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Comparison of Cytotoxic Properties of Neonatal and Adult Neutrophils and Monocytes and Enhancement by Cytokines

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We studied cytotoxic capabilities of newborn polymorphonuclear leukocytes (PMNs) and monocytes and their enhancement by cytokines and antibodies. Umbilical cord PMNs were assessed for their ability to kill various target cells spontaneously, after activation with phorbol myristate acetate, in the presence of antiserum (antibody-dependent cellular cytotoxicity), and in the presence of dually specific antibody (heteroantibody-mediated cytotoxicity). Target cells included the K562 cell line (natural killer cell target), chicken erythrocytes (CRBCs), and herpes simplex virus-infected CEM cell lines. Newborn PMNs were equivalent to adult PMNs in their cytotoxic capacity in several cytotoxicity assays. Neither adult nor newborn PMNs lyse tumor cell targets (i.e., K562 cells) spontaneously, but both lyse K562 cells following activation with phorbol myristate acetate. Both adult and newborn PMNs lyse CRBCs and herpes simplex virus-infected CEM cells in antibody-dependent cellular cytotoxicity assays, and this lysis could be enhanced by the cytokines granulocyte-macrophage colony-stimulating factor and gamma interferon. PMN heteroantibody-mediated cytotoxicity, resulting from the use of an antibody with dual specificity to CRBCs and immunoglobulin G FcRII, was greater in newborn PMNs than in adult PMNs; however, monocyte heteroantibody-mediated cytotoxicity, resulting from the use of an antibody to CRBCs and monocyte immunoglobulin G FcRI, was lower in newborn monocytes than in adult monocytes. The percentage, but not the density, of PMNs expressing FcRII was significantly reduced in newborn PMNs compared with that in adult PMNs, while the percentages and densities of FcRI expression were equivalent in newborn and adult monocytes. We conclude that the cytotoxic capability in term newborn PMNs is equivalent to that in adult PMNs, that the activity of newborn PMNs can be enhanced by antibody and/or cytokines, and that PMNs can contribute to the newborn's ability to kill virus-infected cells.

Phagocytic cells, the polymorphonuclear leukocyte (PMN) and the monocyte, are involved primarily in the acute response to bacterial invasion (10). Their ability to phagocytize and kill microorganisms by means of an oxidative burst is their most important and best-defined function (10), although nonoxidative mechanisms may also play an accessory role in microbial killing (14).

In a viral infection, the role of phagocytic cells, particularly the PMN, is less defined. PMNs concentrate at the site of a viral infection (7, 33) and limit viral replication by release of reactive oxygen intermediates or antimicrobial peptides (7, 26). PMNs may also ingest free virus (34) or lyse a virus-infected antibody-coated cell by antibody-dependent cellular cytotoxicity (ADCC) (12).

Past studies have identified impaired cytotoxic and cytokine responses in neonatal lymphocytes, which make newborns particularly susceptible to viral infection (2, 15, 36). Only a few studies examining PMN cytotoxicity in newborns relevant to PMN antiviral activities are available (9, 11, 13). Therefore, we studied spontaneous antibody-dependent PMN cytotoxicity against several target cells, including virus-infected cells. We also studied the ability of newborn PMNs (and monocytes) to lyse target cells coated with a heteroantibody (28), a dually specific antibody in which one specificity is directed against an Fc receptor and the other is directed against the target cell.

The studies reported herein indicate that neonatal PMN cytotoxic functions are, for the most part, intact and under certain circumstances exceed those of adult cells; furthermore, these functions can be enhanced by pharmacologic means.

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MATERIALS AND METHODS

Cell preparation. PMNs and mononuclear cells were obtained from normal adult volunteers and from the umbilical cords of newborn infants from normal vaginal deliveries following term gestations. Cord blood was collected into sterile tubes and processed within 4 to 10 h of birth. Guidelines established by the UCLA Human Subject Protection Committee were followed. Leukocytes were separated from heparinized blood by mixing the blood with 15% Plasmagel (Laboratoire Roger Bellon, Neuilly sur Seine, France) and allowing the erythrocytes to settle (23, 25). Mononuclear cells and PMNs were separated from the leukocyte-rich supernatant by using Ficoll-Hypaque density gradient centrifugation. The two cell populations were collected and washed separately. Erythrocytes were lysed with cold hypotonic phosphate-buffered saline (PBS).

The cells were stained with Wright's stain to verify the composition of each fraction. Mononuclear preparations were >96% lymphocytes and monocytes, and PMN preparations were >97% neutrophils and eosinophils.

