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Diagnostic value of CD45RO expression on circulating T lymphocytes of fetuses and newborn infants with pre-, peri- or early post-natal infections

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SUMMARY

We examined the expression of the CD45RO antigen, which characterizes the antigen primed/memory phenotype of T lymphocytes, as a marker for congenital infection in blood samples of newborns and fetuses. CD45RO expression on T cells was determined by triple-colour fluorescence flow cytometry. In total 537 blood samples of newborns and infants up to an age of 3 months and 89 fetal blood samples from gestational weeks 19–31 were analysed. Of the newborns and infants, 74 had a clinically, serologically and/or antigenically evident infection, and four of the fetuses had a confirmed intra-uterine infection. In 35 infants with acute predominantly bacterial infections such as sepsis or pneumonia, 17 (48·6%) had elevated CD45RO^{bright} expression. In 39 infants with proven pre-, peri- or early post-natal infections with toxoplasmosis, cytomegalovirus (CMV), rubella, herpes simplex virus (HSV) or human herpes virus type 6 (HHV6), 25 (64·1%) exhibited enhanced CD45RO^{bright} expression. Three of four fetuses with confirmed intra-uterine infection (three with CMV, one with parvovirus B19) exhibited elevated CD45RO^{bright} expression. The specificity of the CD45RO assay for detecting microbial infections was 94·6% for newborns and infants up to 3 months and 90·6% for fetuses. It is concluded that elevated numbers of CD45RO^{bright} T cells in infants up to 3 months of age strongly suggest an infection. However, the sensitivity of the CD45RO assay is not sufficient to enable the test to be used as a general marker for prescreening infants to detect pre-, peri- or early post-natally acquired infections.

Keywords CD45RO T lymphocytes flow cytometry congenital infection neonate

INTRODUCTION

Diagnosis of congenital infections in newborns and infants is associated with several problems. The clinical signs of infections, which can be caused by different organisms such as bacteria, viruses or protozoa, vary greatly. Since specific IgM antibody responses may be lacking, a large panel of time consuming and expensive specific tests including antigen detection, virus isolation in cell culture and polymerase chain reaction (PCR) methods is often required to identify a certain organism. In general, screening is only performed for the most likely infectious organisms, so that uncommon organisms may go undetected. Since non-specific markers of infection such as C-reactive protein (CRP) can often give false positive and negative results, an efficient and inexpensive screening test for congenital infection is needed for prenatal diagnosis. Candidates for markers of congenital infections include certain cell surface molecules, which appear on the surface of T lymphocytes upon antigenic contact. When activated by microbial antigens, certain T cell subsets expand and the T lymphocyte changes its repertoire of surface molecules from a virgin T cell phenotype to an activated phenotype (e.g. enhanced expression of CD69, CD25, HLA-DR, CD29) and to a phenotype characteristic for memory cells [1–3]. A number of cell surface molecules are differentially expressed on virgin and memory cells and their expression on T cells increases or decreases with age [4,5]. Some of these molecules, such as CD2, LFA-1 (CD11a/CD18), LFA-3 (CD58) and CD44, whose expression is enhanced on memory cells, are involved in cell–cell interactions or in transmitting activation signals (CD11a/CD18, CD58) or function as homing receptors (CD44). The best studied markers that delineate virgin and memory T cells are the variant isoforms of CD45, the CD45RA isoform (mol. wt 190 000–220 000) and the CD45RO isoform (mol. wt 180 000). CD45RO^{+} T cells are defined as memory cells because they are derived from CD45RA^{+} T cells after antigenic or mitogenic stimulation and provide the recall response to antigen [1]. Thus, healthy newborns who have not experienced infection express CD45RO on a small minority of the T cells [2,6–8]. However, in newborns who have come into contact with infectious organisms transmitted from the mother, the number of CD45RO^{+} T cells should be increased, as has been reported for HIV-infected children [9]. A recent study showed an enhanced

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expression of CD45RO in eight cases of proven congenital infection [8]. A negative result could therefore reduce the number of further specific diagnostic tests required to confirm or exclude congenital infection. Here we have evaluated the sensitivity and specificity of the CD45RO assay as an inexpensive infection marker in newborns and fetuses.

