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Ability of Human Cord Blood Lymphocytes to Mediate Antibody-Dependent Cellular Cytotoxicity Against Influenza Virus-Infected Cells

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Cord blood lymphocytes, monocytes, and neutrophils from newborns were shown to mediate antibody-dependent cellular cytotoxicity (ADCC) against influenza virus-infected cells. Antibody mediating ADCC was detectable in cord plasma, indicating that all components necessary for ADCC against influenza virus-infected cells are present in newborns. Among adult lymphocytes, two effector cell populations of influenza ADCC are recognized: non-T and Tγ cells. Each of these cell types expresses an antigen recognized by monoclonal HNK-1 antibody. The proportion of HNK-1 antigen-positive lymphocytes in cord blood was markedly lower than in adult blood; furthermore, ADCC was mediated by cord blood lymphocytes which were HNK-1 negative. By lymphocyte fractionation, the effector lymphocytes in cord blood were, as in adults, non-T and Tγ cells, suggesting that HNK-1 antigen is not expressed on these cell lineages in newborns.

We have recently shown that three major immunocompetent cell types in adult peripheral blood (lymphocytes, monocytes, and neutrophils) mediate antibody-dependent cellular cytotoxicity (ADCC) against influenza virus-infected cells (G. Hashimoto, P. F. Wright, and D. T. Karzon, J. Infect. Dis., in press). The effector lymphocytes were characterized as E rosette-forming and -nonforming cells which bear immunoglobulin G Fc receptors and express an antigen recognized by a monoclonal antibody, HNK-1. The surface HNK-1 antigen is a marker for human natural killer and ADCC effector lymphocytes (1). Abo et al. (2) have recently shown that a low percentage of peripheral blood lymphocytes express the surface HNK-1 antigen in neonates. Using K-562 cells to measure natural killer activity and sensitized chicken erythrocytes to measure ADCC, they found that neonatal lymphocytes mediate only low levels of cytotoxicity. There are conflicting observations on the ability of human neonatal lymphocytes to mediate ADCC (4, 8, 10, 11, 14) and natural killer cytotoxicity (3, 7, 13, 17). In the present study cord blood lymphocytes were only slightly less effective than adult lymphocytes in mediating ADCC against influenza virus-infected cells. This suggests that neonatal lymphocytes, although not expressing the HNK-1 antigen, can mediate ADCC in a viral system.

MATERIALS AND METHODS

Cell preparation. Umbilical cord blood was obtained from 11 normal newborns at delivery. Peripheral blood was obtained form seven healthy adult donors. Plastic-nonadherent lymphocytes, monocytes (plastic adherent), and neutrophils were prepared as previously described (Hashimoto et al., in press). Briefly, heparinized blood was subjected to Ficoll-Hypaque density centrifugation. Mononuclear cells were taken from the interface and separated into adherent and nonadherent populations by being placed for 2 h at 37°C in a plastic flask precoated with fetal calf serum. Neutrophils were obtained from the Ficoll-Hypaque pellet, with contaminating red cells lysed hypotonically.

Plastic-nonadherent and nylon wool-passed lymphocytes were depleted or enriched for HNK-1 antigen-expressing cells. The monoclonal HNK-1 antibody was kindly supplied by T. Abo, University of Alabama, Birmingham. To deplete HNK-1-positive cells, 3×10^6 lymphocytes were incubated with 200 μl of HNK-1 antibody (250 μg/ml) for 20 min at room temperature. Fresh rabbit serum was then added at a final dilution of 1:8, and incubation was carried out for 60 min more at 37°C.

HNK-1-positive cells were enriched by adherence to an antibody-coated plastic dish. A plastic dish (no. 3506; Costar, Cambridge, Mass.) was filled with 1 ml of HNK-1 antibody (1 mg/ml), left overnight at 4°C, and washed well with phosphate-buffered saline before use. Lymphocytes (2×10^7) were allowed to adhere to the antibody-coated dish at 37°C for 60 min. After removal of nonadherent cells, adherent cells were scraped, washed once, and used in the assay. Cord

