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Rapid re-expression of CD45RC on rat CD4 T cells *in vitro* correlates with a change in function*

Rat $CD4^+$ T cells are divided phenotypically by the anti-CD45RC monoclonal antibody OX22 into subsets with contrasting functions. Stimulation of T cells *in vitro* is known to induce a change in isoform from $CD45RC^+$ to $CD45RC^-$. We have investigated the *in vitro* conditions which promote a switch in isoform in the opposite direction. We observed that a majority of $CD45RC^-$ CD4 T cells (> 90%) spontaneously re-expressed CD45RC during the first 1–3 days of culture in both the presence and absence of alloantigen. The T cells remained $CD45RC^+$ when cultured for 7 days in serum-free growth medium. However, alloantigen-activated lymphocytes, expressing the interleukin-2 receptor (IL-2R), down-regulated CD45RC by day 4 and remained $CD45RC^-$ during the course of the experiment. Using mixtures of allotype-marked $CD45RC^+$ and $CD45RC^-$ T cells, it was demonstrated that each subset showed comparable survival, IL-2R expression and time courses of activation in response to alloantigen. The repertoire of neither subset was, therefore, deficient in terms of allorecognition. The rapid re-expression of CD45RC in culture was accompanied by a change in function: $CD45RC^+$ "converts", obtained by overnight culture of $CD45RC^-$ T cells, induced significantly higher graft-versus-host responses. Thus, the transition in culture from $CD45RC^-$ to $CD45RC^+$ reflects a major functional reprogramming of the cell and not a trivial modulation of a surface antigen.

1 Introduction

The leukocyte common antigen, CD45, can exist in a number of isoforms generated by alternative splicing of three (or possibly more) variable exons A, B and C [1–4]. While B cells express a CD45 molecule encoding all three exons, CD45R isoforms are differentially expressed on T lymphocytes. Thus, higher molecular weight isoforms may be the products of different combinations of the three exons. A low molecular mass (180 kDa) isoform is generated by splicing out all three variable exons. In humans, rats, mice and sheep, the expression of different isoforms of CD45R defines functionally distinct subsets of CD4 T cells [5–11]. This was first documented in the rat using the mAb OX22 [8] which identifies an epitope of the CD45RC exon product [12]. $CD45RC^+$ CD4 T cells produced more IL-2 [9], responded well to alloantigen and mitogen *in vitro* [8, 9], induced high graft-versus-host (GVH) responses [8, 13] and were the most active in inducing skin allograft rejection *in vivo* [14]. In contrast, $CD45RC^-$ CD4 T cells provided help for B cells in secondary antibody responses [9, 15], produced higher levels of IL-4 [16] and were deficient in inducing allograft rejection [14]. It was not clear whether the different responses induced by $CD45RC^+$ and $CD45RC^-$ CD4 T cells to GVH and graft rejection represented differences in allorecognition repertoires (a TcR deficiency) or in changes in the physiology of the T cell.

Many groups have shown that T cells, when stimulated *in vitro*, lose the high molecular mass isoforms (CD45RA, human; CD45RB, mouse) and synthesize the 180-kDa (CD45RO) molecule [6, 17–20]. It was suggested that this change in isoform expression represented a unidirectional transition from naive to memory T cells [21–23]. There are instances *in vivo*, however, when a switch in isoform occurs in the opposite direction. Recent work in the rat showed that CD4 T cells leaving the thymus as recent thymic emigrants were $CD45RC^-$ and re-expressed CD45RC in the periphery within 7 days [14]. In addition, adoptive transfer to athymic nude rats established that the progeny of $CD45RC^-$ T cells re-expressed CD45RC some months later [13, 24]. These observations indicated that the expression of CD45RC could be cyclical *in vivo* [25, 26] and not unidirectional as suggested by *in vitro* studies.

In view of this apparent discrepancy, we have re-examined CD45RC isoform changes *in vitro*. Were there, for example, conditions that could be defined which favored a change from $CD45RC^-$ to $CD45RC^+$? We examined the expression of this isoform in both the presence and absence of alloantigen. The investigation documents a rapid *in vitro* switch of CD4 T cells from $CD45RC^-$ to $CD45RC^+$ and links this change to the acquisition of functional properties, demonstrable *in vivo*, associated with the higher molecular weight isoform.