PATIENTS AND METHODS

Patient categories and samples

Blood samples ($n = 537$) from newborns and infants up to 3 months of age were selected on the basis of suspected infection and screened for infectious agents as described below. The blood of these infants with and without clinical and/or serological signs of infection was drawn and collected in tubes containing 1·5 mg EDTA-K_2/ml. These samples included 214 (40%) cord blood samples obtained at birth. Four groups of patients were defined based on the following parameters: group 1 ($n = 18$), healthy infants on whose results the 'healthy' cutoff for percent $CD45RO^+$ T cells was defined; group 2 ($n = 463$), patients without any serological evidence of infection; group 3 ($n = 35$), patients with acute infections such as pneumonia or sepsis predominantly caused by bacteria; group 4 ($n = 39$), patients with serologically, virologically or antigenically confirmed infection.

Fetal blood ($n = 89$) was obtained by cordocentesis, performed at weeks 19–31 of gestation and collected in EDTA tubes. Prenatal diagnosis was performed for rubella, cytomegalovirus (CMV), varicella zoster virus (VZV), parvovirus B19 or *Toxoplasma gondii* infection according to the type of maternal infection or fetal ultrasound abnormalities. The fetal origin of the blood was confirmed by the determination of fetal haemoglobin. Eight fetal blood samples were found to be contaminated with maternal blood and were therefore excluded from the study.

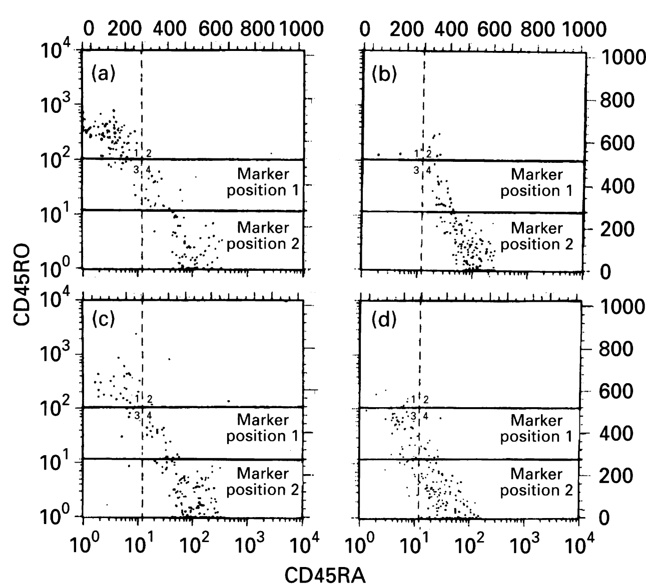

Fig. 1. Representative dot plot analyses of CD45RA and CD45RO expression on T lymphocytes. Cells were stained and analysed as described in Patients and Methods. Markers were set to discriminate between $CD45RO^{bright^+}$ (marker position 1), $CD45RO^{low, bright^+}$ T cells (marker position 2) and $CD45RO^-$ T cells. (a) Healthy adult (36 years old): marker position 1, 44·2% $CD45^{bright^+}$ T cells; marker position 2, 68·0% $CD45RO^{low, bright^+}$ T cells. (b) Healthy infant (6 weeks old): marker position 1, 4·2% $CD45^{bright^+}$ T cells; marker position 2, 18·7% $CD45RO^{low, bright^+}$ T cells. (c) Infant with proven prenatal cytomegalovirus (CMV) infection (6 weeks old), IgM-positive, CMV early antigen test-positive, virus isolation in cell culture from urine sample positive: marker position 1, 15·3% $CD45^{bright^+}$ T cells; marker position 2, 36·8% $CD45RO^{low, bright^+}$ T cells. (d) Fetus without evidence of microbial infection (gestational week 21): marker position 1, 2·8% $CD45^{bright^+}$ T cells; marker position 2, 32·0% $CD45RO^{low, bright^+}$ T cells.