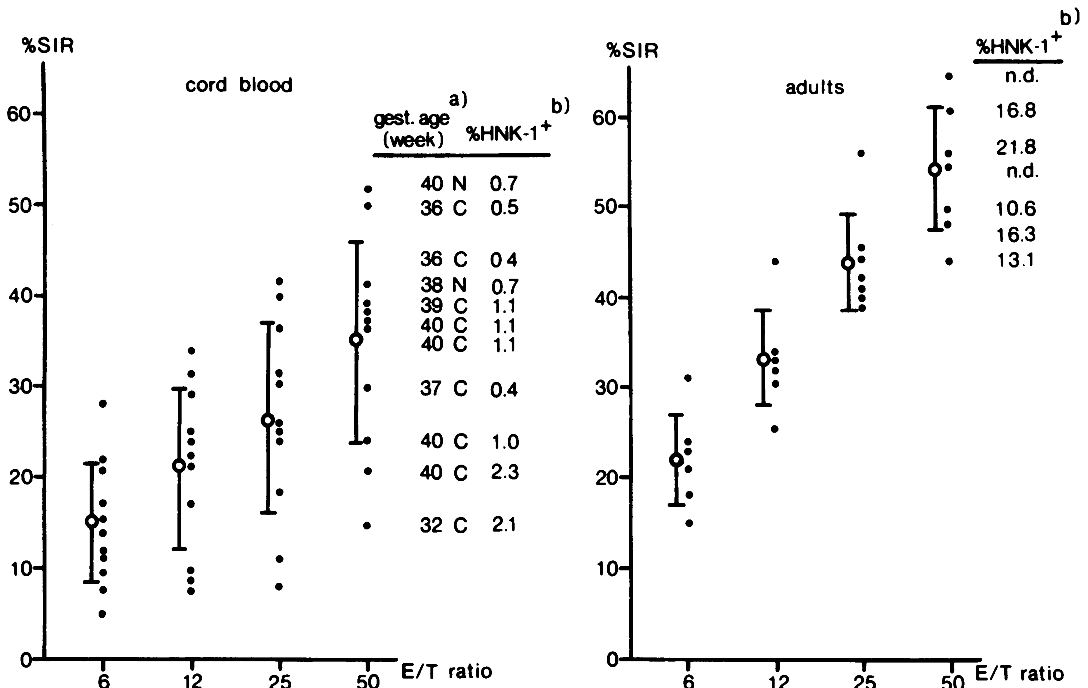

FIG. 1. ADCC activity and percentage of HNK-1-positive cells of cord blood lymphocytes and adult peripheral blood lymphocytes. Each dot represents the percentage of SIR of an individual sample, and open circles and bars represent mean ± standard deviation. (a) Gestational age (weeks) and types of delivery. N represents normal delivery, and C represents Caesarean section. (b) Percentage of HNK-1-positive cells. n.d., Not determined.

blood lymphocytes were separated into T and non-T cells with E rosette fractionation (5), using neuraminidase-treated sheep erythrocytes as E (5). Each cell population was suspended in RPMI 1640 with 10% agamma calf serum (KC Biologicals, Lenexa, Kansas).

Virus. The ADCC assay was performed by using ^{51}Cr-labeled and influenza A/Alaska/6/77 (H3N2) virus-infected BHK-21 cells, as described previously (Hashimoto et al., in press). Briefly, 100 μl of infected cells (5×10^3) was incubated with 25 μl of influenza A H3N2 immune human serum and 100 μl of effector cells at effector/target (E/T) ratios of from 6 to 50 for 6 h at 37°C. The percentage of specific immune release (SIR) = $(A - B)/(M - B) \times 100$, where A is the count from the cell mixture with antibody, B is the count from the cell mixture without antibody, and M is the average count of maximum release. Samples were assayed in triplicate, or in duplicate when the number of effector cells was insufficient.

Immunofluorescence assays. Cells reactive with HNK-1 antibody were enumerated by an indirect immunofluorescence assay as described elsewhere (1). The secondary antibody was fluorescein isothiocyanate-conjugated goat anti-mouse μ chain-specific antibody (Cappel Laboratories, Cochranville, Pa.) absorbed with washed, pooled human lymphocytes.

RESULTS

Although the mean ADCC activity of cord blood lymphocytes was significantly lower than that of adult lymphocytes at all E/T ratios ($P < 0.01$ at E/T = 50, 25, and 12, and $P < 0.05$ at E/T = 6), cord blood lymphocytes from all 11 newborns showed considerable ADCC activity against influenza virus-infected cells, with 14.7 to 52.0% SIR at an E/T ratio of 50:1 (Fig. 1). Additionally, in Fig. 1, the gestational age and percentage of HNK-1-positive cells for each newborn's cord blood is shown ranked by his or her percentage of SIR at an E/T ratio of 50:1.The percentage of HNK-1-positive cells in cord blood was strikingly lower (1.0 ± 0.6% [mean ± standard deviation]) than in adult peripheral blood (15.7 ± 3.8%). The lowest SIR was seen