2 Materials and methods

2.1 Animals

The following inbred and congenic strains of rat were bred and maintained under conventional conditions in the

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Abbreviations: **TDL:** Thoracic duct lymphocytes **PLN:** Popliteal lymph node

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Animal Unit, University of Manchester Medical School: AO ($RT1^u$), BN ($RT1^n$), PVG-$RT7^b$ ($RT1^c$), PVG-$RT7^a$ ($RT1^c$), (BN × PVG)F_1 and (PVG × AO)F_1 hybrids. Animals of 3–6 months in age were used.

2.2 Monoclonal antibodies

The following mAb were used for phenotyping and cell separation: W3/25 (anti-CD4), W6/32 (anti-human HLA, control or blocking mAb), OX8 (anti-CD8), R73 (anti-αβ TcR), OX12 (anti-Ig κ chain), OX6 (anti-class II MHC), OX22 (anti-CD45RC), OX32 (anti-CD45RC, non-competing with OX22), OX7 (anti-Thy-1), OX39 (anti-IL-2R, p55), OX40 (against a molecule with some homology to CD40 and nerve growth factor receptor [27] present on activated CD4 T cells), OX27 (anti-$RT1A^c$), NR5/10 (rat anti-$RT1A^u$), 8G6.1 (rat anti-$RT7^b$) and NDS58 (rat anti-$RT7^a$). Mouse mAb were purchased from Serotec, (Bicester, Oxon, GB) or were produced at Manchester University as ascites by injection of the relevant hybridoma cell line into BALB/c mice. NDS58 and 8G6.1 were produced as tissue-culture supernatants from rat/mouse hybridomas. The R73 clone was a gift from Dr. T. Hünig. Other hybridoma cell lines were obtained from ECAAC, Porton Down, GB. mAb were purified using protein G Sepharose column (mAbTrap; Pharmacia Ltd) and biotinylated (bio-) or conjugated to FITC as previously described [28].

2.3 Fluorescence staining and cytofluorographic analysis

Cells were stained as previously described [28]. Dual fluorescence staining was either a two-stage procedure: (1) mouse mAb + bio-rat mAb, and (2) phycoerythrin-streptavidin (PE-SA, Sera-lab, Crawley Down, GB) and FITC-$F(ab')_2$ anti-mouse Ig (Dako, High Wycombe, GB) + 1% normal rat serum (F-anti-MIg), or a five-stage procedure: (1) mouse mAb, (2) F-anti-MIg, (3) W6/32 (blocking step), (4) bio-mouse mAb, and (5) PE-SA. In experiments using cells purified by positive sorting, FITC-anti-mouse Ig was found to persist on cells in culture for up to 4 days. In these instances, staining was preceded by the addition of W6/32 mAb to block binding sites on the residual FITC conjugate. Stained cells were fixed in 1% formaldehyde and analyzed using a Becton Dickinson FACScan and Consort 30 software. Forward and side scatter gates were set to exclude debris and dead cells. In some cases propidium iodide (PI) exclusion was used to gate dead cells. Populations excluded by PI gating showed close correlation to those excluded by forward and side scatter gates.