Laboratory procedures

All blood samples from newborns and infants were either referred to our routine screening programme for congenital infections ('STORCH'), or in case of known maternal infection in pregnancy referred for diagnosis for specific infections. Acute bacterial infections included newborns and infants with sepsis and bacterial pneumonia. An acute infection with CMV was diagnosed by detection of specific IgM by ELISA and virus isolation in cell culture and/or positive PCR, and in some cases using the pp65 early antigen immunofluorescence test. Rubella virus infection was diagnosed by detection of specific IgM antibodies using ELISA techniques. Infections with *T. gondii*, herpes simplex virus (HSV), human herpes virus type 6 (HHV6) and VZV were diagnosed by detection of specific IgG and IgM antibodies. Parvovirus B19, rubella virus, VZV and *T. gondii* infections were also confirmed by PCR techniques.

Flow cytometric analysis

Triple-colour fluorescence flow cytometry was used to determine the expression of the CD45RO antigen on the surface of T lymphocytes. The following MoAbs were used: PerCP-conjugated anti-CD3 (Leu-4), FITC- and PE-conjugated irrelevant mouse immunoglobulins, FITC-conjugated CD45RA and PE-conjugated CD45RO. All antibodies were obtained from Becton Dickinson (Heidelberg, Germany).

The samples were prepared using a whole blood procedure. Briefly, EDTA-blood containing $2·5 \times 10^5$ leucocytes was incubated for 20 min in the dark with the following antibody combinations: (i) CD3-PerCP/FITC-/PE-conjugated isotype control; and (ii) CD3-PerCP/CD45RA-FITC/CD45RO-PE. Erythrocytes were then lysed and the cells fixed for 10 min at room temperature using the Becton Dickinson lysing reagent, centrifuged at 300 ***g*** at 10°C and washed once with 3 ml PBS.

The cells were analysed on a FACScan (Becton Dickinson) with five-parameter analysis including two scatter and three immunofluorescence channels. $CD3^+$ lymphocytes were gated using the PerCP-fluorescence. Before analysis of patients, normal values for CD45RO expression on $CD3^+$ T lymphocytes were obtained for healthy adults ($n = 59$) and for healthy newborns up to 3 months of age ($n = 18$). Only the cells with CD45RO bright fluorescence ($CD45RO^{bright}$; marker position 1) were defined as 'positive'. These $CD45RO^{bright}$ T cells did not express CD45RA (Fig. 1a).

Statistical analysis

For statistical analysis of the data an analysis of variance procedure (Scheffe's test for pairwise comparison) was applied. The operation was preceded by a Proc Univariate test on normal distribution values and an arc sin transformation, as necessary for calculation with percentage values. The level of significance was $P = 0·05$.

RESULTS

Normal values for CD45RO$^{bright+}$ T cells for adults, healthy newborns and fetuses

Fifty-nine samples from healthy adults (mean age 29·5 ± 6·0 years) were analysed for CD45RO$^{bright+}$ T cells. In this control group CD45RO^{bright} was expressed on 35·7 ± 9·3% (mean ± s.d.) of CD3^{+} cells. A representative dot plot of a 36-year-old healthy adult with 44·2% CD45RO$^{bright+}$ T cells is shown in Fig. 1a.

In healthy newborns up to 3 months old (group 4), the mean CD45RO^{bright} expression on T cells was 5·1 ± 1·7% ($n = 18$) (Fig. 2, column 1). This group was used as the healthy control reference group. Based on these results we defined newborns with a CD45RO^{-} expression < 9% as negative (no prior antigenic contact caused by a microbial infection) and those with > 9% of CD45RO$^{bright+}$ T cells as positive (suggestive of a microbial infection). As it is normally unlikely that an antigenic contact causes a severe infection, or that immunization occurs in the early post-natal period, cord blood samples as well as blood samples up to 3 months of age were included in this study. Representative dot plots of the CD45RO expression of a healthy infant (6 weeks old) with 4·2% CD45RO$^{bright+}$ T cells and of an infant with proven congenital CMV infection (6 weeks old) with 15·3% CD45RO$^{bright+}$ T cells are shown in Fig. 1b and c, respectively.