Cytotoxicity. The cytotoxicity of PMNs against K562 cells, chicken erythrocytes (CRBCs), and CEM cells infected with herpes simplex virus (HSV) type 1 was measured by chromium release (24). The K562 and CEM tumor cell lines were maintained in culture and washed extensively prior to cytotoxicity assays. Assays were done in 96-well U-bottom microtiter

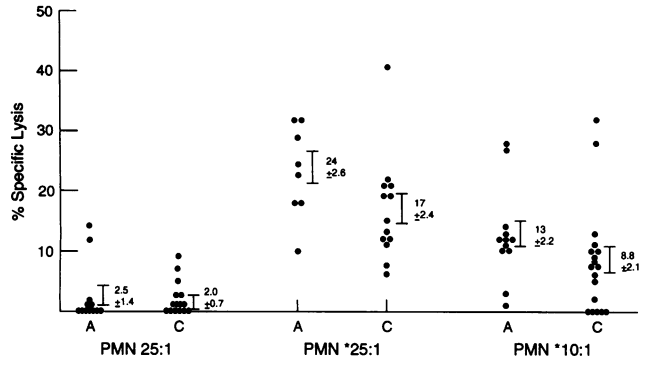

FIG. 1. PMA-activated cytotoxicity of cord and adult PMNs. Lysis of K562 cells by PMNs from adult (A) and cord (C) blood at 18 h without or with (*) activation of cells by prior incubation in PMA (see Materials and Methods). There was little lysis without activation by either cord or adult cells at a 25:1 E/T ratio. With activation, both cord and adult cells demonstrated considerable lysis at both 10:1 and 25:1 E/T ratios. The bars indicate ±1 standard error.

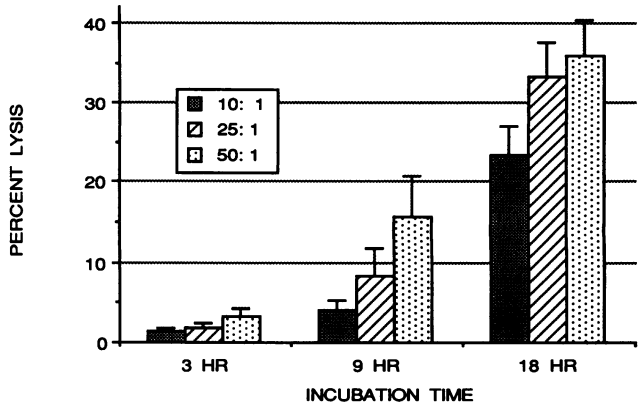

FIG. 2. PMA-activated spontaneous cytotoxicity. The effect of incubation time and E/T ratio on the degree of lysis of K562 target cells by normal adult PMNs activated by PMA. Each column represents the mean of 5 to 15 determinations made with PMNs from normal adults. The bars indicate ±1 standard error.

plates as described previously (24). PMNs were activated by incubation in 100 ng of phorbol myristate acetate (PMA) per ml for 10 min at 4°C. The cells were then washed in cold PBS and resuspended in cold RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. PMNs exposed to PMA by this procedure were fully activated when warmed to 37°C, as indicated by chemiluminescence assays. The cell suspensions were kept in an ice bath until the start of the assay, i.e., at the time they were combined with the labelled target cells. The PMN-target cell mixtures were incubated at 37°C for 18 h unless otherwise stated. We utilized various effector/target cell (E/T) ratios as indicated in the figures.

The specific lysis of target cells was calculated by the following formula: specific lysis = [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100, where cpm is counts per minute. Student's *t* test (two tailed; unpaired unless otherwise stated) was used to compare adult and cord populations.

The virus-infected CEM cells for the cytotoxicity assays were prepared by incubating the CEM cells with the MacIntyre strain of HSV (multiplicity of infection = 40) for 18 h (20). These conditions allowed 75% of the cells to express viral antigen, as indicated by fluorescence with a polyvalent anti-HSV serum. These target cells were used in an ADCC assay utilizing polyvalent rabbit anti-HSV serum diluted 1:10,000. The coated, chromated target cells were incubated for 18 h with the PMN effector cells at a 20:1 E/T ratio.

