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Comparison of T Cell Functional Changes during Childhood with the Ontogeny of CDw29 and CD45RA Expression on CD4+ T Cells

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ABSTRACT. The ontogeny of the peripheral blood mononuclear cells' responsiveness to various activators during childhood was studied and compared to the expression of CDw29 and CD45RA molecules at the surface of CD4+ T cells. The results show that newborn peripheral blood mononuclear cells are characterized by a responsiveness to mitogens that is higher than that observed in adults, at least shortly after stimulation. This contrasts with a clear decreased response to CD2 and CD3 MAb at any time after stimulation. These functional characteristics correlate with a low density of CDw29 antigen on virtually all CD4+ T cells and a high density of CD45RA antigen on most CD4+ T cells at birth. These patterns of reactivity and phenotype are similar to those found among naive adult T cells. When ageing, the response to mitogens becomes rapidly similar to the adult's values, whereas the responses to CD2 or CD3 MAb are more gradually acquired. This slow rate of functional changes grossly parallels the increase of CDw29+ CD4+ and the decrease of CD45RA+ CD4+ T cell subsets. These changes finally lead to the immunophenotypic and functional characteristics that are typical of adult memory T cells. These results suggest that iterative antigenic stimulations both induce memory T cells and create the conditions to improve the overall immune competence. (*Pediatr Res* 32: 81–86, 1992)

Abbreviations

CD, cluster of differentiation
PBM, peripheral blood mononuclear cell
PHA, phytohemagglutinin
PWM, pokeweed mitogen
(3H)TdR, tritiated thymidine

The immune system of human newborns at term is partly immature, as determined by studying Ig synthesis, natural killer activity, and cutaneous reactions of delayed hypersensibility. This immaturity in both humoral and cell-mediated immunity (1–8) is likely to contribute to an increase in the newborn's vulnerability to infections.

During an immune response, T cells interact with foreign antigens through T cell receptors of appropriate specificity (9, 10). The CD3 molecule, which is the nonpolymorphic part of the "T cell receptor complex," transduces activation signals leading to the T cell functions. Among the T lymphocytes, CD4+ T helper cells are specialized in producing T cell growth factors such as IL2. The binding of IL2 to the newly synthesized IL2 receptor (11) induces T lymphocyte proliferation. Autocrine proliferation of T cells can also be triggered through the CD2 molecule (12), which is the receptor for sheep red blood cells, expressed by all T lymphocytes. This alternative antigen-independent activation pathway is presumed to play a role in the thymic maturative pathway (13).

The development of MAb directed against the CD2 molecule or CD3/T cell receptor has proven to be very helpful for exploring the function of different T cell subsets in adults (9, 14, 15). Indeed, two cell types have been described by virtue of their responsiveness to these MAb. These two cell subsets express differentially CD45RA and CDw29 antigens at their surface and might be related to different stages of maturation (16).

In the newborn, several different experimental systems have been used to study the helper T cell function. However, until recently, the immaturity of neonatal T cells has been difficult to substantiate. Therefore, in the present study, to get further insight into the characteristics of T cells in the newborn and the maturation of this subset after birth, we tested from birth to adulthood the T cell proliferation induced by various reagents, including mitogens, alloantigens, and MAb specific for CD3 or CD2 antigens. Additionally, we tested the relationship between the functional changes and the ontogeny of CD45RA and CDw29 expression on CD4+ T cells.

MATERIALS AND METHODS

Subjects. Children's blood samples were either cord blood from healthy newborns ($n = 8$) or residues of peripheral blood from infants devoid of immunologic disorders who were admitted to hospital for orthopedic or plastic surgery ($n = 55$). Adult blood samples were collected from healthy volunteer donors who were more than 25 y old ($n = 12$).

Cell preparation. Resting PBM were obtained as follows. Cord heparinized blood samples were collected by puncture from the umbilical vein before delivery in uncomplicated pregnancies at term. Newborn children or adult heparinized blood samples were collected by venipuncture. PBM were isolated by Ficoll-Hypaque density gradient centrifugation and resuspended in RPMI culture medium. All PBM from each PBM sample were then frozen and kept in nitrogen according to a standard procedure until use.