In most fetal blood samples analysed, in contrast to samples of newborns, a relatively high percentage (up to 35%) of fetal T cells expressed the CD45RO antigen in low concentrations (low fluorescence; CD45RO^{low}). A representative dot plot is shown in Fig. 1d. In this case, only 2·3% of the T cells expressed CD45RO^{bright}, but 32·0% of T cells were CD45RO$^{low+bright+}$. As described above, these CD45RO^{low+} T cells were below the defined marker position 1 and thus were not regarded as CD45RO^{+}.

We investigated whether the amount of CD45RO$^{bright+}$ cells (marker position 1) may be a marker to discriminate between non-infected newborns and fetuses, and those who had experienced a microbial infection. Blood samples ($n = 537$) of newborns and infants up to 3 months of age and 89 fetal blood samples, which also underwent our routine diagnostic tests, were tested for correlation with a congenital or early post-natal infection.

CD45RO^{bright} expression on T cells of newborns and infants up to 3 months old with suspected pre-, peri- or early post-natal infection

As shown in Table 1, we investigated 463 samples of newborns and infants up to 3 months old without any serological or antigenic evidence of congenital infection (group 2). Included were 245 samples of newborns with suspected congenital infection according to clinical abnormalities, which were referred to our routine 'STORCH' screening programme (a). Of the samples, 96·3% ($n = 236$) had a CD45RO^{bright} expression within the normal range. Only 3·7% ($n = 9$) had T cells with a CD45RO^{bright} expression on more than 9% of T cells. The mean of 5·9 ± 1·4% CD45RO$^{bright+}$ T cells is not significantly different from the control group 1 of healthy newborns ($n = 18$, 5·1 ± 1·7%).

Furthermore, 218 samples of newborns from mothers with a known history or suspected infection in pregnancy were analysed (b). The maternal infections included toxoplasmosis ($n = 88$), CMV ($n = 46$), parvovirus B19 ($n = 26$), VZV ($n = 23$), rubella virus ($n = 15$), HSV ($n = 5$) and several other infections ($n = 15$) such as coxsackie virus, hepatitis C virus, HIV, HHV6, measles, pertussis and borrelia. The results were comparable to those of group 2a: only 7·3% of the samples tested had a CD45RO^{bright} expression on > 9% of T cells. Furthermore, there was no significant difference in the mean of CD45RO$^{bright+}$ T cells (4·7 ± 1·9%) compared with the control group 1 of healthy newborns. The results of these two groups of infants having no evidence of infection are summarized in Table 1. The test for CD45RO^{bright} expression on T cells as a marker for microbial infection exhibited a specificity of 94·6%.

Figure 2 summarizes the 74 cases with either clinically evident, mainly bacterial acute infections such as sepsis and pneumonia (group 3, $n = 35$) or with confirmed congenital or early post-natal predominantly viral and toxoplasmosis infections (group 4, $n = 39$). Among the 35 cases with clinically evident predominantly bacterial infections the percentage of CD45RO$^{bright+}$ T cells ranged from 1·7% to 19·1% (Fig. 2). The mean of 8·4 ± 4·2% CD45RO$^{bright+}$ T cells was significantly different ($P \leq 0.05$)