2.4 Cell separation

Thoracic duct lymphocytes (TDL) were obtained by cannulation of the thoracic duct as previously described [29]. Cervical and mesenteric lymph nodes were excised and passed through a stainless steel mesh to obtain cell suspensions (LNC). B cells were stained with OX6 and OX12 and removed by mixing with anti-mouse Ig-coated ferric Biomag particles (Metachem Diagnostics Ltd., Piddington, GB). Particles with bound cells were separated using a magnetic particle concentrator (Dynal Ltd., New Ferry, GB) as detailed previously [30]. The resulting populations were >97% αβ TcR^+. CD4 T cells were prepared by immunomagnetic separation as described above after staining with OX6, OX12 and OX8. The resulting populations were >97% $CD4^+$. $CD45RC^+$ CD4 T cells were purified from CD4 T cells prepared as above. After a second round of immunomagnetic depletion using anti-mouse Ig coated Dynabeads (Dynal), the remaining cells (>99% $CD4^+$) were stained with OX22 followed by F-anti-MIg. $OX22^+$ cells were purified by fluorescence-activated cell sorting (FACS) [13, 24] using FACS IV and Coulter Epics V sorters maintained at the Paterson Laboratories, Christie Hospital, Manchester. The resulting $CD45RC^+$ population was >99% pure. $CD45RC^-$ CD4 T cells were purified by three rounds of immunomagnetic separation (two with Biomag particles and one with Dynabeads) after staining with OX6, OX22, OX32, OX8 and OX7 (mAb OX7 removes $Thy\text{-}1^+$ recent thymic emigrants [14]).

2.5 Cell culture

LNC or TDL were cultured routinely in RPMI 1640 supplemented with 1 mM L-glutamine, 25 mM Hepes, 5×10^{-6} M 2-mercaptoethanol and 50 IU/ml each penicillin and streptomycin (Gibco, Paisley, GB). 10% heat-inactivated FCS (Sera-lab) was also added, unless otherwise specified. In some experiments, $CD45RC^-$ T cells were cultured in medium where normal rat serum was substituted for the FCS and in others a serum-free growth medium, CG (Serotec) was used without further addition. Cells were cultured in a humidified incubator in a 5% CO_2 atmosphere.

2.6 Mixed lymphocyte reaction (MLR)

Responder T cells (1.25×10^6 cells/ml) were mixed with 2.5×10^6 unseparated stimulator LNC/ml (allogeneic cells irradiated with 2000 rad or unirradiated F_1 hybrid cells) and cultured as described above.

2.7 GVH activity

GVH activity of LNC was measured by a standard parental to F_1 popliteal lymph node (PLN) weight assay as described previously [29]. Cells (1.5×10^6 in 0.1 ml PBS) were injected into the footpads of (PVG × BN)F_1 recipients. Seven days later, PLN were removed and weighed. R1 values (the increase in lymph node weight in mg if a dose of 10^6 cells had been injected) were calculated as previously described [29]. Background PLN weights (4.5–7.2 mg, obtained after injection of PBS alone) were subtracted to give net increases in PLN weight, ΔR1. Statistical significance of differences in GVH response induced by the various cell populations was determined using a Student's *t*-test.

3 Results

3.1 Parental → F_1 MLR

Evidence of an *in vitro* change in isoform expression from $CD45RC^-$ to $CD45RC^+$ was originally observed in a

parental to F_1 hybrid MLR as part of another investigation. Analysis of responder AO LNC and stimulator (PVG × AO)F_1 hybrid LNC indicated that about a third of cells were CD45RC$^-$ at the start of culture. By day 2 very few (10%) CD45RC$^-$ T cells were still present (data not shown). However, by day 4 a substantial CD45RC$^-$ subpopulation reappeared.

3.2 MLR with irradiated stimulators

To further substantiate CD45RC isoform switching *in vitro*, purified PVG T cells were used as responders in an MLR with irradiated allogeneic AO strain LNC. The phenotype of the responders was monitored on day 0 before the addition of allogeneic cells and daily thereafter (not all days shown, Fig. 1). Using mAb to polymorphic class I MHC determinants, it was ascertained that by day 1 > 97% of the allogeneic stimulators in suspension culture had died and were not detected during the course of the experiment (data not shown). Thus, responder phenotypes could be monitored in suspension cultures without significant interference from the stimulator population. Adherent allogeneic cells that are not in suspension may, of course, persist for longer.

In agreement with previous reports [8, 13, 24], CD45RC was expressed on CD4 T cells as a continuum from negative to positive (Fig. 1, day 0). However, after culture or when using purified subsets, the distinction between CD45RC$^+$ and CD45RC$^-$ subsets was more pronounced; an intermediate staining population, probably representing cells in transition [14], was absent. As far as possible reagents and flow cytometer settings were kept constant during analysis to facilitate comparisons of flow cytometer profiles obtained on separate days. We have also used a common marker to define the CD45RC$^-$ subset based on the profile of a non-staining control mAb (W6/32).