When indicated, activated T cells rather than resting PBM were used, and they were obtained as follows. One million PBM were seeded into flat-bottomed 24-well plates (linbro, Labsystem, France) in 1 mL culture medium. They were stimulated by PHA

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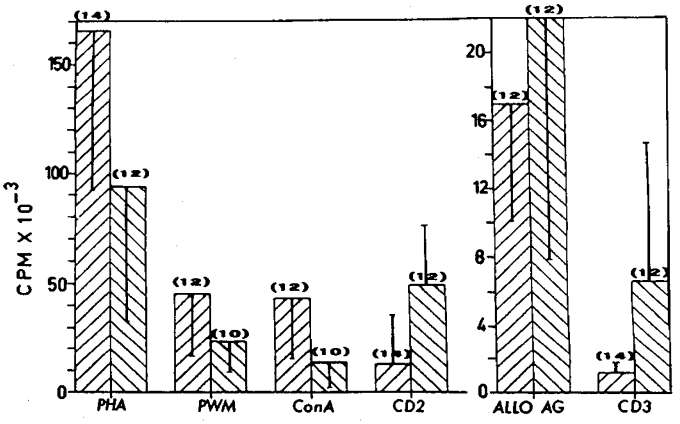

Fig. 1. Functional characteristics of newborn PBM. PBM reactivity to PHA, PWM, concanavalin A (*ConA*), CD2 MAb (*CD2*), CD3 MAb (*CD3*), or alloantigens (*ALLO AG*) was assessed as indicated in Materials and Methods. Results are given as mean value of (3H)TdR incorporation in newborn (▨) and in adult (▧) with SD. The number of infants and adults studied for each stimulator is indicated in *parentheses*.

at a concentration of 10μg/mL for 48 h. After this time, recombinant IL2 (Boehringer, France) was added at a final concentration of 10 IU/mL during 5 additional days. Activated T cells were then recovered from these primary cultures and washed twice in RPMI before being tested for their phenotype and capacity to respond to CD2 MAb.

MAb. MAb used for stimulation were either CD3(CD3×3) of IgG2a subtype that is specific for CD3 molecule or a combination pair of CD2 MAb specific for CD2 molecule and made of CD2(CD2×11) and CD2(D66), both of IgG2a subtype. These MAb were provided by Professor A. Bernard (Nice, France). The requirements for T cell proliferation induced by these MAb in the adult have been extensively characterized elsewhere (17). They were used as filtered (0.22 μm) ascitic fluids at a concentration found to be optimal in adults. MAb used in immunofluorescence studies were fluorochrome conjugated. CD4 and CD45RA MAb were purchased from Becton-Dickinson (Grenoble, France) and CDw29 MAb was purchased from Coulter (Margency, France).

Cell proliferation. PBM (500 000/mL) were cultured in triplicate in round-bottomed microtiter plates in RPMI culture medium supplemented with glutamine (1% vol/vol), antibiotics, and heat-inactivated, pooled human AB sera (20% vol/vol). They were activated by PHA (Wellcome SA, Vallebone, France; 10 μg/mL), concanavalin A (Sigma, St. Quentin Fallavier, France; 10 μg/mL), PWM (Sigma; 10 μg/mL), or MAb at a final dilution of 1% of ascitic fluids each. Unless otherwise indicated, (3H)TdR was added on d 3. The plates were incubated in a 5% CO_2 humidified atmosphere at 37°C. Cell proliferation was measured by (3H)TdR incorporation (Amersham, les Ullis, France; sp act 5 mCi) during the last 6 h of culture. Cells were then harvested with a cell harvester (Skatron, Lier, Norway) and counted in a liquid scintillation counter (Beckman, Fullerton, CA). Mixed leukocyte reactions were performed according to a standard procedure. Briefly, 50 000 PBM were seeded into round-bottomed microtiter plates with 50 000 allogeneic, irradiated (2600 rad) PBM in a total volume of 200 μL culture medium. (3H)TdR was added on the 5th d for 18 h.

