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The null T cell in pig blood is not an NK cell

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SUMMARY

Up to 50% of the blood lymphocytes in young pigs are thymus-derived, lack all subset-specific markers and appear immunologically unresponsive, with no known functional role. In an examination of their possible role in natural killing, NK activity was found in unpurified mononuclear cells and in preparations of unselected and nylon non-adherent lymphocytes (T cells and Null cells). However, NK activity was abolished by removing the E rosette forming T cells using a rat IgM anti-pig CD2 monoclonal antibody and rabbit complement, but not by control treatments with a non-binding rat IgM monoclonal reagent and complement or with any other reagent alone. Thus the resting Null T cell appears not to play a significant role in natural killing.

INTRODUCTION

Young pigs have high blood lymphocyte counts compared to most other mammals ($\sim 10^7$/ml). Up to 50% of these blood lymphocytes are Null cells, which lack all surface markers specific to lymphocyte populations [surface Ig(sIg), γFc receptor (FcR), complement receptor (C'R), major histocompatibility complex (MHC) class II, CD2, CD4, CD8] bearing only MHC class I and leucocyte common antigen (Binns, 1982). These cells are thymus-dependent, disappearing after thymectomy (Binns *et al.*, 1977) and being enriched among thymus emigrant cells (Binns, Pabst & Licence, 1988). They are peculiar in that they are unresponsive to mitogens (Outteridge, Binns & Licence, 1982), do not play a part in antibody or direct cellular cytotoxicity response *in vitro* (Binns, 1982) and do not recirculate (Binns & Pabst, 1988). However, they are highly non-adherent for nylon-wool (Binns *et al.*, 1979; Binns & Licence, 1981; Binns, 1982) and this property allows their isolation in bulk using treatment of non-adherent blood lymphocytes with a rat IgM anti-pig CD2 monoclonal antibody and rabbit complement. The functional role and fate of this large population of Null T lymphocytes is entirely unknown. Spontaneous cytotoxicity of blood lymphocytes for tumour or virus-infected target cells, which is thought to be an important natural defence mechanism, has been described in many species, including the pig (Kim & Ichimura, 1986). This communication examines the possible involvement of Null T cells in natural cell killing (NK) as a potential protective function in blood.

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MATERIALS AND METHODS

Effector cells

Standard methods were used to prepare sterile mononuclear cells from blood taken into acid citrate dextrose (ACD) (Binns, Blakeley & Licence, 1981) and lymphocytes from defibrinated blood treated with carbonyl iron (Sigma C-3518; Poole, Dorset) (Outteridge *et al.*, 1982), using dextran (Dextraven 110, Fisons PLC, Loughborough, Leics) sedimentation of red cells and centrifugation of the leucocyte-rich supernatant on Ficoll–triosil (Pharmacia, Milton Keynes and Nyegaard, Oslo, Norway). The donors were Babraham Large White female pigs aged 3–6 months. These cell suspensions were kept in RPMI-1640 medium (Flow Labs, Irvine, Ayrshire) containing bicarbonate, 20 mM HEPES, 100 HU/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated fetal calf serum (FCS), termed complete tissue culture medium (TCM). The medium without HEPES is shown as TCM'. Nylon-wool non-adherent (NWNAD) cells, which include classical CD2$^+$ T cells and Null T cells, were isolated from both these preparations as described previously (Binns *et al.*, 1979; Outteridge *et al.*, 1982) using Fenwall Leuco-pak Lot No. 4B466R6 (Baxter Health Care, Thetford, Norfolk). The Null T cells were separated from T cells in these preparations by adding together equal volumes of $2{\cdot}5 \times 10^7$ NWNAD cells/ml and 1/50,000 ascites of a rat IgM anti-pig CD2 mAb (AFRC MAC 83; R. M. Binns, G. Butcher, D. J. Davies and A. Larkins, manuscript in preparation), incubating them for 15 min at 4° and then incubating them for a further 20 min at 37° after adding one volume of 1/4 rabbit serum complement (RC'). The undiluted low toxicity RC', collected from selected rabbits, and the monoclonal reagents diluted to 1/1000 in TCM, were stored at −70° after freezing in single drops in liquid nitrogen, to avoid repeated thawing and

waste of reagent. Control treatments were with MAC 83 alone, with a non-binding monoclonal rat IgM ascites (AFRC MAC 232; anti-spinach nitrate reductase; Notton *et al.*, 1988) and RC′, or with medium alone. The efficiency of killing CD2$^+$ T cells was assessed by E rosette formation of the cell suspensions with sheep red blood cells in the presence of dextran (Binns, 1978). The titre of anti-CD2 mAb chosen (1/50,000) would safely kill all T cells with RC′ but only partially block E rosette formation without RC′ (see Fig. 5 and Table 1) and so allow a check of the efficiency of T cell depletion.

