (Wilson *et al.*, 1986).

Histology

Cytospin smears of approximately 10^5 cells were prepared, air-dried and fixed for 30 seconds in methanol. Slides were stained with Alcian blue and Toluidine blue using standard methods (Bancroft, 1982). For electron microscopy, a pellet of cells was fixed in glutaraldehyde, post-fixed in somium tetroxide, embedded in Epon, and ultrathin sections stained with lead acetate.

IEL were stained for surface immunoglobulin using anti-pig IgA monoclonal antibody by incubating for 1·5 hr at a concentration of 1 μml/ml in PBS, followed by incubation with rabbit anti-mouse horse radish peroxidase conjugate 1/50 for 1·5 hr. Cells were washed again and finally developed using diaminobenzidine, 0·5 mg/ml × 3 μl/ml 30% H_2O_2 in Tris-HCl at pH 7·4 for 15 min. After a final wash, the slides were mounted in DPX and examined under a light microscope. All immunological reagents were prepared by the Department of Veterinary Medicine, Bristol University School of Veterinary Science.

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Media

RPMI was obtained from Flow Laboratories, Irvine, Ayrshire. It was supplemented with 10 mM HEPES, glutamine, 2·5 μg/ml amphoterecin B, 5 μg/ml gentamycin and 10% fetal calf serum (Flow) and 10^{-5} mM 2-mercaptoethanol (Sigma, Poole, Dorset). This is referred to as complete medium in the text. For washing, Hanks' balanced salt solution (HBSS; Flow) was used. Minimal essential medium was purchased as a dry powder (K. C. Biological, KS) and made up in H_2O, 5% fetal calf serum, 10 mg/l bicarbonate, and penicillin (60 mg/l), streptomycin (100 mg/l) and polymixin (50,000 i.u.) were added. This is referred to as MEM.

Production of interferon and T-cell growth factor by IEL

Test supernatants from IEL were prepared by culturing IEL for 36 hr in complete media containing 5 μg/ml concanavalin A (Con A). Two cell densities were used: 2×10^6/ml for T-cell growth factor (TCGF) and 1×10^7/ml for interferon (IFN) production. The supernatants were harvested and the Con A inactivated with 20 mg/ml methyl α-D-mannopyranoside. Control cultures without Con A were prepared in the same way and reconstituted by adding Con A along with the methyl α-D-mannopyranoside. Aliquots of Con A-stimulated interferon supernatants were dialysed at pH 2 for 18 hr in 2% KCl.HCl buffer. All samples were frozen at −70° until assayed.

Porcine growth factor was demonstrated using pig lymphocyte blasts. Peripheral blood lymphocytes (PBL) were isolated from 10 ml heparinized blood samples by spinning at 400 *g* for 30 min, resuspending the buffer coat cells in 2 ml RPMI and overlaying on 2 ml Ficoll–Hypaque 1·077 g/ml (Ficoll–Paque, Pharmacia, Uppsala, Sweden). The resulting gradients were

spun for 30 min at 400 *g* and the lymphocytes collected from the interface, washed three times in HBSS, suspended at 2×10^6/ml in complete medium containing 5 μg/ml Con A (Sigma), and incubated for 4 days at 37°, 5% CO_2. The cells were then harvested and washed twice in HBSS containing 20 mg/ml methyl α-D-mannopyranoside (Sigma), then resuspended at 1×10^6/ml in complete media. One-hundred μl of this cell suspension were added to 96-well plates containing doubling dilutions of test supernatant in complete media; each supernatant was assayed in triplicate. The plates were incubated overnight at 37° in a 5% CO_2 atmosphere, pulsed for 8 hr with 0·5 μCi of [^{3}H]thymidine (Amersham Radiochemical Centre, Amersham, Bucks) in 50 μl complete media, harvested using an automatic cell harvester (Skatron Titertek, Flow Laboratories), then counted on a scintillation counter using Scintron Cocktail T (BDH, Poole, Dorset).