Double label immunofluorescence. An FITC-conjugated MAb was used on one arm, and on the other a phycoerythrin-conjugated MAb was used. After staining according to manufacturer's recommendations, samples were analyzed for two-color fluorescence with a FACStar plus (Becton Dickinson, San Jose, CA). Cells were gated on the lymphocyte population according to their size and granulation content. Red fluorescence (phycoerythrin) and green fluorescence (FITC) were analyzed at 488 nm; red into green and green into red spectral overlap was compensated using the FACStar plus compensation controls.

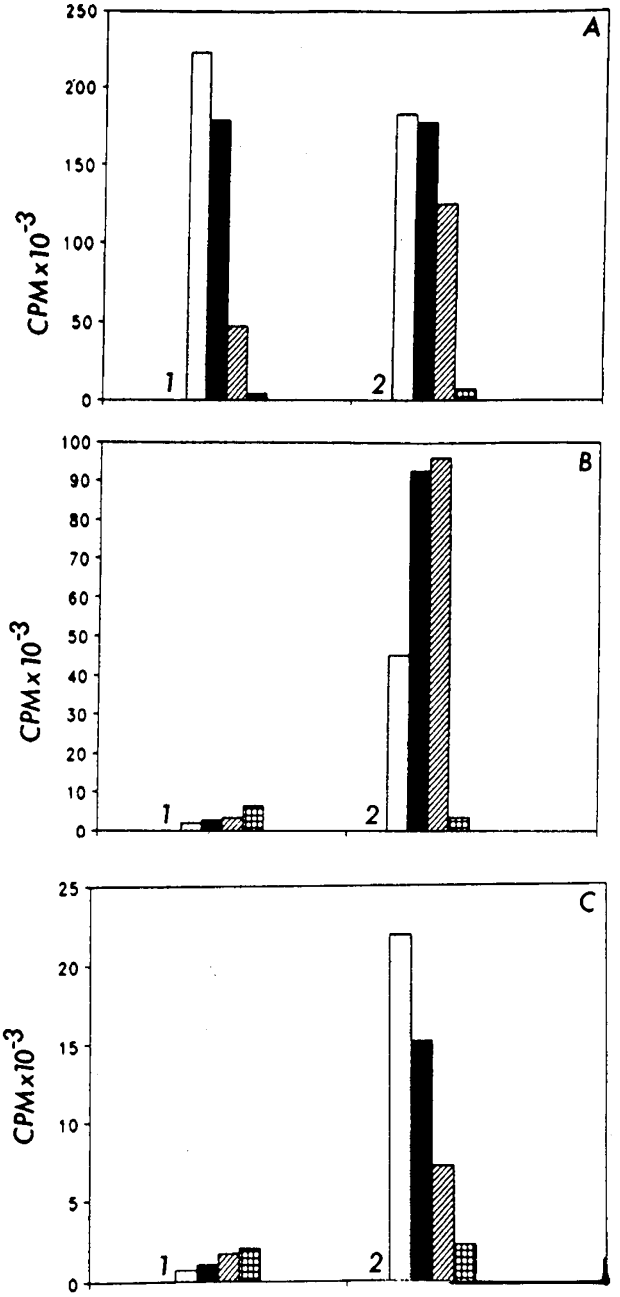

Fig. 2. Kinetics of the newborn and adult PBM responses to PHA, CD2 MAb, and CD3 MAb. Reactivity of cord (*1*) or adult (*2*) PBM to PHA (*A*), CD2 MAb (*B*), or CD3 MAb (*C*) was tested as described in Materials and Methods. (3H)TdR was added during the last 6 h of culture after 3 d (□), 4 d (■), 5 d (▨), and 8 d (▩) of stimulation. Results are presented as cpm values of (3H)TdR incorporation and are representative of the results obtained in several experiments.