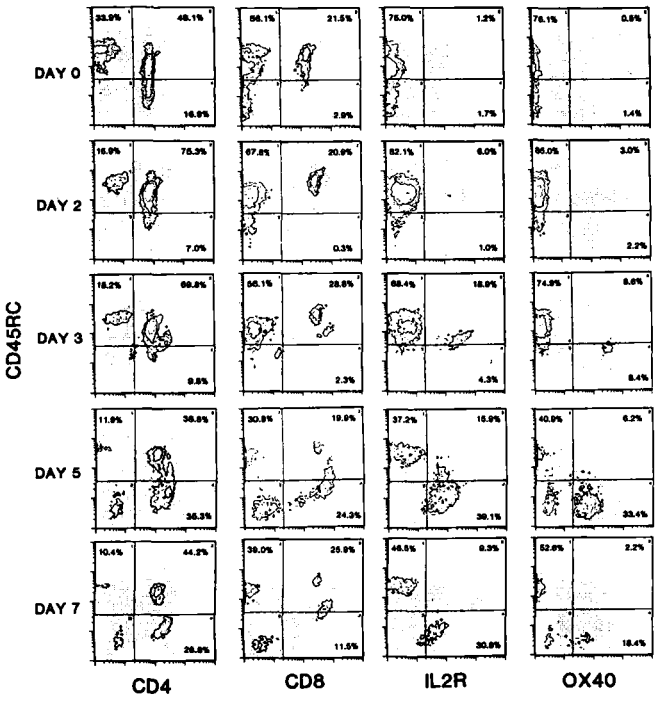

Figure 1. Sequential changes in CD45RC isoform expression during a one-way allogeneic MLR. PVG strain (RT1^c) LNC, depleted of B cells before culture, were mixed with irradiated AO (RT1^u) unseparated stimulator cells. Two-color cytofluorographic analysis using mAb against CD45RC (y axis, PE) and CD4, or CD8, or IL-2R, or a T cell activation molecule (OX40) (x axis, FITC).

The percentage of CD45RC$^-$ CD4 T cells decreased steadily during the first two days of culture (Fig. 1) but this population increased again from day 4 onwards, reaching a peak at around days 5–6 (Fig. 1). Although at day 0 the CD45RC$^-$ cells were small cells as determined by forward and side scatter measurements, the CD45RC$^-$ cells emerging after day 4 were large blast cells. A few (approx. 15%) CD45RC$^+$ small lymphocytes persisted. It was not clear whether the CD45RC$^+$ and CD45RC$^-$ subsets of CD4 T cells were dynamically interchanging or selectively surviving.

The entire population of CD8 T cells was CD45RC$^+$ at the initiation of culture and remained so until day 4, at which point a subpopulation of CD45RC$^-$ CD8 LT cells appeared (Fig. 1, second column). Again by forward and side scatter measurements, these CD45RC$^-$ CD8 T cells appeared to be large blast cells. As with the CD4 T cells a small population of CD45RC$^+$ CD8$^+$ cells persisted.

At day 0 only low levels of IL-2R expression were observed, but the expression of this T cell-activation marker was up-regulated by days 3–4, initially on CD45RC$^+$ cells, but subsequently on CD45RC$^-$ T cells (Fig. 1, third column). The expression of a CD4 T cell activation marker recognized by the mAb OX40 followed a similar course except that the antigen was up-regulated slightly later than IL-2R and was first detected on lymphocytes that had become CD45RC$^-$ (Fig. 1, last column).