Target cells and NK cytotoxicity assay

K562 human myeloid leukaemia cells, cultured in TCM′ in 5% CO_2 in humid air at 37°, were maintained in log phase growth. A volume of cell suspension providing $3–5 \times 10^6$ cells was centrifuged down and the cells resuspended in 100 μl TCM′. These cells were then labelled by addition of 100 μCi ^{51}Cr-sodium chromate (CJS4 Amersham International, Amersham, Bucks) and incubation at room temperature for 60–90 min before thorough washing. In the cytotoxicity assay, modified from Koren, Amos & Kim (1978), 1/2 log dilutions of effector cells in 100 μl TCM′ (starting at $3{\cdot}16 \times 10^7$/ml) were added to 1×10^4 labelled K562 target cells in 100 μl TCM′ in triplicate, in flat-bottomed 96-well microtitre plates (Falcon Cat. No. 3072, Becton-Dickinson UK Ltd, Oxford) to give effector to target cell (E:T) ratios of 316:1, 100:1, 32:1, 10:1, 3:1 and 1:1 and, unless otherwise stated, were incubated overnight (12–16 hr) at 37° in 5% CO_2 in humid air. The cells were then pelleted by centrifuging the plates at 400 *g* and 80 μl supernatant removed for counting in an LKB Wallac Clini Gamma 1272-003 (LKB, Milton Keynes) and calculation of specific ^{51}Cr release. In control wells target cells in 100 μl TCM′ were incubated with 100 μl TCM′ (background medium release) or with 100 μl 5% Triton X-100 (maximum release). Percentage specific ^{51}Cr release was calculated using the standard formula:

$$\% \text{ specific release} = \frac{\text{c.p.m. experimental} - \text{c.p.m. medium}}{\text{c.p.m. maximum} - \text{c.p.m. medium}} \times 100.$$

The data are shown as mean and standard error.

RESULTS

Initial studies (Fig. 1) showed that, although freshly isolated pig blood lymphocytes do show some NK cell activity in short-term

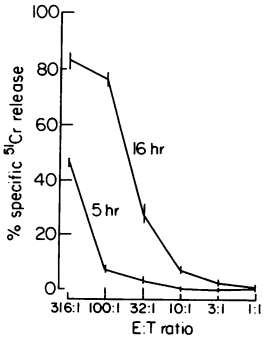

Figure 1. Specific ^{51}Cr release in 5- and 16-hr NK cell killing of labelled K562 target cells using ACD blood mononuclear effector cells in varying effector to target (E:T) ratios.

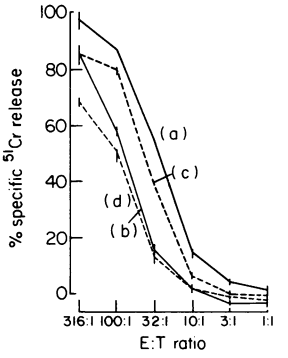

Figure 2. Comparison of the killing of labelled K562 target cells by unseparated (a) and nylon-wool non-adherent (c) ACD blood mononuclear cells and by unseparated (b) and nylon-wool non-adherent (d) lymphocytes (14-hr NK cell assay).

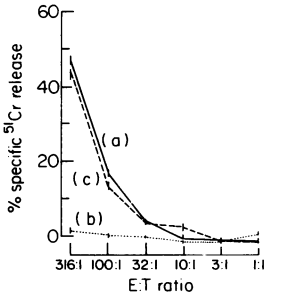

Figure 3. Null cells show no significant NK cell activity. Treatment of nylon-wool non-adherent cells (a) with anti-CD2 mAb and rabbit complement removes NK cell activity (b) in contrast to the lack of effects of control treatment with non-binding IgM mAb and rabbit complement (c) (12-hr NK cell assay).

assays (~5 hr), a longer period is necessary for consistent killing of K562 target cells (overnight for 12–16 hr).