RESULTS

Newborn and adult PBM proliferation in response to mitogens. Newborn mononuclear cells were analyzed for their ability to proliferate in response to different mitogens. Because labor has been reported to influence the newborn's immunity (18), PBM were collected either from cord at birth or by venipuncture during the 1st wk of life. It must be pointed out that no difference was found between these two populations (not shown). In each experiment, PBM from healthy adults were treated at the same

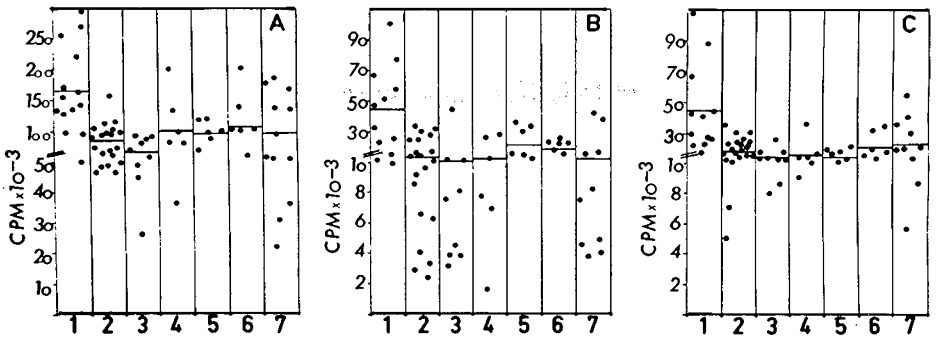

Fig. 3. Ontogeny of T cell proliferation to mitogens. PBM from infants were subdivided into seven groups numbered as follows: group 1 (neonates; $n = 14$); group 2 (infants from 1 wk to 6 mo of age); group 3 (6 mo to 1 y of age); group 4 (1 to 5 y); group 5 (5 to 10 y); group 6 (10 to 15 years); and group 7 (more than 25 y). *Circles* indicate individual values of (3H)TdR incorporation after a 3 d stimulation with PHA (*A*); concanavalin A (*B*), or PWM (*C*). Mean values are represented as *horizontal bars.*

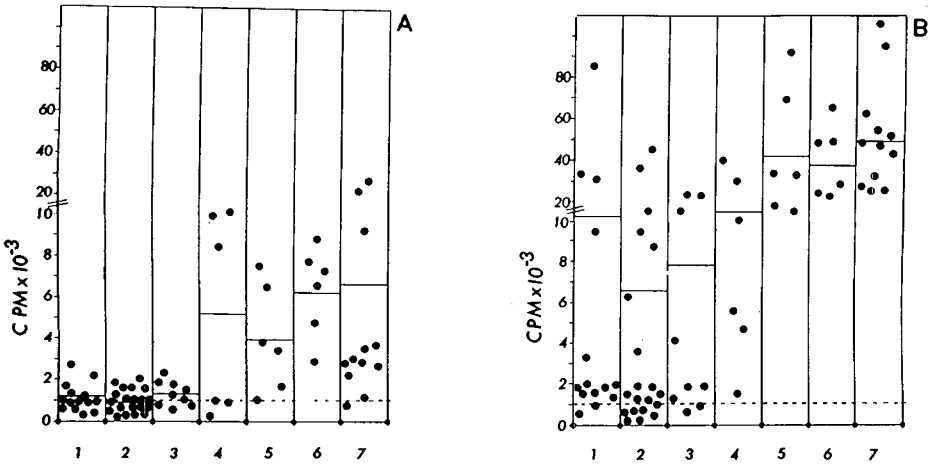

Fig. 4. Ontogeny of T cell proliferation to CD2 and CD3 MAb. *Circles* indicate individual values of (3H)TdR incorporation after a 3-d stimulation with CD3 MAb (*A*) or CD2 MAb (*B*). Ages of each group are identical to those in Figure 3. *Values under broken lines* are considered as negative according to (3H)TdR incorporation by PBM in the absence of stimulation.

time. As shown in Figure 1, newborn PBM proliferated better than adult PBM on d 3, whatever the mitogen used, of note and illustrated in Figure 2. This increase was observed shortly after stimulation only, in repeated experiments. As determined by *t* test, differences observed on d 3 are highly significant because *p* values are less than 0.02, 0.01, and 0.05 in response to PHA, concanavalin A, and PWM, respectively.