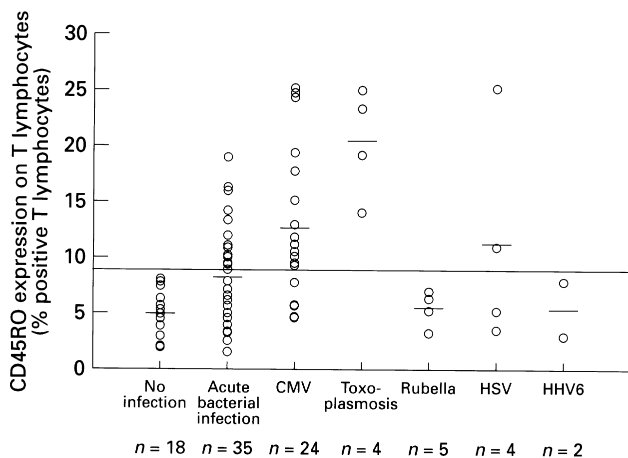

Fig. 2. Relative numbers of CD45RO$^{bright+}$ T cells in infants up to 3 months old with proven congenital or early post-natal infection. Cells were stained and analysed as described in Patients and Methods. Samples with CD45RO^{bright} expression < 9% were defined as negative, > 9% as positive (horizontal line at 9%). Mean values are indicated (–) for each group. No infection, Control group of healthy newborns; acute bacterial infections, newborns with clinically evident predominantly bacterial acute infections such as sepsis or pneumonia; CMV, toxoplasmosis, rubella, HSV, HHV6, newborns with proven congenital or early post-natal infection with the indicated infectious agent.

Table 1. CD45RO^{bright} expression on T cells of newborns and infants up to 3 months of age with no evidence of microbial infections based on serological and antigenic tests (group 2)

CD45RO$^{bright+}$ T cells	< 9% (negative)	> 9% (positive)
a. $n = 245$; mean 5·9 ± 1·4%		
Newborns with suspected congenital infection based on clinical findings ('STORCH')	236 (96·3%)	9 (3·7%)
b. $n = 218$; mean 4·7 ± 1·9%		
Newborns from mothers with known or suspected history of infection in pregnancy	202 (92·7%)	16 (7·3%)
Sum, $n = 463$	438 (94·6%)	25 (5·4%)

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compared with the control group of healthy newborns (5·1 ± 1·7%). However, CD45RO^{bright} expression was elevated and thus considered to be positive in only 17 (48·6%) of these 35 cases. Therefore in this group the sensitivity of the assay as a marker of acute infection was 48·6%.

Newborns with proven congenital or early post-natal infection showed an enhanced CD45RO^{bright} expression in 64·1% of cases (25 out of 39) with a range of 4·8% to 25·3% CD45RO$^{bright+}$ T cells.

The mean of 12·0 ± 6·9% CD45RO$^{bright+}$ T cells was significantly different ($P \leq 0·05$) from the control group. The sensitivity of the test for detecting congenital or early post-natal infections was 64·1%.

As shown in Fig. 2, newborns with connatal *T. gondii* infection exhibited elevated CD45RO^{bright} expression in all cases ($n = 4$). In newborns with proven congenital, peri- or early post-natal CMV infection, CD45RO^{bright} expression was elevated in 19 out of 24 cases (79·2%). However, CD45RO^{bright} expression was not enhanced in five cases of confirmed congenital rubella syndrome and in two proven cases of early post-natal HHV6 infection.

CD45RO^{bright} expression on T cells of fetuses between weeks 19 and 31 of gestation

We tested 89 fetal blood samples for CD45RO^{bright} expression on T cells (Table 2). In 85 cases intra-uterine infection was excluded by negative serology or negative PCR results in fetal blood. In 9·4% ($n = 8$) of these cases enhanced numbers of CD45RO^{bright} T cells were found, resulting in an assay specificity of 90·6%. In four cases a fetal infection was diagnosed by serological and virological methods. In two cases of CMV infection and one case of parvovirus B19 infection, CD45RO^{bright} expression on T cells was elevated with 16·9%, 20·7% and 17·0%, respectively. However, the proportion of CD45RO$^{bright+}$ T cells in an additional case of intra-uterine CMV infection lay within the normal range (3·2%).