The heteroantibody experiments utilized chromated CRBCs as targets. The heteroantibodies were prepared as described by Shen et al. (29). The monocyte studies utilized the 32.2 monoclonal antibody, specific for FcRI (CD64) on monocytes, conjugated to an Fab fragment of a rabbit antibody to CRBCs and used at a final concentration of 1:80 (with an undiluted heteroantibody preparation defined as having an optical density of 0.1) (8). The ratios of effector monocytes to targets were 4:1, 2:1, and 1:1. The PMN studies utilized the IV-3 monoclonal antibody specific for FcRII (CD32) of human PMNs, conjugated to the rabbit anti-CRBC Fab fragment (8). E/T ratios of 20:1, 10:1, and 5:1 were used. The durations of incubation, based on preliminary experiments, were 18 h for the PMN heteroantibody experiments and 4 h for the monocyte heteroantibody experiments.

Flow cytometry. Adult and cord blood cells were stained with monoclonal antibodies following purification on Ficoll-Hypaque gradients. Five microliters of the appropriate fluorescein-conjugated reagent was incubated with 5×10^5 mononuclear cells for 30 min at 4°C. The antibodies used were LeuM3 (CD14), Leu 11c (CD16), HLA-DR, and Leu 15 (C3bi and CD11b), all purchased from Becton Dickinson, Inc. (Mountain View, Calif.), and monoclonal antibodies 32.2 (FcRI and CD64) and IV-3 (FcRII and CD32), prepared by one of us (M.W.F.) (8). The cells were then washed twice with 1 ml of PBS containing 2.0% fetal calf serum (Irvine Scientific, Santa Ana, Calif.) and 0.1% azide.

The fluorescent staining was analyzed on an EPICS C (Coulter Electronics, Inc., Hialeah, Fla.) flow cytometer. Electronic gates were set to enable analysis of the fluorescence of the lymphocytes or monocytes in each preparation. Dead cells and cell types other than lymphocytes or monocytes were thereby excluded from the analyses. The percentage of cells staining with each monoclonal antibody was determined by comparing each histogram with one from control cells stained with fluorescein-labeled myeloma proteins of the same isotype.

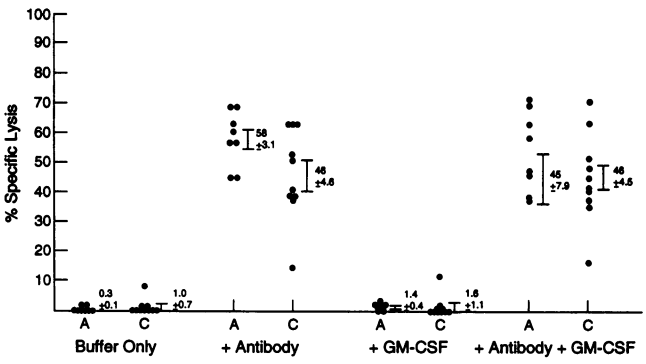

FIG. 3. Antibody-dependent cytotoxicity of cord and adult PMNs and the effect of GM-CSF. Lysis of CRBCs by PMNs from adult (A) and cord (C) blood with the addition of buffer alone, specific antibody (rabbit anti-CRBC at 1:10,000), GM-CSF (50 pM), and the combination of specific antibody and GM-CSF. The E/T ratio was 5:1, and the PMNs and CRBCs were incubated together for 18 h. The bars indicate ±1 standard error.