Allogeneic responses of newborn and adult PBM. The capacity of newborn PBM to respond to alloantigens was tested in mixed leukocyte reactions as described in Materials and Methods. The intensity of (3H)TdR incorporation of newborn PBM after activation by newborn allogeneic irradiated PBM was compared with that of adult responding cells activated by adult stimulator cells. Results given in Figure 1 show that no significant difference could be noticed.

Responses to CD3 and CD2 MAb in newborn and in adult. Either an MAb directed against the CD3 complex of the T cell receptor complex, *i.e.* CD3(CD3×3), or a combination pair of two MAb directed against the CD2 molecule, *i.e.* CD2(CD2×11) and CD2(D66), was used for stimulation. As shown in Figure 1, most newborns have, compared to adults, a dramatic defect of proliferative responses to CD2 and CD3 MAb. The results are statistically significant according to the *t* test: *p* values are less than 0.001 and 0.02 for CD2 and CD3 MAb, respectively. This defect does not depend on the concentration of MAb that we used. Indeed, when we studied different dilutions of CD2 or CD3 MAb, no proliferation was observed whatever the dilution used (data not shown). Additionally, no proliferation could occur at any time of culture from d 3 to 8 (Fig. 2). It is worth noticing that in all these assays the PBM reactivities against MAb and mitogens were tested simultaneously from the same samples of cell suspensions.

Ontogeny of T cell responsiveness to mitogens and CD2 and CD3 MAb. PBM from infants of various ages were also tested for their reactivity to mitogens (Fig. 3) and CD2 or CD3 MAb (Fig. 4). (3H)TdR incorporation was determined on d 3 because that time was optimal to test all stimulators simultaneously. Moreover, clear differences between newborn and adult responsiveness to mitogens were observed on d 3 only (see kinetic experiments, Fig. 2). Results show that during the 1st mo of life the intensity of proliferation induced by mitogens significantly decreased to reach adult values before 6 mo of age. In contrast, reactivity to CD2 or CD3 MAb was acquired at a slower rate, the maximal values for both reagents being reached after 5 y of age only. The *p* values of the regression curves observed with ageing are significant because they are all less than 0.001 (PHA, PWM, concanavalin A, CD3 MAb, and CD2 MAb.

Changes of CD45RA and CDw29 expression on CD4+ T cells with ageing. We further investigated the intensity of CD45RA and CDw29 expression on CD4+ T cells in infants of various ages and in adults. This was achieved by two-color fluorescence analysis using phycoerythrin-conjugated CD4 on one arm and FITC CD45RA or CDw29 MAb on the other. Results showed that CD45RA and CDw29 antigens appeared to be present on virtually all CD4+ T cells, albeit at different levels. This enables us to divide CD4+ populations into CD45RA antigen high or low and CDw29 antigen high or low subsets. Only CD4+ T cells expressing a high level of CD45RA or CDw29 antigens were considered and are henceforth referred to as CD45RA+ or CDw29+. As illustrated in Figure 5, data indicate that more than