3.3 MLR using allotype-marked CD45RC$^+$ and CD45RC$^-$ CD4 T cells

These observations did not answer two key questions: (i) did the disappearance of CD45RC$^-$ CD4 T cells result from a conversion to CD45RC$^+$ or from death during culture? and (ii) was there any evidence of a difference in allorecognition by the CD45RC CD4 T cell subsets that could reflect a variation in TcR repertoire? To address these questions, we established MLR cultures using mixtures of purified CD45RC$^+$ and CD45RC$^-$ CD4 T cells, each bearing a distinct RT7^a or RT7^b allotype marker (RT7A or RT7B) to enable subsequent identification. The mixtures were stimulated with irradiated AO strain LNC as before. Only responder phenotypes were monitored. This approach enabled us to investigate the alloreactive T cell repertoire of each subset unconstrained by deficiencies in cytokine production [9] of one or other subset.

RT7B allotype-marked T cells, which were CD45RC$^-$ at the initiation of culture, did not die, but re-expressed CD45RC after 1 day in culture (Fig. 2). By day 6 these cells (or their progeny) had become CD45RC$^-$ again, corresponding to a similar shift in the phenotype of RT7A cells which were initially CD45RC$^+$. These phenotypic changes suggested that activation of both subsets had occurred. This

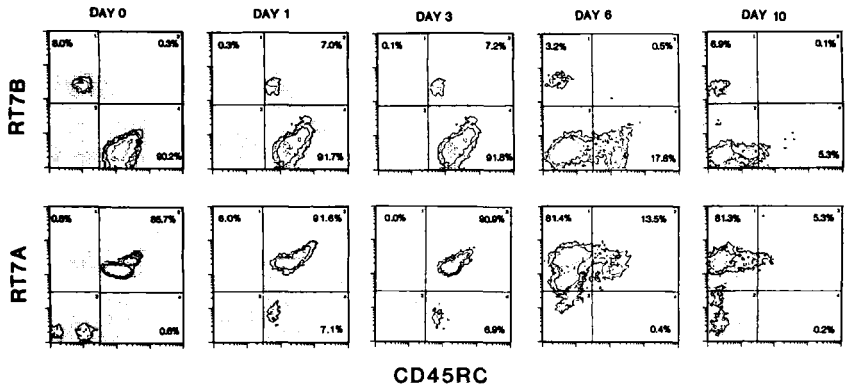

Figure 2. CD45RC$^-$ CD4 T cells re-express the CD45RC isoform during the early stages of an allogeneic MLR but subsequently lose it. PVG-RT7^b (RT7B) allotype-marked CD45RC$^-$ CD4 T cells were mixed with PVG strain (RT7A) CD45RC$^+$ CD4 T cells and stimulated *in vitro* with irradiated AO LNC. Samples for two-color cytofluorographic analysis were removed from the cultures on various days and stained for RT7^a or RT7^b (y axis, PE) and CD45RC expression (x axis, FITC).

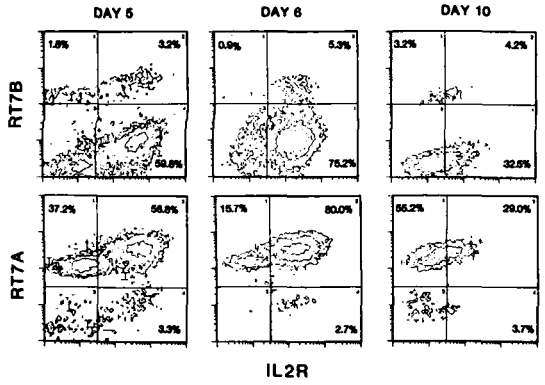

Figure 3. CD45RC$^-$ and CD45RC$^+$ CD4 T cells are activated (IL-2R expression) with similar kinetics in a one-way MLR. Cells from the same culture as in Fig. 2 were stained for RT7^a or RT7^b allotype (y axis, PE) and the IL-2R (x axis, FITC).