Interferon was measured by a modification of the method of McWilliams *et al.* (1971) using the inhibition of RNA synthesis by Semliki Forest virus grown in a pig embryo kidney (PEK) cell line. The PEK line (donated by Dr Lukey, Central Veterinary Laboratories, Weybridge, Surrey) was grown in MEM 5% FCS in 75 ml tissue culture flasks (Sterilin, Teddington, Middlesex); the cells were split twice weekly. Semliki Forest virus was grown up and passaged twice through PEK cells, the supernatant was harvested after 12 hr growth and filtered through 0·22 μm filters (Midlex GV, Millipore, Molsheim, France). For the assay, 7×10^4 cells were added to each well of a 96-well flat-bottomed tissue culture plate (Titertek, Flow) and incubated overnight to form a monolayer. The media were aspirated and semi-log dilutions of test supernatant in MEM were made. The plate was incubated for a further 24 hr and the media removed and replaced by 25 μl of MEM containing 1:64 dilution of stock virus, and 3 μg/ml actinomycin D were added to each well and removed after 2 hr then replaced with 50 μl MEM containing 3 μg/ml actinomycin D and 0·5 μCi [^{3}H]uridine 29 Ci/mmol (Amersham). The cells were harvested after a further 6 hr incubation by removing the supernatant and replacing it with 200 ml phosphate-buffered saline containing trypsin–versene, incubating for a further 10 min and then harvesting on an automatic cell harvester before counting as above.

Natural killer cell activity

Natural killer activity was measured in an 18-hr chromium-release assay as described by Norley & Wardley (1983) using K562 human myeloid cells as targets.

Mitogen-mediated cytotoxicity was measured by the method of Bevan & Cohn (1975). Briefly, 10^4 chromium-labelled P815 cells were incubated for 4 hr with varying numbers of IEL in the presence of 2·5 μg/ml PHA. In both assays, the percentage release of chromium was calculated using the formula:

$$\frac{\text{specific} - \text{spontaneous}}{\text{total} - \text{spontaneous}} \times \frac{100}{1}.$$

RESULTS

History and immunohistology

Stained smears of IEL showed a range of sizes from small lymphocytes with little cytoplasm to larger cells with distinct cytoplasm. Eosinophils were commonly seen as well as epithelial cells, and form up to 5% of the isolate. The presence of cytoplasmic granules was demonstrated by Alcian blue or

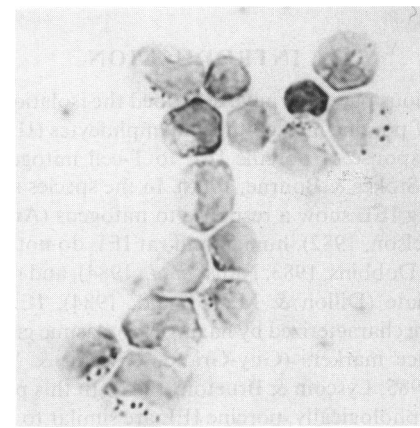

Figure 1. Photomicrograph of pig intraepithelial lymphocytes stained with Toluidine blue. Note the large dark staining granules and variable cytoplasmic volume (magnification × 320).

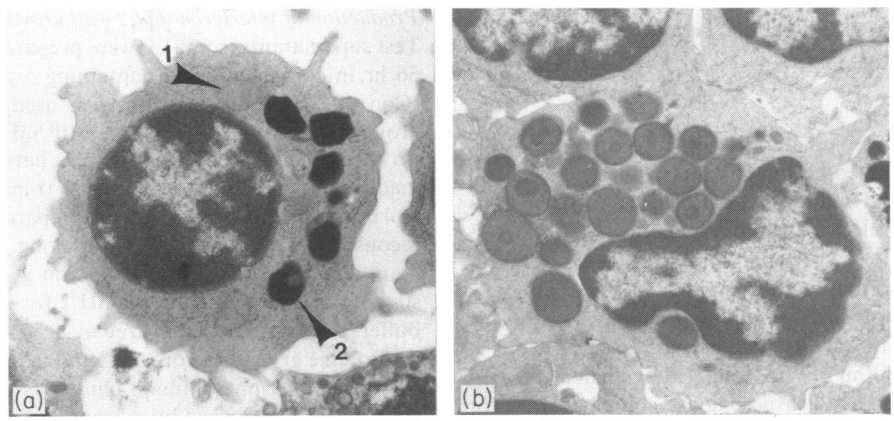

Figure 2. (a) Electron micrograph of a pig IEL showing mitochondria (arrow 1), cytoplasmic granules (arrow 2) and variable staining of the nucleus (magnification × 8000). (b) Electron micrograph of eosinophil at the same magnification. It is larger with more numerous granules containing round central bodies.