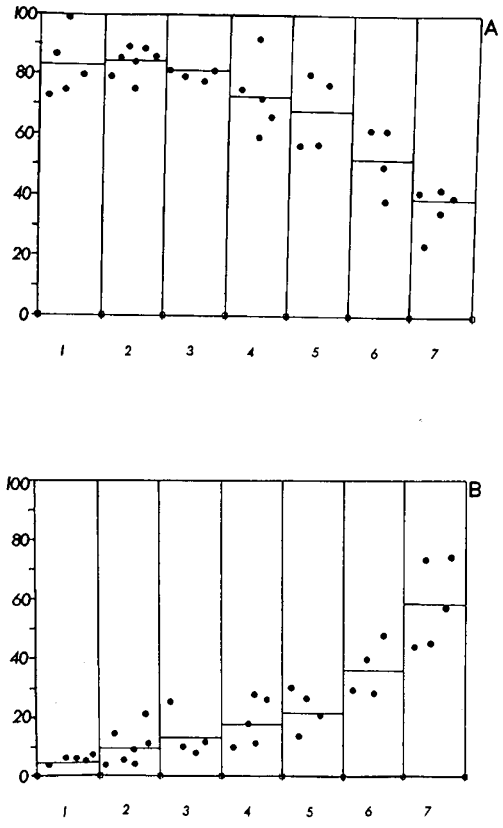

Fig. 5. Ontogeny of CDw29 and CD45RA expression on CD4+ T cells. Double labeling with fluorochrome conjugated CD4+ and CD45RA+ or CD4+ and CDw29+ was performed as described in Materials and Methods. Results are indicated as an individual percentage of CD4+ lymphocytes for each infant that are double labeled with CD4 and CD45RA MAb (*A*) or CD4 and CDw29 MAb (*B*). Mean values are represented as *horizontal bars*. Ages in each group are identical to those in Figure 3.

70% of CD4+ resting T cells are CD45RA+ and less than 10% are CDw29+ at birth. This contrasts with the results found in adults, whose CD4+ T cells are mostly CDw29+. Data further indicate that even if phenotypic changes start during the 1st y of life, the adult values are reached only during the 2nd decade of life.

Maturation of newborn PBM phenotype and function upon in vitro activation by PHA. We then tested the effect of *in vitro* stimulation on the phenotype of CD4+ T cells and the T cell function. PBM were stimulated by PHA at 10 μg/mL, which led to PBM proliferations in both the newborn and the adult. After 7 d of culture performed as indicated in Materials and Methods, T cells were recovered and washed twice. At that time, monocytes were not detectable in the cell suspension as determined by an immunofluorescence analysis (not shown). CD4+ T cells contained in this cell preparation were tested for the expression of CD45RA and CDw29 antigens and for their responsiveness to the CD2 MAb. This MAb was selected for stimulating in the secondary culture because it has been previously shown that it was not dependent on the accessory signals provided by monocytes for T cell activation in adults. A representative experiment is shown in Figure 6. As expected, before stimulation, the percentages of CD4+ CD45RA+ and CD4+ CDw29+ cells in newborns were 75.4 and 13.4, respectively, which contrasted with the percentages found in adults, *i.e.* 28.3 and 68.3, respectively (Fig. 6*A*). More interestingly, after *in vitro* stimulation by PHA, most newborn-activated CD4+ T cells converted into the CDw29+ CD4+ phenotype to reach percentages of double-labeled T cells similar to those found in adults, *i.e.* 99.1 and 85.3, respectively (Fig. 6*B*). This change correlated with the acquisition of a high responsiveness to CD2 MAb. Indeed, as shown in Table 1, activated T cells from newborns proliferated vigorously in response to CD2 MAb as did adult resting PBM or activated T cells. This contrasted with a poor responsiveness of newborn resting PBM to CD2 MAb. This clearly indicates that newborn T cells can acquire the phenotype and the functional characteristics of memory T cells upon *in vitro* stimulation. Moreover, similar to what has been found in adults, newborn activated T cells that could not be stimulated by CD3 MAb in the absence of monocytes fully proliferated when freshly isolated monocytes were added to the culture (data not shown).

DISCUSSION

The present study documents the ontogeny of the responsiveness of T cells to various activators from birth to more than 15 y of age. Results clearly show that lymphocytes from newborns proliferated normally in a mixed leukocyte reaction. As tested on d 3 after stimulation, proliferation in response to all mitogens was found to be higher in newborns. These differences might be related to different kinetics rather than to differences in the intensity of the response, inasmuch as they were observed only shortly after stimulation (Fig. 2). In contrast, a clear defect of activation by CD3 or CD2 MAb was observed in PBM cells at birth at any time after stimulation.