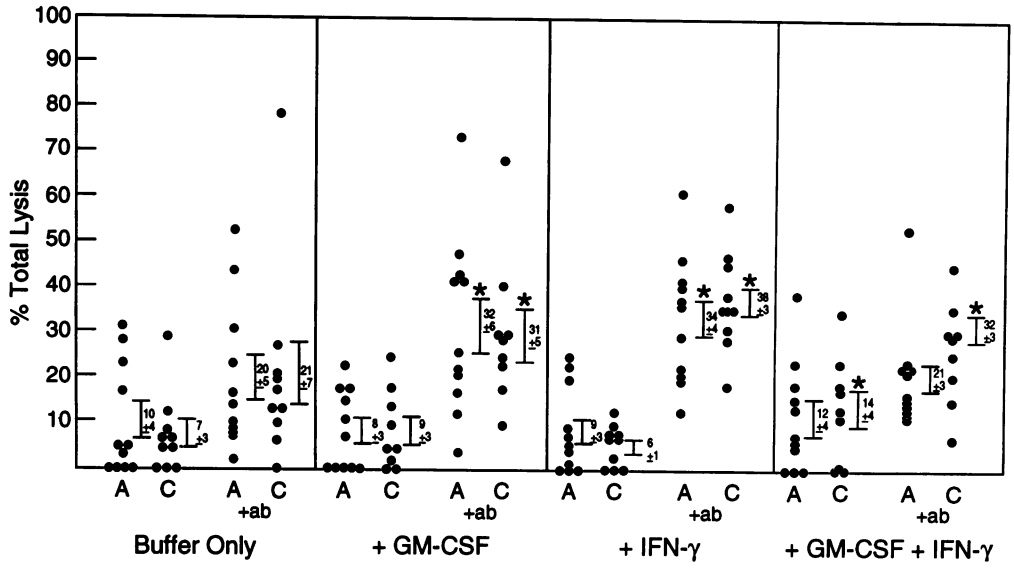

FIG. 4. Antiviral spontaneous and antibody-dependent cytotoxicity of adult and cord PMNs. Lysis of HSV-infected CEM cells by adult (A) and cord (C) PMNs in the absence or presence of specific antibody. The E/T ratio was 50:1, and the time of incubation was 18 h. The bars indicate means ± 1 standard error. The asterisks indicate a P of <0.05.

Relative linear fluorescence intensity was determined by calculating 1.02712^n for each sample, where n is the mean channel number and 1.02712 is the theoretical base. Net relative linear fluorescence intensity was determined by subtracting the value for the isotype control from that for the corresponding sample (27).

RESULTS

Cytotoxicity. Spontaneous PMN cytotoxicity without activation was minimal (Fig. 1) at any E/T ratio for either adult or cord blood against K562 cells. However, activation after exposure to PMA resulted in strong and equivalent cytotoxicities of both adult and newborn PMNs at either a 10:1 or a 25:1 E/T ratio against this target. The degree of activated PMN cytotoxicity at 18 h approached that of spontaneous (natural killer) mononuclear cell cytotoxicity at 4 h. Similar results were noted when HSV-infected CEM cells were used for targets (data not shown).

PMN cytotoxicity proceeds considerably more slowly than lymphoid (natural killer)-mediated cytotoxicity (22). Maximal lysis by activated PMNs of K562 cells required 18 h even when high E/T ratios were employed, as shown in Fig. 2. This delay is not due to delayed activation since this is achieved as soon as the PMNs are warmed to 37°C (24). This slower onset and time of activated PMN lysis was also noted for PMN ADCC against CRBCs and contrasts with spontaneous mononuclear cell (natural killer) activity, which is maximal in 4 to 6 h (data not shown).

PMN ADCCs directed against antibody-coated CRBCs were equivalent in newborn and adult PMNs (Fig. 3). Human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; gift of Judith Gasson, University of California, Los Angeles). GM-CSF did not enhance ADCC activity. The degree of ADCC lysis is equivalent to that achieved by PMA-activated PMNs against this target (24).

Figure 4 shows the cytotoxicities of adult and cord PMNs against HSV-infected CEM cells alone (spontaneous) or in the presence of anti-HSV antibody (ADCC) and the effect of cytokines. The antibody had no effect on augmenting cytotoxicity against noninfected CEM cells (not shown). The presence of anti-HSV antibody increased the cytotoxicity of both adult and cord cells in medium alone or in the presence of the cytokines GM-CSF or gamma interferon by paired t test. The cytokines alone or in combination did not enhance the spontaneous cytotoxicity of the adult PMNs but did enhance their ADCC activity. The combination of GM-CSF and gamma interferon also enhanced the spontaneous and ADCC activity of the cord cells.

The next group of cytotoxic experiments utilized heteroantibody with dual specificity for CRBCs and either the immunoglobulin G Fc receptor on monocytes (FcRI) or PMNs (FcRII). Figure 5 shows that efficient cytotoxicity could be achieved with heteroantibody by using either PMNs (Fig. 5A)

TABLE 1. Fc receptors on cord and adult mononuclear and polymorphonuclear phagocytes

Type of phagocytes	Monoclonal antibody	Cord cells		Adult cells	
		% ± SE (n)	Density[a] ± SE (n)	% ± SE (n)	Density ± SE (n)
Polymorphonuclear	IV-3 (FcRII-CD32)	81 ± 4.5 (7)[b]	8.7 ± 3.4 (6)	94 ± 1.7 (9)	6.2 ± 2.7 (6)
Mononuclear	32.3 (FcRI-CD64)	69 ± 6.0 (10)	4.3 ± 1.5 (11)	76 ± 2.8 (6)	6.4 ± 1.5 (5)

[a] Net relative linear intensity.
[b] Significantly different from adult cells ($P < 0.01$).