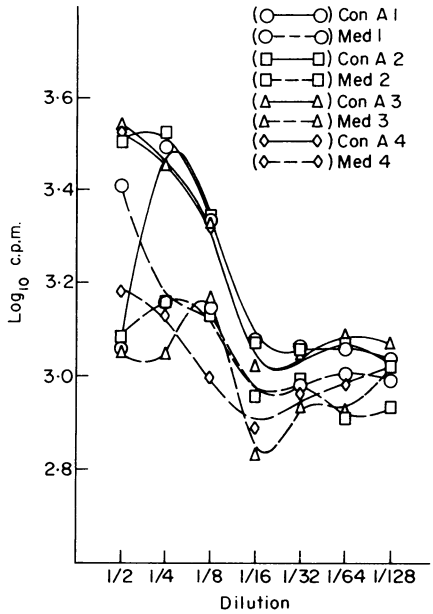

Figure 3. TCGF production by IEL isolated from four different pigs. Cells were incubated for 36 hr in the presence (solid lines) or absence (dotted lines) of Con A (5 μg/ml) and the resulting supernatants assayed for TCGF.

Toluidine blue staining (Fig. 1), which showed 24·5 ± 11% and 26·8 ± 11% positive cells, respectively ($n = 6$); less than 1% of IEL stained positively for IgA.

Electron microscopy of IEL (Fig. 2a) confirmed the presence of granules in the cytoplasm of approximately half the cells, and also mitochondria, ribosomes and nuclei with prominent nucleoli. By comparison, eosinophils (Fig. 2b) were larger and had more numerous granules, which contained round central bodies.

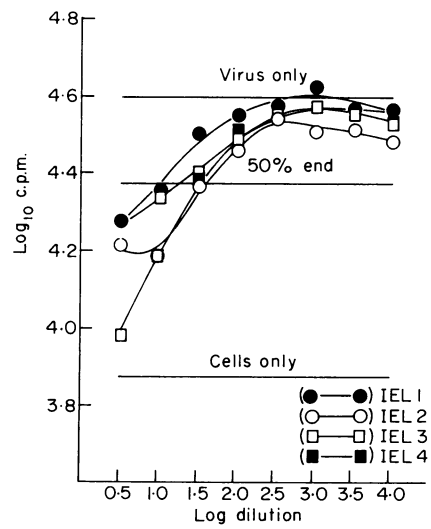

Figure 4. Interferon activity in supernatants of IEL cultures. The *y* axis indicates $\log_{10}$ counts per minute of [^{3}H]uridine uptake. The horizontal lines show the uridine uptake of cells treated with actinomycin-D (cells only) of virus grown in actinomycin-D-treated cells (virus only) and the point of 50% reduction in virus RNA uptake (50% end).

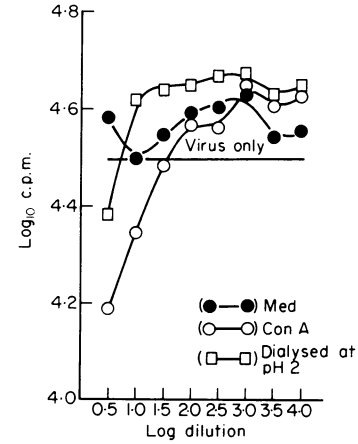

Figure 5. Effect of IEL supernatants on replication of Semliki Forest Virus. Supernatant grown with media alone and then reconstituted with Con A and methyl α-D-mannopyranoside apparently enhanced virus replication. Supernatant grown from IEL stimulated with Con A and then inactivated with methyl α-D-mannopyranoside inhibited virus replication at high concentrations by 50% but enhanced virus replication at low concentrations. The dialysed sample falls between the two.