Conflictive results on newborn T cell responsiveness have been reported so far. The responses to PHA have been found to be increased (19–21) or normal (22, 23). The responses to CD3 MAb, which were determined by proliferation or IL2 production, have been found to be decreased (24, 25) or normal (26, 27). Finally, in contrast to adult T cells, newborn T cells have been shown to be dependent on accessory signals provided by monocytes during T cell proliferation through CD2 molecules (28). These accessory signals could be overcome by recombinant IL2. Related to these observations, we also found that recombinant IL2 restored the unresponsiveness of newborn PBM to CD3 and CD2 MAb (data not shown). This suggests that the defect of T cell proliferation through CD2 and CD3 molecules is due to a deficiency of IL2 production rather than an IL2 receptor expression. Interestingly, the patterns of reactivity that we found in newborns and adults are highly related to those that differentiate between two adult cell populations (19). One of these populations, which expresses CDw29 antigen at a high density, has been proposed to correspond to memory T cells because this is the only subset that proliferates when boosted by antigens such as tetanus toxoid. Additionally, this population fully responds to soluble CD2 and CD3 MAb but responds to PHA to a lesser extent (29–34). The second subset, which expresses CD45RA antigen at a high density, is supposed to correspond to naive unprimed T cells. Upon *in vitro* stimulation, they commutate to a CD45RA+ low, CDw29+ high phenotype and acquire a full responsiveness to CD2 and CD3 MAb (16, 29–34). These two subsets are designated as naive T cells and memory T cells, respectively (16). The observation of a predominant population of CD45RA+ CDw29− cells among circulating lymphocytes in neonates has been considered strong enough to support the above distinction, inasmuch as neonates have not experienced antigen (33, 34). Our present results are consistent with the idea that CD45RA+ CDw29− so-called naive T cells from adults are indeed unprimed lymphocytes, inasmuch as their pattern of reactivity is very close to that which we found in PBM of neonates (this report). Because CD45RA+ T cells in adults have been described initially as suppressor inducer cells for Ig production (16), this functional status might also contribute to explaining a neonatal susceptibility to infectious diseases and a low Ig production. The persistence of naive T cells at a high frequency during the first years of life as shown here might also be responsible for the deficient responsiveness to CD2 and CD3 MAb even though antigenic challenges have occurred as it is demonstrated by the increment of CDw29+ memory T cells soon after birth.