TABLE 1. Effect of cord blood lymphocytes on ADCC activity of adult lymphocytes

No. of adult lymphocytes ($\times 10^5$)	No. of cord blood lymphocytes added ($\times 10^5$)	% SIR (mean ± SD)
5	0	64.2 ± 1.6
5	0.3	64.3 ± 0.7
5	0.6	61.8 ± 2.1
5	1.2	62.5 ± 0.4
5	2.5	61.1 ± 1.4
0	2.5	36.6 ± 2.6
0	5.0	43.1 ± 0.6

TABLE 2. Effect of monocytes on lymphocyte-mediated ADCC

Effector cells	% Monocytes[a]	% SIR at the following E/T ratio:				
		100	50	25	12	6
Nonadherent	3.3 ± 0.8[b]	49.9	40.1 ± 11.4[b]	31.5 ± 9.7	25.3 ± 9.6	16.5 ± 9.2
Nonadherent NWP[c]	0.8 ± 0.3	50.4	41.1 ± 7.2	33.5 ± 7.0	29 ± 5.5	20.2 ± 6.8
Adherent	89.7 ± 2.7	13.4	19.1 ± 7.0	14.3 ± 5.3	8.9 ± 4.5	3.8 ± 3.5

[a] Determined by Giemsa and esterase staining.
[b] Mean ± SD of four experiments.
[c] NWP, Nylon wool-passed lymphocytes.

TABLE 3. ADCC activity of cord blood and adult neutrophils

Neutrophil type	% SIR (mean ± SD) at the following E/T ratio:				
	100	50	25	12	6
Cord blood (*n* = 5)	46.9 ± 10.2	36.7 ± 6.9	25.9 ± 4.9	13.4 ± 2.9	5.1 ± 1.8
Adult (*n* = 6)	40.9 ± 11.8	31.2 ± 9.0	25.6 ± 7.2	17.9 ± 7.2	11.5 ± 3.4

TABLE 4. ADCC activity of HNK-1-positive cell-depleted fraction

Donors	Treatment	% HNK-1$^+$ cells (mean ± SD)	% SIR (mean ± SD) at the following E/T ratio:			
			50	25	12	6
Newborns (*n* = 5)	None	0.7 ± 0.34	36.9 ± 9.1	25.8 ± 13.2	23.4 ± 9.3	16.8 ± 7.2
	Complement alone	0.6 ± 0.3	34.5 ± 11.7	26.7 ± 12.4	20.1 ± 0.8	14.8 ± 5.8
	HNK-1 + complement	0.1 ± 0.1	35.1 ± 7.9	26.7 ± 8.1	20.1 ± 8.1	14.4 ± 6.1
Adults (*n* = 4)	None	15.5 ± 4	58.7 ± 8.1	46.2 ± 7	35.5 ± 6.4	24 ± 5.5
	Complement alone	12.2 ± 1.7	54.4 ± 3.8	48.9 ± 5.8	33.2 ± 5.4	26.1 ± 7.0
	HNK-1 + complement	0.6 ± 0.2	16.7 ± 4.5	11.5 ± 3.5	7.2 ± 2.1	4.2 ± 1.2

with a premature infant born at 32 weeks of gestation, but further studies would be necessary to determine an effect of gestational age or type of delivery on ADCC activity as has been suggested with herpesvirus ADCC (6). Cord blood lymphocytes did not suppress the cytotoxicity of adult lymphocytes (Table 1), nor was cord blood cytotoxicity diminished by adherence to fetal calf serum-coated plastic plates and subsequent passage through a nylon wool column (steps designed to eliminate as many contaminating monocytes as possible from the lymphocyte population) (Table 2). Neutrophils (Table 3) and, to a lesser extent, monocytes (Table 2) from cord blood mediate ADCC. A considerable amount of antibody capable of mediating ADCC was detectable in each sample of cord blood. Cord blood serum with adult lymphocytes, at an E/T ratio of 100:1, could be diluted to from 1:90 to 1:3,780 before SIR dropped below 10%.

Depletion of HNK-1-positive cells by treatment with HNK-1 antibody and complement has previously been shown to diminish ADCC activity in adult peripheral blood lymphocytes (Hashimoto et al., in press). Therefore, cord blood and adult lymphocytes were depleted of HNK-1-positive cells by complement lysis (Table 4). Greater than 90% of HNK-1-positive cells were effectively eliminated from adult lymphocytes, with a concomitant decrease in ADCC activity. In cord blood, the HNK-1-positive cells were reduced to the lower limit of detection, i.e., less than 1 HNK-1-positive cell in 200 lymphocytes, without an alteration in the SIR at multiple E/T ratios. Treatment with complement or HNK-1 antibody alone did not alter the cytotoxicity (the latter data not shown).