T-cell growth factor and interferon production

Figure 3 shows a graph of the results of TCGF production by IEL isolated from four pigs with and without Con A stimulation. There was some resting production of TCGF but this was augmented by Con A stimulation. Interestingly, the stimulated cultures plateaued at a higher level of activity than the unstimulated cultures. Pig 1 showed a pro-zone of inhibition in the stimulated culture and also had the highest titre in an unstimulated cultre.

The production of γ-interferon was less easily demonstrated. Figure 4 shows the curves of four IEL samples stimulated with Con A: clearly there is some inhibition of virus at high concentrations. In a further experiment on eight separate pigs we showed that the inhibition of virus was dependent on Con A stimulation and was at least partially inactivated by dialysis at pH 2, both properties of γ-interferon. Figure 5 shows the results from one such pig.

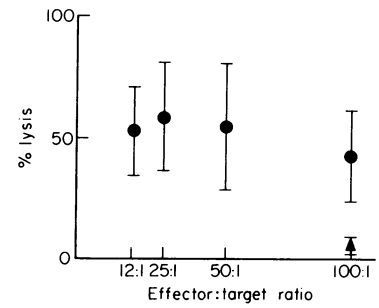

Figure 6. Cytotoxicity of IEL for P815 targets after 4 hr in the presence of 2·5 μg/ml PHA. The dots are means from four pigs ± standard deviation. The triangle represents the same cells at 100:1 effector:target ratio in the absence of PHA.

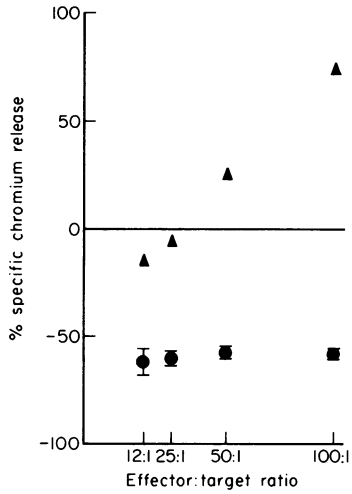

Figure 7. Cytotoxicity of pig blood lymphocytes and IEL for K562 targets in an 18-hr assay. IEL represent mean ± standard deviation of four pigs.

Mitogen-mediated cytotoxicity

IEL are able to kill P815 targets in the presence of PHA (Fig. 6). The triangular point at 100:1 effector:target ratio shows that this assay is dependent on the presence of PHA, and no significant spontaneous cytotoxicity is present. The reduced activity at higher effector:target ratios is probably due to reabsorption of chromium by IEL.

Natural killer cell activity

Figure 7 shows the results of an 18-hr NK assay using K562 cells as a target. The triangular points show typical results with pig peripheral blood lymphocytes. In contrast, there was no lysis of K562 target by IEL: indeed, there is a negative specific release, probably due to reabsorption of spontaneously released ^{51}Cr by the IEL themselves.

DISCUSSION

The morphology of porcine IEL described in this study shows a broad similarity to the description of IEL in mice (Marsh, 1975; Guy-Grand *et al.*, 1978), rats (Mayerhoffer, 1980) and man (Marsh, 1985), namely the presence of granules containing glycosaminoglycans staining with Alcian blue or Toluidine blue and also visible in electron micrographs (Figs 1 and 2). The numbers of IEL that contained Alcian blue staining granules were lower than reported in rats, but this may be due to the fixing and staining methods used (Mayerhoffer, 1980). The lack of immunoglobulin-containing cells suggests that the IEL population is free of lamina propria or Peyer's patch contamination, whilst the presence of eosinophils is not surprising as these can be seen within the epithelium of intact intestinal sections.