Using such an assay, populations both of mononuclear cells isolated from ACD blood (a) and of lymphocytes from carbonyl iron treated defibrinated blood (b), showed strong NK cell activity (Fig. 2) that was dependent on E:T ratio. However, the lymphocyte preparation (b) showed significantly reduced NK cell activity, paralleling the loss of monocytic and other cells. These lymphocytes (b) showed little further reduction in NK cell activity following the passage down nylon-wool columns (d) to remove B cells. In contrast, passage of the mononuclear cells (a) down nylon-wool resulted in some loss of NK activity. So some NK cell activity was present in the phagocytic adherent cells but a major proportion of the NK cell activity in pig blood is in preparations containing only classical CD2$^+$ T cells and Null T cells.

Figure 3 shows that isolation of the Null T cells from the classical T cells in nylon-wool non-adherent populations (a), by killing the latter with the IgM anti-CD2 mAb (MAC 83) and RC′, resulted in complete loss of NK cell activity (b), even in E:T ratios of >300 Null T cells per target cell. In contrast, control treatment of the NWNAD cells with a non-binding IgM mAb and RC′ did not alter the NK activity (c). Further experiments (Fig. 4) showed that control treatment of non-adherent populations with MAC 83 alone did not result in loss of NK cell activity (c compared with b), although a possible

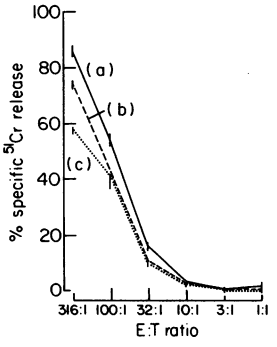

Figure 4. Passage of lymphocytes (a) through nylon-wool columns results in only slight reduction in NK cell activity (b) and further treatment with anti-CD2 mAb alone has little more effect (c) (13-hr NK cell assay).

Table 1. The effect of treatment of blood lymphocytes with combinations of control IgM mAb or IgM anti-CD2 mAb and rabbit complement on E rosette formation

mAb	RC′	% E rosettes
–	–	37·8
–	+	36·4
MAC232 – Control IgM	–	37·9
MAC232 – Control IgM	+	34·9
MAC83 – IgM anti-CD2	–	13·8
MAC83 – IgM anti-CD2	+	<0·3

Percentages of dead cells and cell yields were as expected from reagents used.

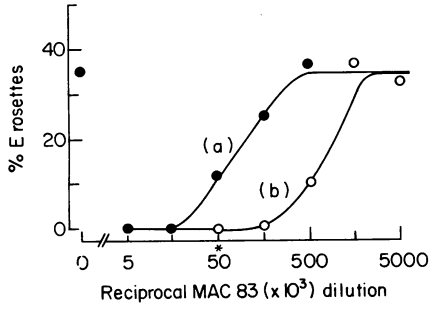

Figure 5. Differences in the titres of E rosette inhibition (a) and complement-mediated killing (b) of T cells by ascites MAC83 IgM anti-pig CD2 mAb. The titre used (*1/50,000) killed all T cells at a level giving only partial inhibition of E rosette formation.

slight reduction may have resulted from steric blocking of T-cell function. Thus the loss of NK cell activity after MAC 83 and RC′ treatment could not be a direct effect of MAC 83 binding.

The purity of the Null cell preparations used in these experiments was confirmed by showing that only when both MAC 83 and RC′ were present was there complete disappearance of E rosetting T cells (Table 1). All the control treatments resulted in negligible or, in the case of MAC 83 alone, only partial reduction in E rosette numbers. Control experiments (Fig. 5) had shown that the level of anti-CD2 mAb we routinely used to kill T cells (*) caused only partial blocking of the receptors for E rosette formation on living cells when used in the absence of RC′ (a) but with RC′ removed all E rosetting T cells (b).

DISCUSSION

This study has shown that NK cell activity in the blood of young Large White pigs is found both in the adherent mononuclear cells and the non-adherent lymphocytes. The non-adherent lymphocyte NK cell function was found entirely in the CD2$^+$ T-cell population. Purified non-adherent Null T cells showed no significant activity, even at effector to target (E:T) ratios of >300:1.