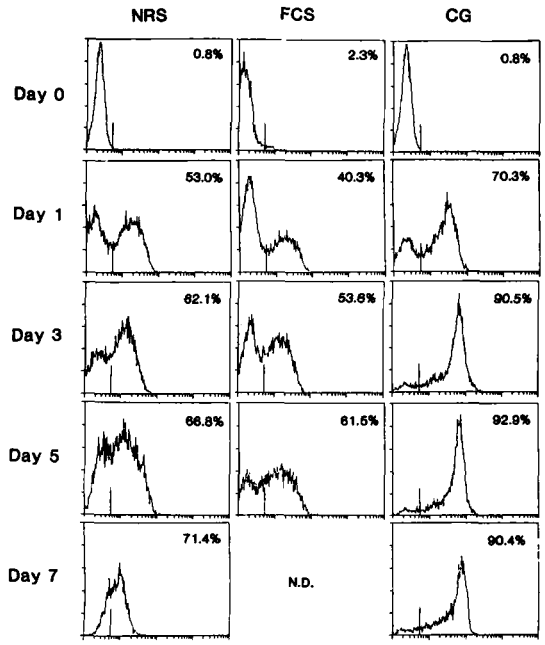

Figure 4. Purified CD45RC$^-$ CD4 T cells (day 0) re-express and maintain the CD45RC isoform *in vitro* in the absence of allogeneic or mitogenic stimuli. Cells were purified from TDL and cultured in RPMI containing 10% normal rat serum (NRS, left-hand column) or containing 10% fetal calf serum (FCS, middle column) or in serum-free growth medium (CG, right-hand column). y axis = relative cell number; x axis = CD45RC (log fluorescence FITC). Numbers represent percent of cells positive for CD45RC (vertical marker shown).

was supported by changes in IL-2R expression monitored during days 5–10 (Fig. 3). IL-2R was up-regulated on both allotype-marked populations with similar kinetics reaching a peak on day 6 (Fig. 2). This demonstrated that T cells derived from either CD45RC$^+$ or CD45RC$^-$ CD4 T cells were activated equally in an MLR. It should also be noted that the proportion of RT7A and RT7B staining cells remained relatively constant throughout the culture period, indicating that both CD45RC$^+$ andd CD45RC$^-$ subsets survived equally well *in vitro*.

3.4 CD45R isoform switching in the absence of stimulation

A rapid switch from CD45RC$^-$ to CD45RC$^+$ was also observed in the absence of allogeneic stimulation when T cells were cultured in RPMI 1640 medium containing FCS or normal rat serum (Fig. 4). Thus, 40–60% of purified CD45RC$^-$ T cells became CD45RC$^+$ after 1 day of culture. The CD45RC$^+$ phenotype was, however, retained in the absence of stimulation and up-regulation of the IL-2R did not occur. Not unexpectedly, survival of T cells under these conditions was poor. Re-expression of CD45RC was also observed in a serum-free growth medium (CG medium), a rather higher proportion of cells becoming CD45RC$^+$ (> 90%) and retaining this phenotype (Fig. 4). Cell viability was also substantially improved in CG medium.

When CD45RC$^-$ IL-2R$^+$ CD4 T cells from MLR or PHA-stimulated cultures were washed and transferred to fresh flasks (at day 6 of culture) to remove the "stimulus", they maintained an activated CD45RC$^-$ phenotype during a further 8 days of culture (results not shown). At later times poor cell viability precluded further phenotypic analysis.

3.5 Effect of CD45R isoform switching on T cell function

To determine whether the rapid change in phenotype observed *in vitro* was simply an up-regulation of a surface molecule or whether it involved a more fundamental change in cell function, the ability of CD45RC$^-$ CD4 T cells to induce a GVH response either before or after 20 h in culture was tested. Our previous work showed that CD45RC$^+$ CD4 T cells induced much larger GVH responses than did CD45RC$^-$ CD4 T cells [13]. It is impor-

Table 1. CD45RC$^-$ CD4 T cells re-express the high molecular weight isoform and increase their GVH activity after overnight culture