The nature and origin of epithelial lymphocytes have been the subject of controversy (Mayerhoffer, 1980; Mayerhoffer & Whatley, 1983; Marsh, 1985; Guy-Grand *et al.*, 1978; Dillon & Macdonald, 1984); however the majority of studies on surface markers agree that in man (Greenwood *et al.*, 1983; Selby *et al.*, 1983) and mice (Dillon & MacDonald, 1984; Lyscom & Brueton, 1982), the majority of IEL exhibit cytotoxic T-cell markers with a lesser number of T-helper phenotypes present. The number of cells with pan T-cell markers is 85–95% in man, but less than 45% in rats and mice. This latter observation leaves a number of cells without a pan T-marker but positive for cytotoxic T-cell marker, a phenotype apparently unique to IEL. The phenotypic markers used in such studies are not generally available for pigs, and we have therefore examined the nature of porcine IEL by their functional characteristics.

Interleukin-2 has been demonstrated in supernatants from mouse IEL (Cerf-Bensusan *et al.*, 1984). The production of a lymphocyte growth factor by Con A-stimulated IEL is consistent with the view that pig IEL also produce interleukin-2. Experiments using mouse spleen (Pfizenmaier *et al.*, 1984) or human peripheral blood (Mingari *et al.*, 1984) have shown that although IL-2 production is much greater in cells with helper T-cell phenotype, a significant proportion of phenotypically cytotoxic cells also produce this lymphokine. Thus, one cannot conclude that IL-2 production is definitive proof of the presence of phenotypic helper lymphocytes in pig IEL populations. Nevertheless, IEL are clearly capable of some helper functions when stimulated by mitogens, and presumably also antigens.

γ-Interferon has not been fully characterized in the pig. Small amounts were detected in PHA-stimulated lymphocyte cultures using ovine choroid plexus cells and vesicular stomatities (Yilma, 1983). Other workers have also only been able to demonstrate small amounts of interferon activity in pig blood lymphocyte cultures (B. Charley and C. La Bonardier, personal communication). The supernatants of IEL cultures were clearly able to inhibit viral RNA synthesis (Fig. 2). Furthermore, there is greater inhibition in Con A-stimulated cultures than in unstimulated cultures (Fig. 3). This is not a direct effect of Con A on virus as the control cultures were reconstituted with Con A after harvesting. This observation and the reduction of virus inhibition following dialysis at pH 2 are consistent with the properties of γ-interferon. Normally, interferons are assayed by interpolation of a 50% end-point and comparison with a standard interferon. It was initially our intention to compare the supernatants with a standard pig γ-interferon. However, the assay used is extremely sensitive, and 1000 units/ml of γ-interferon have a 50% end titre great than 1:10,000, suggesting a titre of less than 1 unit/ml of interferon and rendering comparison insensitive to changes in titre.

Both IL-2 and IFN have been demonstrated in the supernatants of rat IEL cultures (Cerf-Bensusan *et al.*, 1984), and the presence of IFN may be important in the regulation of class II major histocompatibilty antigens expression by the intestinal epithelial cells. Unfortunately, it was not possible to address this question directly in the pig, due to a lack of suitable anti-IA antibodies.

Finally, we have examined the cytotoxic properties of pig intraepithelial lymphocytes in NK- and mitogen-mediated assays. By comparison to blood lymphocytes, pig IEL lacked NK activity. In other species, NK activity has been reported in IEL from rats (Flexman, Shellman & Mayerhoffer, 1983; Nauss *et al.*, 1984) and mice (Tagliabue *et al.*, 1982; Mowat *et al.*, 1983), but it is distinct from that in spleen (Mowat, 1983) and is not mediated by phenotypic NK cells (Tagliabue, 1982) in the mouse. Thus, it may not be surprising that an assay that detects natural killing in blood lymphocytes of a pig is not effective with epithelial lymphocytes. On the other hand, we detected strong cytotoxicity in a mitogen-mediated assay. This type of assay has

been reported to be mediated by cytotoxic T cells in mice (Bevan & Cohn, 1975) and has been demonstrated in human PBL T-cell clones (Moretta *et al.*, 1983). Cytotoxic T cells may also be responsible in the pig, and the use of phenotypic markers to clarify this point would be useful.

Overall, these results indicate that pig IEL have a range of morphological and functional characteristics similar to those of other species. The characteristics are also consistent with the view that some of them at least are T lymphocytes capable of both cytotoxic and helper functions.

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