Table 2. CD45RO^{bright} expression on T cells of fetuses (19–31 gestational week) with and without evidence of intra-uterine infection ($n = 89$)

I. *No evidence of an intra-uterine infection*

CD45RO^{bright} T cells	<9% (negative)	>9% (positive)
a. $n = 49$		
Fetuses with suspected prenatal infection (abnormal ultrasound)	45 (91·8%)	4 (8·2%)
b. $n = 36$		
Fetuses from mothers with known history of infection	32 (88·9%)	4 (11·1%)
Sum, $n = 85$	77 (90·6%)	8 (9·4%)

II. *Evidence of intra-uterine infection*

CD45RO^{bright} T cells	<9% (negative)	>9% (positive)
CMV	1*	2†
Parvovirus B19	0	1‡
Sum, $n = 4$	1	3

Diagnosis:

* Fetal ascites diagnosed at week 26 of gestation, prenatal diagnosis positive for cytomegalovirus (CMV) (amniotic fluid, CMV-IgM in fetal blood): preterm delivery at gestational week 31 with hydrops fetalis, intracranial calcifications.

† Hydrocephaly, ventricular asymmetry, pericardial effusion diagnosed at week 21 of gestation, prenatal diagnosis positive for CMV (amniotic fluid, CMV-IgM in fetal blood): termination of pregnancy (week 23): disseminated CMV infection of the fetus.

Primary maternal CMV infection in first trimenon, no fetal ultrasound abnormalities in weeks 22 and 24 of pregnancy, fetal blood positive for CMV (CMV-PCR, CMV-IgM), total IgM (40·4 g/dl) elevated, newborn healthy, at age of 5 months bilateral deafness diagnosed, urine CMV positive, serum CMV-IgG levels elevated.

‡ Mother with acute parvovirus B19 infection in pregnancy, excessive non-infectial hydrops fetalis (NIHF) at week 26 of gestation, prenatal diagnosis from fetal blood positive for parvovirus B19 (PCR, Parvo-IgM).

DISCUSSION

In order to define normal values for CD45RO expression on T lymphocytes using our laboratory methods, we tested 59 healthy adults and 18 healthy newborns. The normal ranges of 35·7 ± 9·3% CD45RO$^{bright+}$ T cells for adults and 5·1 ± 1·7% for healthy newborns are in good agreement with values from previous studies [5,8,10,11].

The analysis of 89 fetal blood samples obtained between 19 and 31 weeks gestation from fetuses without any evidence of intra-uterine infection revealed higher numbers of CD45RO^{+} T cells than in cord blood. However, CD45RO^{+} cells of fetuses predominantly exhibited a low positive fluorescence signal (CD45RO^{low+} T cells) (Fig. 1d). When comparing the numbers of T cells with CD45RO^{bright} staining, no difference was observed between cells from fetuses and newborns without evidence of an infection. Bofill *et al*. [12] described a subset of resting T cells with expression of low levels of CD45RA and CD45RO molecules in cord blood. Their data suggest that these cells are the relatively immature antecedants of CD45RA^{+}RO^{-} T cells which may be recent emigrants from the thymus. The relatively high number of CD45RO^{low+} T cells found in fetal blood in our study might be the T cell subset described by Bofill *et al*. [12], which is more predominant in the immature blood of fetuses than in that of the neonate. In agreement with these findings, Paganelli *et al*. [11] found significantly higher numbers of CD45RO^{+} CD4^{+} T cells in fetuses (7–27%) than in newborns (4–9% of CD4^{+} T cells). In combination with the presence of considerable numbers of activated T cells (IL-2-Rα) they suggest that these populations arise as a consequence of antigenic stimulation during intra-uterine life, which leads both to activation and to immunologic memory. The decreased numbers of CD45RO^{+} cells in newborns compared with fetuses may be the result of dilution by continuous production of CD45RA^{+} lymphocytes [13,14]. Alternatively, reversal to the high mol. wt CD45RA isoform [15] may explain the low levels of CD45RO^{+} T cells in the blood of newborns. Furthermore, the limited capacity for proliferation of intrathymic CD45RO^{+} T cells consistent with a commitment to death may contribute to the absence of larger numbers of CD45RO^{+} T cells in newborn blood [16].

Based on our results of newborns and fetuses with proven infection, we consider that the CD45RO$^{bright+}$ T cell population can distinguish the child who experienced intra-uterine infection from children with abnormalities resulting from, for example, genetic, metabolic or developmental defects.