As the Null T cells may resemble an immature form, we felt it important to assay their activity directly after isolation and not after a long period of potential maturation *in vitro*. So rather than preincubating our cells overnight and assaying them for only 4 hr (Koren *et al.*, 1978; Kim *et al.*, 1980), we assayed all our freshly prepared cells immediately for 12–16 hr. An immediate 4–5 hr assay would not have been long enough (Fig. 1; Norley & Wardley, 1983; Yang, Schultz & Spano, 1987), 12 hr minimum being necessary (Pinto & Ferguson, 1988). Nevertheless, pre-incubation did not alter their responsiveness (data not shown).

In this study, the prime aim was to ascertain the NK cell activity of the Null T cell, not the phenotype of NK cells. It was evident that considerable NK cell activity was present among adherent mononuclear cells, which include both monocytes and high affinity Fc receptor$^+$ large granular leucocytes. Both cell types have been shown (Pospisil *et al.*, 1986) to include NK$^+$ cells and would be markedly depleted on nylon-wool (Binns & Licence, 1981). So these results are consistent with the notion that NK cells, probably of different lineage and phenotype, are present in both the adherent and non-adherent populations of mononuclear cells.

However, our data are clear that the NWNAD NK$^+$ lymphocyte is a CD2$^+$ T cell, as shown by the disappearance of NK cell activity after treatment with IgM anti-CD2 mAb and RC′ but not a control IgM mAb and RC′. Pescovitz, Lowman & Sachs (1988) showed that NK cell activity in the adult minipig is present mainly in a CD2$^+$ CD8$^+$ MHC class II$^+$ cell population, which would have been present in our preparations. However, that study was not designed to test the NK cell activity of Null T cells adequately because their adult minipigs have few sIg$^-$CD2$^-$ blood lymphocytes (Pescovitz, Lunney & Sachs, 1984), their preparations included monocytic cells and the Null T-cell residuum was not enriched to the high E:T ratio necessary for an NK assay (Pescovitz *et al.*, 1988).

Our results are in marked contrast to those of Norley & Wardley (1983) who suggest that it is the NWNAD E$^-$ cell in pig blood which shows the highest level of NK activity. We must assume that these findings result from incomplete removal either of adherent cells or of T cells. Our method of adherent cell depletion is designed to leave a small but significant number even of the more adherent E rosette forming T cells on the column (Outteridge *et al.*, 1982; Binns & Licence, 1981). The subsequent T-cell depletion using anti-CD2 mAb and RC′ was also very effective. The use of a dilution of anti-CD2 mAb known to kill all T cells in the presence of RC′ but not block all E

receptors, showed the effectiveness of the T-cell depletion. The appropriate control also showed that IgM anti-CD2 on the cell surface of T cells did not block their capacity to kill, incidentally also showing that the CD2 molecule is not closely associated with or part of the NK receptor. Putative effects on the killing through Ig binding to Fc receptors were ruled out by the lack of effects of rabbit serum C′ (~3mg Ig/ml) and of irrelevant (MAC 232) and anti-CD2 (MAC 83) IgM.

The Null and NK^+ populations in the blood of young pigs clearly differ from those in most other species (i) because the blood contains an unusually large population of Null T cells, (ii) because pig NK cell activity is predominantly found in small non-granular lymphocytes (Pinto & Ferguson, 1988), as opposed to large granular leucocytes (Timonen, Ortaldo & Herberman, 1981), and (iii) because the only non-adherent lymphocyte, which is NK^+, is a $CD2^+$ cell (also found in man; Zarling *et al.*, 1981), the major Null population being NK^-, as shown by our study. On the other hand, recently described Null T cells in sheep (MacKay, 1988) and cattle (Baldwin, Morrison & Naessens, 1988) have not been examined for NK cell activity.

So we contend that the resting Null T cell is not responsible for NK cell activity in pig blood. In this respect its negativity supplements the list of its similar unresponsiveness to PHA, Con A and allogeneic cells, its lack of involvement in antibody or direct cytotoxicity *in vitro*, and its lack of subset-specific markers (Binns, 1982; Outteridge *et al.*, 1982). It remains to be shown whether Null T cells are precursors of an NK cell population given appropriate stimuli *in vitro* or *in vivo*. Experiments are in progress to try and establish this and the functional role and fate of these enigmatic cells.

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