Activation of unprimed CD4+CD45RA+/RO- T cells results in a gradual loss of CD45RA expression concomitant with the acquisition of CD45RO. It has been suggested that this conversion occurs in vivo through a CD45RAbrght/RObright stage. Next to this small CD45RAbrght/RObrght subset (Dbright), a larger subpopulation that expresses both RA and RO isoforms at low levels (Ddull) can be found in the circulating CD4+ T-cell population of all donors. The properties of the latter population are largely undefined. Here, we show that Ddull cells have an intermediate phenotype for antigens such as CD31, CD62L, CD58, and CD95 that are differentially expressed on unprimed versus primed T cells. In addition, they are able to provide help for B-cell differentiation and contain substantial numbers of tetanus toxoid (TT)-specific precursor cells. Remarkably, both intracellular cytokine staining and analysis of T-cell clones showed that Ddull cells and CD45RO T cells produce comparable high amounts of both interferon (IFN)-γ and interleukin (IL)-4, which clearly distinguishes them from CD45RA+ and Dbright T cells. Finally, prolonged culture of sorted Ddull cells in a mixture of IL-2, IL-6, and tumor necrosis factor (TNF)-α showed that about half of the population retained the Ddull phenotype. Part of the cells upregulated the CD45RA isoform, whereas only a minority switched to single CD45RO expression. Our findings indicate that the Ddull population contains primed T cells, some of which may reacquire an “unprimed” phenotype in the absence of antigenic stimulation.

MATERIALS AND METHODS

Reagents. The monoclonal antibodies (MoAbs) CLB-T11.1/1, CLB-T11.2/1, CLB-HIK27 (all CD2), CLB-CD4/1, CLB-CD8/1, CLB-CD14/1, CLB-FCγ graln1 (CD16), CLB-CD19/1, CLB-HeC/75 (CD31), CLB-CD25/1, and CLB-CD27/1 were all produced at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB). CD95 MoAb was kindly provided by Dr Yonehara (Tokyo, Japan) and CD58 MoAb (TS2/9) was a gift of Dr T.A. Springer (Boston, MA). CD62L MoAb (Leu-8) was from Becton Dickinson (San Jose, CA). CD45RA MoAbs 2H4 and 2H4-RD1 were obtained from Coulter Immunology (Hialeah, FL). CD45RO MoAb UCHL1-FITC was purchased from DAKO (Glostrup, Denmark) and UCHL1-Quantum Red from Sigma (St Louis, MO).

Biotinylated CLB-IL-4/1 MoAb (antihuman IL-4, mouse immu-
noglobulin G1 (IgG1), kindly provided by Dr T. van der Pouw-Kraan, CLB) and biotinylated MD-1 MoAb3 (antihuman IFN-γ, mouse IgG1, obtained from Dr P. van der Meide, TNO, Rijswijk, The Netherlands) were used for intracellular staining of cytokines. Mouse IgG1-B (biotinylated mouse IgG1, CLB) was used as a control reagent. Streptavidin Red 670 was obtained from Life Technologies (Gaithersburg, MD). Antibodies for IL-5 and IL-10 enzyme-linked immunosorbent assay (ELISA) were generously provided by DNAX Institute (Palo Alto, CA).

Tetanus toxoid (TT) was purchased from RIVM (Zeist, The Netherlands).

Cell preparation and flow cytometry. Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors by Ficoll-Isopaque density centrifugation (Pharmacia, Uppsala, Sweden). CD4+ T cells were purified by incubating the PBMC with CD8, CD14, CD16, and CD19 MoAbs followed by negative depletion with Dynabeads-MU0 (Dynal A.S., Oslo, Norway). CD4+ T cell blasts (5 × 10⁶ cells/mL) were cocultured with 5 × 10³ autologous irradiated (3,000 rad) plastic-adherent cells in the presence of TT (15 limit dilution [LF]/mL). No antigen was added to control wells. Cells were cultured in IMDM supplemented with 10% human pooled serum (HPS) for 9 days, and during the last 18 hours, 0.2 µCi/well of [³H]thymidine (2 Ci/mmol; Amersham, Buckinghamshire, UK) was added. Twenty-four replicate cultures were set up at each cell concentration. Only those cultures were scored as positive in which the counts per minute incorporated exceeded the mean ± 3 SD in the set of corresponding nonstimulated cultures. Frequency estimates were made using the “single-hit Poisson model” described by Stirrnsbach et al.23