We have extended the study of Michie & Harvey [8] by using a larger patient collection to estimate the specificity and sensitivity of the CD45RO flow cytometric assay. Considering the 463 samples of newborns and infants up to 3 months old with no evidence of microbial infection, the specificity of the assay was 94·6%. Only 25 samples exhibited elevated numbers of CD45RO$^{\text{bright}+}$ T cells. Since a routine screening for infectious organisms cannot include all organisms, in some of these 25 cases the elevated numbers CD45RO$^{\text{bright}+}$ T cells may be due to an infection with an uncommon organism, which went undetected. In addition, common infections in the screening programme may also occasionally go undetected, so that the specificity may perhaps be even higher than 94·6%.

However, the sensitivity of the assay was low. In 14 of 39 cases with proven congenital or early post-natal viral or toxoplasmosis infections (Fig. 2), no enhancement of CD45RO$^{\text{bright}+}$ T cells could be observed; this resulted in a sensitivity of 64·1%. When considering the 32 cases of acute mainly bacterial infections like sepsis or pneumonia, the sensitivity was even lower (48·6%).

It must be considered that the significant increase in CD45RO^{+} T cells which can be detected in peripheral blood after antigenic contact may take several days. Therefore, shortly after the onset of clinical signs the numbers of circulating CD45RO$^{\text{bright}+}$ T cells may still be within the normal range. This may account for the relatively low sensitivity in the case of bacterial infections. In cases where the numbers of CD45RO$^{\text{bright}+}$ T cells are not elevated despite a proven infection (especially with regard to rubella and HHV6 infections), further basic investigation is necessary. However, the absence of the memory phenotype CD45RO^{+} T cells in newborns with congenitally acquired rubella infections in our study is compatible with the well established immune paresis present in congenital rubella, and thus would also correlate with data from the lymphocyte transformation assay of O'Shea *et al.* [17]. Lymphocyte proliferation assayed with the ^{3}H-thymidine test, which is dependent on the presence of 'memory' cells, was negative in children with congenitally acquired rubella virus infection [17].

Of the 89 fetuses investigated, a prenatal infection was diagnosed in only four cases (three CMV, one parvovirus B19). Three of these samples exhibited elevated numbers of CD45RO$^{\text{bright}+}$ T cells and one had normal levels. Further fetal blood samples are necessary to evaluate the sensitivity of the assay to detect prenatal infections in fetuses. However, the specificity of 90·6% is nearly as high as in newborns.

In conclusion, although inexpensive and easy to perform, we think that the determination of CD45RO on T cells is of limited use as a non-specific marker for a prescreening of the neonate for detection of pre-, peri- or early post-natal infections. The presence of normal numbers of CD45RO$^{\text{bright}+}$ T cells cannot exclude infections due to the relatively low sensitivity of the test, so that in most cases the normal routine specific diagnosis tests still need to be performed. Further work has to be done to show if the determination of CD45RO expression on other lymphocyte subsets such as natural killer (NK) cells [18] or $\gamma\delta$ T cells [19] can improve the sensitivity for detecting microbial infections. In three cases of intra-uterine infection an increased number of NK cells was described, a significant proportion of these NK cells expressing the CD45RO phenotype [18]. In newborn babies with serologically confirmed congenital *T. gondii* infection the $\gamma\delta$ T cell subset is expanded, the activated form of which expresses CD45RO [19]. Besides flow cytometric determination of lymphocyte subsets, the measurement of IgM anti-IgG in cord serum seems to be another interesting marker to detect congenital infections [20].

Elevated CD45RO$^{\text{bright}+}$ T cell levels are nevertheless strongly suggestive of an infectious cause of disease in newborns, infants and fetuses, since the specificity of the CD45RO flow cytometric assay is high. Enhanced CD45RO levels in the absence of evidence for a specific infection warrants a broadening of the search to include infectious agents other than those suspected on the basis of anamnestic information.

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