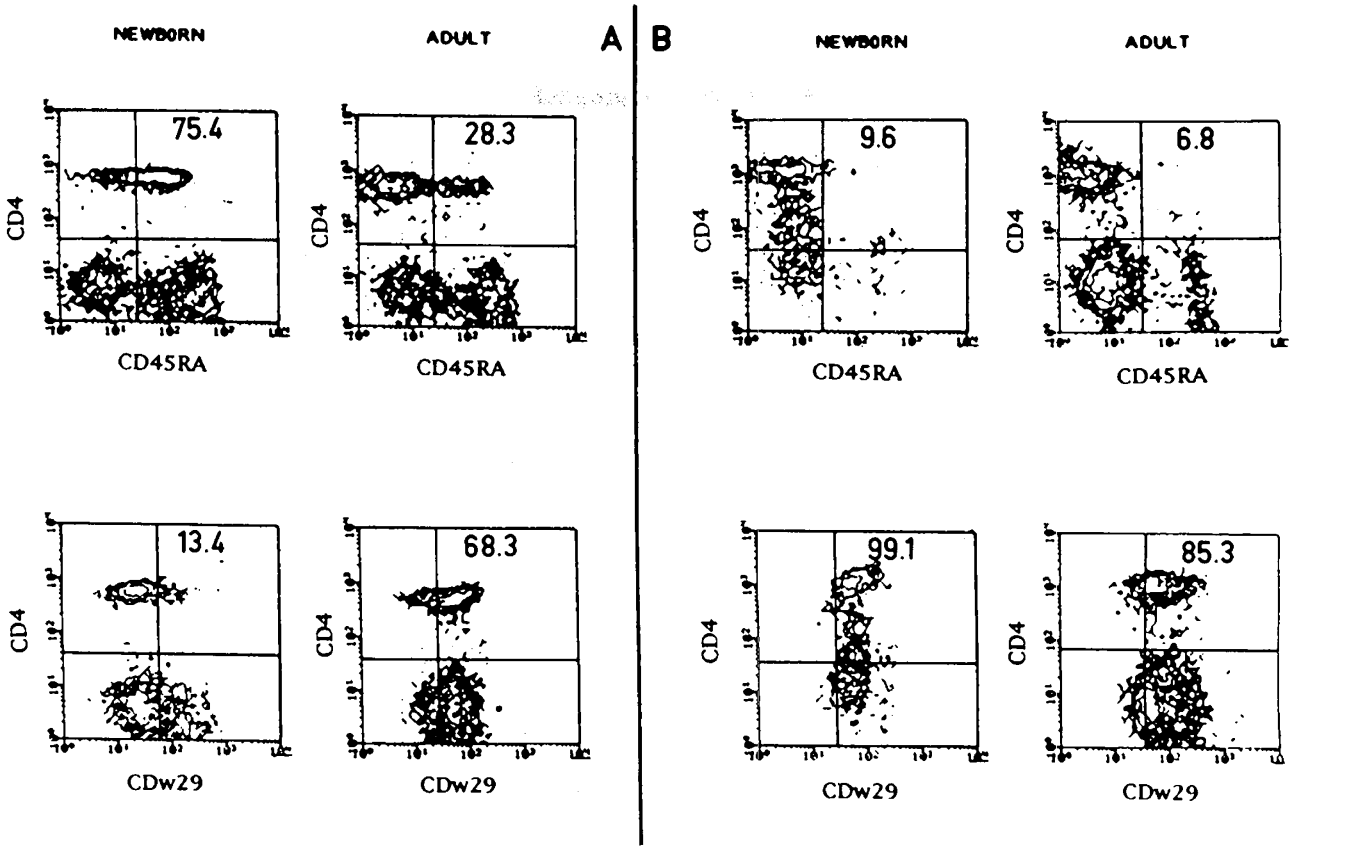

Fig. 6. Analysis for coordinate expression of T cell differentiation antigens. Cells were either resting PBM from newborns or adults (*A*) or activated T cells (*B*) that were obtained as described in Materials and Methods. Values on *x* and *y* axes indicate fluorescence intensity. Cells that are double stained with either phycoerythrin-conjugated CD4 and FITC-conjugated CD45RA MAb or phycoerythrin-conjugated CD4 and FITC-conjugated CDw29 MAb are represented in the upper right quadrant. The percentage of CD4+ T cells expressing either CDw29 or CD45RA molecules is also given.

Table 1. *Responsiveness of newborn PBM to CD2 MAb after activation in primary culture**

Source of PBM	State of activation	Stimulation with CD2MAb†	(3H)TdR incorporation‡
Adult	−	−	58 ± 4.7
Adult	−	+	43 310 ± 3 299
Adult	+	−	321 ± 4
Adult	+	+	79 636 ± 8 557
Newborn	−	−	327 ± 102
Newborn	−	+	2 698 ± 1 509
Newborn	+	−	451 ± 155
Newborn	+	+	75 449 ± 6 255

* Resting PBM were isolated from venipuncture of adult volunteers (Adult) or from cord (Newborn). Activated T cells were obtained as described in Materials and Methods.

† Resting T cells or activated T cells were further stimulated (+) or not (−) by CD2 MAb as indicated in Materials and Methods.

‡ Results are expressed as mean value ± SD of (3H)TdR incorporation measured in triplicate.

This is reinforced by the observation that newborn CD4+ T cells that converted into 100% CDw29+ T cells after a nonspecific stimulation by PHA became fully responsive to CD2 MAb. Iterative antigenic challenges seem therefore to be a major condition needed not only to induce memory T cells but also to create the environment necessary for a global maturation of the immune system. Our results demonstrate that the phenotypic and functional changes accompanying this maturation occur at a slow rate during childhood. According to our data, the apparent immunodeficient status would persist until the age of 4–5 y.

A correlation of the CD45RA and CDw29 antigen expression with the T cell responsiveness to CD3 and CD2 MAb also suggests that the CD45RA and/or CDw29 antigen expression is causally associated with the function of the T cells. Indeed, both of these membrane molecules have been shown to play an important role in the regulation of T cell functions (35–39). However, apart from CD45RA and CDw29 molecules (40–45), other lymphoid surface molecules involved in the regulation of T cell function are also differentially expressed on memory and naive T cells (35–39, 46). Therefore, whether each of these molecules, alone or in association, contributes to improve the intracellular signaling and consequently the T cell function in memory cells remains to be elucidated.

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