Generation of T-cell clones. Purified CD4+ T cells (>95%) were sorted into CD45RA+, CD45RA+/RO+ (Dbright), CD45RA+/RO- (Dull), and CD45RO+ subsets and graded numbers of T cells (40 × 10³ to 3 × 10⁴) were cocultured with 5 × 10³ autologous irradiated (3,000 rad) plastic-adherent cells in the presence of TT (15 limit dilution [LF]/mL). No antigen was added to control wells. Cells were cultured in IMDM supplemented with 10% human pooled serum (HPS) for 9 days, and during the last 18 hours, 0.2 µCi/well of [³H]thymidine (2 Ci/mmol; Amersham, Buckinghamshire, UK) was added. Twenty-four replicate cultures were set up at each cell concentration. Only those cultures were scored as positive in which the counts per minute incorporated exceeded the mean ± 3 SD in the set of corresponding nonstimulated cultures. Frequency estimates were made using the “single-hit Poisson model” described by Strirnsbach et al.23

Statistics. The Mann-Whitney U test was used to compare functional and phenotypic data between the different subsets of T-cell clones.
CD4' T-CELL SUBSETS

Table 1. Phenotypic Analysis of CD4' T-Cell Subsets

<table>
<thead>
<tr>
<th>Subsets</th>
<th>CD45RA'</th>
<th>D dull</th>
<th>CD45RO'</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>47.3 ± 14.6*</td>
<td>15.3 ± 8.4</td>
<td>4.1 ± 2.0</td>
</tr>
<tr>
<td>CD62L</td>
<td>84.9 ± 20.7</td>
<td>72.6 ± 31.0</td>
<td>54.2 ± 26.8</td>
</tr>
<tr>
<td>CD58</td>
<td>14.7 ± 8.1</td>
<td>42.7 ± 19.3</td>
<td>73.8 ± 15.8</td>
</tr>
<tr>
<td>CD95</td>
<td>6.7 ± 3.8</td>
<td>36.0 ± 19.0</td>
<td>73.7 ± 16.4</td>
</tr>
</tbody>
</table>

*Freshly isolated CD4' cells (n = 5) were stained by 3-color immunofluorescence using Quantum Red-conjugated CD45RO and PE-conjugated CD45RA in combination with unlabeled MoAbs that were detected with GAM-FITC.

†Percentage positive cells.

RESULTS

Two intermediate phenotypes exist between CD45RA' /RO' and CD45RA'/RO' T cells. Next to the well-described CD45RA'/RO' (unprimed) and CD45RA'/RO' (primed) cells, two distinct cell populations can be detected within the peripheral blood CD4' T-cell subset that coexpress both CD45 isoforms either at high (CD45RAbright/RObright) or low density (CD45RAnull/ROnull) (Fig 1). In all healthy donors tested (n = 0), both phenotypes were present, although the CD45RAbright/RObright subset that constitutes less than 1% (0.37% ± 0.1%) of the circulating CD4' T-cell population is much smaller than the CD45RAnull/ROnull population (17.4% ± 2.5%). The four subpopulations that can be characterized by differential expression of CD45 isoforms were designated CD45RA', Dbright (CD45RAbright/RObright), D dull (CD45RAnull/ROnull), and CD45RO'.

After antigenic activation, unprimed CD4' T cells not only switch from CD45RA to CD45RO expression, but also undergo coordinate changes in the expression of adhesion molecules and homing receptors.19 Surface antigens that are differentially expressed on unprimed compared with primed T cells were analyzed on the D null population (Table 1). CD45RA' cells contained a higher percentage of cells that expressed the homing receptors CD31 and CD62L compared with CD45RO' cells. CD8, an adhesion antigen, and CD95 were upregulated in the course of transition from unprimed to primed cells. The transitional phenotype of the D null cells resulted either from an intermediate expression level (CD58), the presence of both high and low expressing cells (CD31, CD62L), or both (CD95) (Fig 2).

D null cells have the capacity to provide help for B cells and contain antigen-specific precursor cells. Functionally, CD45RA' and CD45RO' differ in their capacity to provide help for B-cell Ig synthesis.15 To compare helper activity of D null cells with that of the other subsets, sorted T cells were cocultured with E-rosette-negative cells of allogeneic donors in CD3 MoAb-coated wells and Ig synthesis was measured after 14 days. In parallel cultures [1H]-thymidine incorporation was determined on day 3 to test the proliferative capacity of the different subsets to the given stimulus. Despite comparable proliferation of all subsets after optimal stimulation with immobilized CD3 MoAb, CD45RA' cells failed to provide B-cell help. D null cells had considerable helper activity, although the amount of secreted Igs reached only about half the level of that in the presence of CD45RO' cells (Fig 3A). To compare D null and CD45RO' cells in more detail, increasing numbers of sorted cells were cocultured with E-rosette-negative cells. Again, no Ig secretion could be detected in the presence of CD45RA' cells. To generate equal amounts of IgG, approximately five times more D null than CD45RO' cells were required (Fig 3B).