Expt[a]	Before culture % CD45RC$^+$	GVH ΔR1 (mg)[b]	After culture % CD45RC$^+$	GVH ΔR1 (mg)[b]	p[c]
1	6.2	12.5	61.3	17.7	<0.05
2	2.3	8.7	55.0	14.0	<0.05
3A	3.0	4.5	62.5	18.3	<0.001
3B	81.4	35.7	92.1	46.2	NS

a) CD45RC$^-$ CD4 T cells (Expt. 1, 2, 3A) were prepared by depleting TDL of CD8$^+$, Thy-1$^+$, Ig$^+$, MHC class II$^+$ and CD45RC$^+$ cells; a population enriched for CD45RC$^+$ CD4 T cells (Expt 3B) was prepared by depleting TDL of CD8$^+$, Thy-1$^+$, Ig$^+$ and MHC class II$^+$ cells. The purity of the populations is indicated (before culture).

b) Calculated response (net PLN weight increase) induced by 10^6 PVG CD4 T cells injected into the footpads of (PVG × BN)F_1 recipients 7 days earlier. Each value is derived from three to six PLN.

c) ΔR1 values compared before and after culture; Student's t test.

tant to emphasise that the GVH-induced PLN weight increase is primarily due to proliferation of host B cells, not donor T cells [31]. The assay is, thus, a reflection of the lymphokine profile of injected donor cells.

CD45RC$^-$ CD4 T cells free of Thy-1$^+$ recent thymic emigrants [14] were purified by immunomagnetic depletion. A sample of CD45RC$^-$ Thy-1$^-$ cells was removed and tested in a GVH PLN assay, while the remainder were cultured overnight. During the 20 h culture period 55–63% of the CD45RC$^-$ T cells became CD45RC$^+$ (Table 1). There was also a significant increase in GVH activity following overnight culture which correlated with the change in phenotype. The results of three separate experiments are shown in Table 1. When the results were pooled, the mean PLN weights (ΔR1 values) increased from 9.4 ± 5.4 before to 17.7 ± 5.7 after culture, a highly significant change ($p < 0.001$). To control for a possible nonspecific effect induced by overnight culture the GVH activity of CD45RC$^+$ CD4 T cells (Table 1, Expt. 3B), purified from TDL by immunomagnetic depletion (81.4% CD45RC$^+$), was also tested. These cells showed a slight increase both in number of CD45RC$^+$ cells (92.1% CD45RC$^+$) and in GVH activity, but the increase was not statistically significant. In a further experiment purified CD45RC$^-$ CD4 T cells from TDL (98.1% CD45RC$^-$) were cultured overnight to allow re-expression of CD45RC and were then depleted of CD45RC$^+$ T cells. Equivalent numbers of viable cells were tested in a GVH assay before and after depletion of the newly "converted" CD45RC$^+$ T cells. Following 20 h of culture, 82.1% of the recovered cells were now CD45RC$^+$; this population induced a strong GVH response (ΔR1 = 36.4 ± 6.6, n = 6). After depletion of the CD45RC$^+$ "converts", the remaining CD45RC$^-$ T cells (95.3% CD45RC$^-$) responded poorly in the PLN assay (ΔR1 = 5.4 ± 1.3, n = 6). Since purified CD45RC$^+$ and CD45RC$^-$ T cells survive equally well *in vitro* (Fig. 2) there is no evidence to suggest a preferential survival of highly responsive T cells. Therefore, the results indicate that the enhanced GVH activity is a property of T cells that have recently synthesised the high molecular mass CD45RC isoform *in vitro*.

4 Discussion

The present results indicate that the transition from a CD45RC$^-$ to CD45RC$^+$ phenotype occurs on CD4 T cells *in vitro*, whether in the presence or absence of allogeneic stimulation. This does not involve the death of mature CD45RC$^-$ T cells or an overgrowth by CD45RC$^+$ T cells, as clearly indicated by the use of mixtures of allotype-marked CD4 T cells (Fig. 2). This observed change in phenotype was rapid, occurring in 1–2 days. We were careful to exclude from the CD45RC$^-$ subset Thy-1$^+$ recent thymic emigrants, a population which is programmed to become CD45RC$^+$ in the periphery (with the simultaneous loss of Thy-1) as part of a final, non-antigen driven step in the maturation process [14]. We conclude that the change in phenotype represents the re-expression of CD45RC by mature CD45RC$^-$ CD4 T cells. This provides further support for the contention that the antigen-induced change from CD45RC$^+$ to CD45RC$^-$ is neither unidirectional nor irreversible [13, 24, 25].