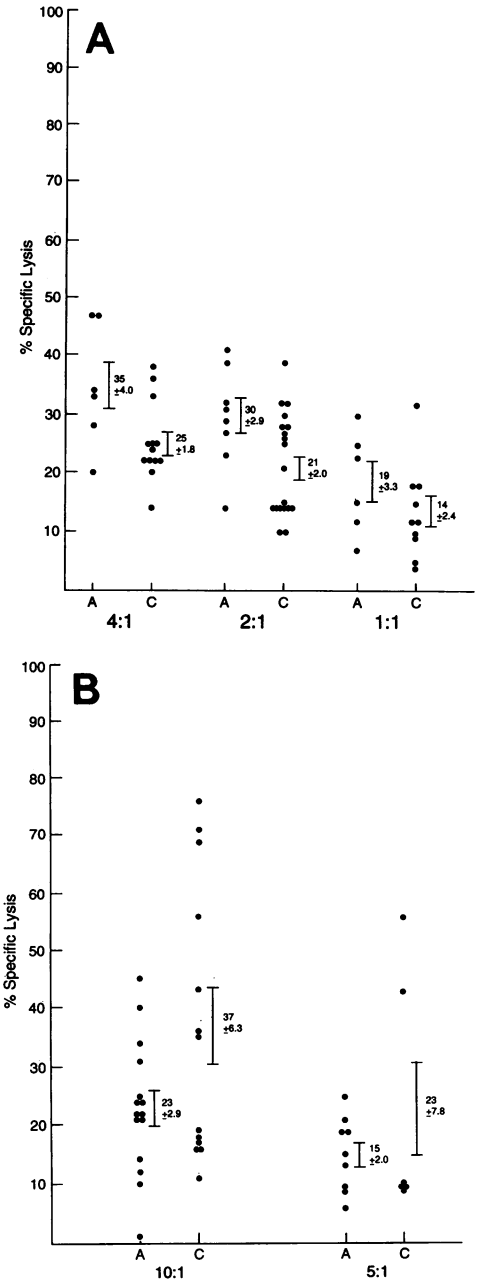

FIG. 5. Heteroantibody-mediated cytotoxicity against CRBCs by newborn and adult PMNs and mononuclear cells. (A) PMN cytotoxicity against CRBCs in the presence of a dually specific antibody (heteroantibody to CRBCs and FcRII). Newborn PMNs had significantly higher cytotoxicity ($P < 0.05$) than adult PMNs at both 10:1 and 5:1 E/T ratios. (B) Mononuclear cell cytotoxicity against CRBCs in the presence of heteroantibody to CRBCs and monocyte FcRI. Adult monocytes had significantly higher cytotoxicity ($P < 0.05$) than neonatal PMNs at 4:1 and 2:1 E/T ratios. The bars indicate ±1 standard error. Abbreviations on *x* axis: A, adult PMNs; C, newborn cord PMNs.

or monocytes (Fig. 5B) as effector cells from either adult or cord blood. Heteroantibody-mediated cytotoxicity was somewhat higher for cord PMNs at both E/T ratios. GM-CSF (500 pM) increased adult PMN heteroantibody cytotoxicity from 23% ± 2.9% to 60% ± 7.5% at a 10:1 E/T ratio and from 15% ± 2.0% to 58% ± 6.3% at a 5:1 E/T ratio and increased cord PMN heteroantibody-mediated cytotoxicity from 37% ± 6.3% to 41% ± 5.8% at a 10:1 E/T ratio and from 23% ± 7.8% to 39% ± 1.5% at a 5:1 E/T ratio.

Fc receptor expression. To determine if ADCC differences in adult and newborn cells were related to Fc receptor expression, we studied the percentage and density (a semiquantitative estimate of the number of receptors on each cell) of Fc receptors (Table 1). Newborn PMNs had a significantly lower percentage of cells expressing FcRII than adult PMNs but a density equivalent to that of adult cells, while newborn and adult monocytes had equivalent numbers and densities of cells expressing FcRI.

DISCUSSION

PMNs play a crucial role in the newborn's defenses against bacteria and may play a supportive role in the defenses against virus infection (10). Furthermore, if immaturity or infection compromises neonatal T-cell defenses, PMNs, in conjunction with passive maternal antibody, may be particularly crucial in mounting a successful challenge to virus invasion. The studies conducted indicate that PMN cytotoxicity, particularly ADCC, is well developed in the newborn period.