Responses to recall antigens such as TT are compartmentalized almost exclusively in the primed CD45RO' subset.16 Subsets of CD4' cells from two healthy blood donors were sorted and a limiting dilution analysis was performed. The
very low number of precursors found in the CD45RA⁺ subset corresponded to the expected low frequency of TT-specific T cells in the primary T-cell repertoire. Both donors tested showed a high precursor frequency within their CD45RO⁺ populations. Notably, also the Ddull subsets contained high numbers of TT-specific precursors (Table 2).

**Ddull cells can secrete both IL-4 and IFN-γ.** The data on the phenotypic and functional characteristics suggested that the Ddull population has mixed properties when compared with CD45RA⁺ and CD45RO⁺ populations. To explore this further, the ability of this subset to secrete cytokines was assessed. Freshly isolated CD4⁺ T cells were sorted into CD45RA⁺, Ddull, and CD45RO⁺ subsets. Subsequently, the sorted populations were stimulated for 5 hours with PMA (1 ng/mL) and ionomycin (1 μmol/L) in the presence of the protein secretion-inhibitor monensin. After fixation and permeabilization of the cell membranes, accumulated IFN-γ and IL-4 were detected with specific MoAbs (Fig 4). In contrast to the CD45RA⁺ population where, as expected, the number of IFN-γ- or IL-4-producing cells was low, the Ddull subset contained substantial numbers of IFN-γ-producing cells compared with the CD45RO⁺ subset. Notable, its frequency of IL-4 producers was comparable to the CD45RO⁺ population.

Thus far, Dbright cells have been only phenotypically characterized. Due to the very low frequency of Dbright cells in the circulation, T-cell cloning is the only possibility to compare functional properties of Dbright and Ddull cells. Purified CD4⁺ T cells were sorted into CD45RA⁺, Dbright, Ddull, and CD45RO⁺ subsets and random cloning was performed with PHA and IL-2 (for the sort windows, see Fig 1). T-cell clones from all subsets were obtained with similar cloning efficiency (30% to 40%) and 24 T-cell clones per subset were analyzed. Although all T-cell clones expressed CD45RO after 3 weeks of culture, the clones were named after the cell population they were generated from. Cytokine secretion of the T-cell clones was analyzed 4 to 6 weeks after their generation. No differences in proliferation of the T-cell clones obtained from the four populations to stimulation either with CD3 MoAb or a combination of three CD2 MoAbs and CD28 MoAb were observed (data not shown). The CD45RA⁺ group of T-cell clones appeared to be mainly composed of IL-2 producers, whereas the CD45RO⁺ group contains high IFN-γ, IL-4, and IL-5, as well as IL-10 producers (Fig 5). The differences between the two subsets were found to be significant for all cytokines measured. T-cell clones derived from Dbright cells had a cytokine secretion that was largely comparable to the CD45RA⁺ group. The main difference was the higher frequency of clones that secreted larger amounts of IFN-γ in the Ddull group. In agreement with the intracellular cytokine staining, T-cell clones generated from Ddull cells displayed a distinct cytokine production that closely resembled the CD45RO⁺ group of clones. The most striking difference between T-cell clones from the Ddull and Dbright subsets is their capacity to secrete IL-4. Both percentage of clones and amount of produced IL-4 are significantly higher in the Ddull group. The same holds to a lesser extent for IL-5 and IL-10 secretion.

Concerning cytokine secretion, both freshly isolated Ddull

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**Table 2. Precursor Frequency of TT-Reactive T Cells**

<table>
<thead>
<tr>
<th>Donor No.</th>
<th>CD45RA⁺</th>
<th>Ddull</th>
<th>CD45RO⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:110,000*</td>
<td>1:900</td>
<td>1:350</td>
</tr>
<tr>
<td>2</td>
<td>1:333,000</td>
<td>1:5500</td>
<td>1:260</td>
</tr>
</tbody>
</table>

Abbreviation: 95% CI, 95% confidence interval.
CD4+ T-CELL SUBSETS

Fig 4. Cytokine production by CD4+ T-cell subsets. Sorted T cells were stimulated for 5 hours with PMA and ionomycin in the presence of the protein secretion-inhibitor monensin. After fixation and permeabilization of the cells, intracellularly accumulated cytokines were stained with specific MoAbs directed against IL-4 or IFN-γ.