Enrichment of HNK-1-positive cells was successfully accomplished with adult lymphocytes by using HNK-1 antibody-coated plates, with the anticipated rise in ADCC activity (Table 5). Insufficient numbers of cord blood lymphocytes attached to the HNK-1 antibody-coated plates to allow analysis, another indication of the lack of expression of this antigen in cord blood.

Two cord blood samples were fractionated into non-T and T cells. It had previously been shown with adult lymphocytes that ADCC activity was present in both fractions. That appeared to be the case in cord blood lymphocytes as well (Table 6), although the relatively low SIR in the

TABLE 5. ADCC activity of adult lymphocytes enriched for HNK-1-positive cells

Expt	Effector	% HNK-1+ cells	% SIR (mean ± SD) at the following E/T ratio:			
			50	25	12	6
1	Nonadherent + NWP[a]	18.9	59.7 ± 0.9	41.1 ± 1.0	27.0 ± 0.9	18.8 ± 0.6
	HNK-1+ cell rich	85.4	80.8 ± 4.7	69.8 ± 2.5	54.5 ± 1.6	ND[b]
2	Nonadherent + NWP	19.5	54.1 ± 1.7	41.9 ± 1.3	29.6 ± 1.9	18.9 ± 1.7
	HNK-1+ cell rich	78.8	ND	77.8 ± 3.5	50.0 ± 1.8	38.9 ± 3.1

[a] Plastic nonadherent and nylon wool-passed lymphocytes.
[b] ND, Not determined.

TABLE 6. ADCC activity of T and non-T cell fractions of cord blood lymphocytes

Case no.	Lymphocytes	% E-RFC[a]	% SIR (mean ± SD) at the following E/T ratio:				
			100	50	25	12	6
5	Unfractionated	ND[b]	ND	38.8 ± 0.2	25.8 ± 3.5	22.5 ± 1.9	15.6 ± 0.2
	T	92.7	ND	16.4 ± 1.0	8.1 ± 1.1	6.6 ± 0.4	4.1 ± 1.6
	Non-T	1.0	ND	39.6 ± 1.1	28.1 ± 1.2	21.6 ± 1.1	17.8 ± 0.6
6	Unfractionated	ND	49.9 ± 1.7	39.0 ± 1.4	25.4 ± 0.3	17.2 ± 0.4	9.6 ± 0.4
	T	94.1	29.1 ± 2.8	13.8 ± 1.9	13.8 ± 1.6	5.5 ± 3.9	ND
	Non-T	1.2	50.0 ± 0.7	39.6 ± 3.5	30.3 ± 1.6	21.7 ± 1.3	13.4 ± 0.8

[a] Percentage of E rosette-forming cells.
[b] ND, Not determined.

T cell population did not preclude it being due to contaminating non-T cells.

DISCUSSION

In contrast to the results of our experiments with adult lymphocytes (Hashimoto et al., in press), HNK-1 antigen was not a marker for ADCC effector lymphocytes in cord blood. Although ADCC activity approaching the levels of adult cells was demonstrated by cord blood lymphocytes, HNK-1-positive cells were rare. Herpes simplex virus-specific ADCC activity of cord blood lymphocytes has been demonstrated (8, 10, 14). However, in a number of nonviral systems, cord blood lymphocytes demonstrate diminished activity when compared with adult cells (2–4, 11, 13, 17). The apparent difference between viral and nonviral systems appears most likely to be due to differing assay systems, many of which employed small numbers of specimens and low E/T ratios, rather than to a fundamental difference in cytotoxic behavior in viral and nonviral systems.

Our observations suggest that lymphocyte populations other than those in the HNK-1-positive lineage mediate ADCC in cord blood or that the HNK-1 antigen is not fully expressed in neonates. Cells mediating natural killer and ADCC reactions have a high cytoplasmic/nuclear ratio and azurophilic granules in the cytoplasm and are called large granular lymphocytes (12, 16). Although we did not attempt to quantitate this cell type, others have reported equivalent percentages of these cells in cord and adult mononuclear cells (15). This observation coupled with our results that, as in adults, neonatal lymphocytes mediating ADCC are in both null cell and Tγ cell populations suggests that cells in the same lineage mediate ADCC at both ages. HNK-1 antigen expression may be a postnatal maturational event.

All of the components necesssary to mediate ADCC should be present in neonates. In spite of this, in an experimental herpes simplex virus infection of newborn mice, adult mononuclear cells and antibody were more effective at preventing disease than were neonatal cells with the same antibody (9). Further such experiments designed to test the competency and importance of the ADCC defense mechanism in both neonates and adults are needed.

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