The current study, in agreement with Roberts et al., shows that unstimulated PMNs have only minimal ability to kill target cells of any type (22). However, upon maximal activation by PMA (which binds to intracellular protein kinases [18]), both newborn and adult PMNs become highly cytotoxic to target cells. PMA-activated PMN cytotoxicity was initially described by Clark and Klebanoff in 1975 with tumor cell line targets (6). PMA stimulates oxidative burst activity which contributes to cytotoxicity; indeed, PMA-induced PMN cytotoxicity is reduced in patients with chronic granulomatous disease, whose cells cannot generate oxygen intermediates (22).

Our findings of normal neonatal PMA-induced cytotoxicity agree with observations that PMA-induced chemiluminescence or nitroblue tetrazolium dye reduction of normal newborn PMNs is equivalent to that of adult PMNs (10).

It is of interest that PMN cytotoxicity is slower than spontaneous mononuclear cytotoxicity inasmuch as PMN activation following PMA is rapid. It is possible that the initial hit is rapid but the lesion is less lethal than the perforin-mediated event in NK cytotoxicity (35) and, therefore, cell lysis is delayed. Alternatively, apoptosis, or programmed cell death, may be initiated by the PMNs.

Only a few studies of neonatal PMN ADCC are available, but these agree with our finding that newborn PMN ADCC is equivalent to adult PMN ADCC. Kohl et al. found no differences between adult PMNs and cord blood PMNs by using antibody-coated HSV type 1-infected Chang liver cells (13). Hashimoto et al. found equivalent ADCC activities for cord blood and adult PMNs when influenza virus-infected BHK-21 cells were used (9). Kohl noted a fall in PMN ADCC activity during the first year of life and suggested possible activation of PMN ADCC in cord cells (11). Nevertheless, the maturational cytotoxic defect in neonatal PMNs is of considerably less magnitude than that present in neonatal lymphocyte-mediated cytotoxicity involving natural killer, antibody-dependent, or cytotoxic T-cell function (19).

We found that GM-CSF had no effect on either spontaneous or antibody-dependent lysis of CRBCs by adult or newborn PMNs (Fig. 3). However, the combination of GM-CSF and gamma interferon did increase the spontaneous cytotoxicity of cord PMNs to HSV-infected CEM cells. GM-CSF and gamma interferon also enhanced the anti-HSV ADCC response of both adult and cord cells. These findings are compatible with

observations that GM-CSF primes cells for enhanced activation by a second stimulus (33). Newborn PMNs may be in a slightly activated state, permitting enhanced cytokine augmentation of lysis. Baldwin et al. (1) and Szelc et al. (32) reported that GM-CSF augmented PMN ADCC in adult subjects against human immunodeficiency virus-coated or human immunodeficiency virus-infected target cells.

PMN ADCC is dependent on immunoglobulin G Fc receptor, particularly FcRII, expression (29). Pross et al. (21), using a rosetting technique, found no difference in expression of Fc receptors in cord and adult cells. Masuda et al. (17), using a similar technique, noted a significantly decreased Fc expression in cord cells (35% ± 8%) compared with that in adult PMNs (60% ± 4%). Rosetting, however, is not a good measure of Fc receptors since this assay is influenced by several factors. Smith et al. (30), using flow cytometry, found diminished expression of both resting and formylmethionylleucyl proline-stimulated FcRIII (CD16) expression in neonatal PMNs. By contrast, Carr and Davies noted that term newborn PMNs had levels of both FcRII and FcRIII expression equivalent to adult levels (4) and levels of soluble FcRIII receptor in the plasma of term newborns and adults (5).

Our findings of strong newborn PMN heteroantibody-mediated cytotoxicity by utilizing a dually specific (hetero)antibody to FcRII and CRBCs are congruent with good expression of this receptor in newborn PMNs. After engagement and activation, the cytotoxic mechanism in newborn PMNs provides a lethal hit equivalent to that of adult cells. This lethal hit is further intensified by GM-CSF.

The diminished heteroantibody cytotoxicity of monocytes of newborn cells mediated through FcRI is congruent with the results of other studies showing impaired neonatal monocyte function, including chemotaxis, and decreased bactericidal capacity (16, 31). However, there is the potential that monocyte-mediated cytotoxicity can be increased with cytokines, possibly by increasing Fc receptor expression.

In summary, these experiments provide evidence that neonatal PMNs have a cytotoxic potential equivalent to that of adult PMNs, including virus-infected targets. This function may be enhanced by cytokines that increase PMN cytotoxicity, antibodies that promote ADCC, and heteroantibodies that increase specificity of PMN killing. Further studies on premature and stressed infants who have impaired cytotoxic function are indicated.

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