CD4+ T cells were sorted into CD45RA+, Ddull, and CD45RO+ subsets. The different cell populations were kept in medium that contained 100 U/mL IL-2, 20 ng/mL TNF-α, and 500 U/mL IL-6 for 14 days. Thereafter, three-color fluorescence was performed to simultaneously analyze expression of CD25, CD45RA, and CD45RO antigens (Fig 6). Approximately 20% of the cells in all three subsets were CD25+ (data not shown). In accordance with this, no significant differences between the studied populations were found when proliferation was measured on day 8. Furthermore, the percentage of living cells after the 14-day culture period was approximately 90% in all subsets (data not shown).

In agreement with published findings, unprimed CD45RA+ cells, although proliferating, did not switch to CD45RO expression, which supports the notion that stimulation of the T-cell receptor is necessary to induce the switch in CD45 isoform expression. The majority of primed cells did not alter their CD45 expression pattern. However, a small subset reexpressed the CD45RA isoform, which indicates that part of the CD45RO+ cells may gain a Ddull phenotype in the absence of T-cell receptor crosslinking. Analysis of the Ddull subset after culture showed a marked heterogeneity in CD45 isoform expression. The original homogeneous population could be subdivided into, first, cells that switched to a CD45RA+/RO− phenotype, second, cells that upregulated the CD45RA isoform, and, third, cells that maintained the Ddull phenotype.

DISCUSSION

Expression of T-cell differentiation and activation antigens can be used to distinguish discrete stages in postthymic T-cell development. Within the CD4+ T-cell compartment, CD45RA and CD45RO antigens are expressed on unprimed and primed T cells, respectively, whereas loss of CD27 expression is a feature of differentiated effector-type T cells. To explore further the human T-cell subset origin in vivo, we analyzed two putative transitional subsets between unprimed and primed T cells in the circulation that can be distinguished on the basis of coexpression of CD45RA and CD45RO either at high (Dbright) or at low (Ddull) levels.

In the secondary lymphoid organs, the development of an unprimed CD45RA+ into a primed CD45RO+ phenotype has
been suggested to occur via a Dbright stage. In the periphery, these cells are only found in low frequency (<1%), and the majority of intermediate cells express the CD45 isoforms at low density. Phenotypically, Ddull cells have intermediate expression of all surface markers that are differentially expressed on unprimed versus primed cells, eg, adhesion antigens such as CD58 and homing receptors such PECAM (CD31) and L-selectin (CD62L). Moreover, also with respect to helper activity for B-cell differentiation and frequency of recall antigen-specific precursors, Ddull cells displayed an intermediate phenotype. However, in marked contrast to these phenotypic and functional findings, Ddull cells were found to have a cytokine secretion pattern that is quite comparable to that of primed CD45RO+ cells. Ddull CD4+ T cells were able to secrete IL-2, IFN-γ, IL-4, IL-5, and IL-10, whereas Dbright cells produced mainly IL-2 and IFN-γ and thus resembled unprimed CD45RA+ cells. The capacity of the Ddull cells to secrete IFN-γ next to IL-2 further indicates that this small population in the peripheral blood could indeed represent recently stimulated cells, since IFN-γ production capacity is rapidly acquired after antigenic stimulation. How may Ddull cells be generated in vivo? First, it might be postulated that next to the transitional Dbright stage, a relatively high number of recently activated CD45RA+ T cells are compartmentalized within this subset. A prerequisite for this notion would be that the capacity to secrete a large array of cytokines is acquired before complete change of membrane phenotype and the acquisition of B-cell-helper activity. However, in contrast to the Dbright T cells, the majority of which express activation markers and are in the S or G2/M phase of the cell cycle, only a few cells of the Ddull subset have these characteristics of recently activated cells. This makes it questionable whether a large proportion of Ddull T cells are actually converting from a CD45RA+/RO- to a CD45RA+/RO+ phenotype due to recent antigenic stimulation.

Another possibility is that the Ddull subset encompasses primed CD45RA+/RO- cells that, upon waning of their specific antigen, reexpress the CD45RA isofrom. Indeed, during in vitro culture, in the absence of stimulation of the T-cell...
CD4+ T-CELL SUBSETS

Fig 6. In vitro culture of CD4+ T-cell subsets. Sorted T cells were cultured with a combination of IL-2 (250 Ul/mL), rTNF-α (20 ng/mL), and rIL-6 (500 Ul/mL). Expression of CD45RA and CD45RO isoforms on the different subsets was analyzed immediately after sorting (A). Fourteen days after initiation of the culture, cells were restained with CD45RA and CD45RO MoAb (B).

ACKNOWLEDGMENT

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Heterogeneity of the human CD4+ T-cell population: two distinct CD4+ T-cell subsets characterized by coexpression of CD45RA and CD45RO isoforms

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