When the rat T cell cultures were extended beyond 3 days in the presence of alloantigen (Fig. 2) or mitogens (data not shown), T cells became CD45RC$^-$, in agreement with observations in other species [6, 17–20, 32–34]. This change did not occur in the absence of stimulation (Fig. 4) and was found to correlate with T cell activation, evidenced by the fact that CD45RC$^-$ T cells were large blasts expressing high levels of IL-2R and the OX40 activation molecule (Figs. 1 and 3). Interestingly, IL-2R was up-regulated on CD45RC$^+$ T cells before CD45RC was down-regulated (Fig. 1, day 3), suggesting that the switch in isoform occurred during a later stage of T cell activation.

Freshly isolated CD45RC$^-$ CD4 T cells (from TDL or LNC, day 0 of culture) were small IL-2R$^-$, OX40$^-$ T cells and apparently not in a state of activation. If the CD45RC$^-$ population was derived from a previous antigen encounter [8, 15], this subset would appear to represent cells at a post-activational stage in which the down-regulation of activation markers occurred faster than a change in isoform. In the absence of continued stimulation *in vitro*, the CD4 T cells reverted to a resting stage and re-expressed CD45RC. We have also observed a rapid *in vivo* change of CD45RC$^-$ CD4 T cells to CD45RC$^+$ occurring on a large percentage of cells within 24 h of transfer into allotype-marked syngeneic euthymic rats (E. B. Bell, S. M. Sparshott and C.-P. Yang, to be published). Freshly isolated CD8 T cells were all CD45RC$^+$ but became CD45RC$^-$ on activation in an MLR (Fig. 1). A similar change in phenotype occurred during the proliferation phase when CD8 T cells were adoptively transferred to athymic nude rats (M.

McDonagh and E. B. Bell, unpublished). We are currently investigating the re-expression of CD45RC by these CD8 T cells.

Previous studies in the rat suggested that CD45RC$^-$ T cells were poor responders in an MLR [9]. In the human, both CD45RA and CD45RO CD4 T cells were able to respond to alloantigens equally well [7, 35]. It is interesting that IL-2-producing CD45RA$^+$ human T cells, when mixed with CD45RO$^+$ T cells, induced a synergistic response to alloantigens [36]. Our strategy of combining the allotype-marked CD45RC$^-$ T cells, a low IL-2-producing population, with IL-2-producing CD45RC$^+$ T cells [9, 16] avoided the complications associated with inadequate levels of IL-2 in the culture reported by others [37].

Previous work in the rat showed that in two *in vivo* tests, GVH activity [8, 13] and graft rejection [14], CD45RC$^+$ CD4 T cells exhibited greater alloreactivity than mature CD45RC$^-$ Thy-1$^-$ T cells. Could this be explained by differences in the T cell repertoire of the two subsets? Since the CD45RC$^-$ subset is thought to be a product of antigen encounter, it would contain T cells bearing a relatively restricted TcR repertoire, determined predominantly by environmental antigens. By contrast, the TcR repertoire of the CD45RC$^+$ subset should be broad. The demonstration here that both CD45RC$^+$ and CD45RC$^-$ CD4 T cells became activated in an MLR to a comparable extent and with similar kinetics suggested that neither subset had a deficient alloreactive TcR repertoire. The activation of CD45RC$^-$ T cells could not be attributed to a nonspecific bystander effect, for when using a non-irradiated parental to F_1 instead of an irradiated allogeneic MLR the F_1 stimulator cells (which would be subjected to the putative bystander influence of the responding T cells) remained quiescent (P. Sutton, unpublished). An explanation of the deficient *in vivo* activity of CD45RC$^-$ T cells to alloantigens awaits further investigation.

To assess whether the switch from CD45RC$^-$ to CD45RC$^+$ was associated with a change in function, the response of each subset was tested in a parental to F_1 PLN GVH assay. After culturing CD45RC$^-$ CD4 T cells for 20 h, during which time 55–63% became CD45RC$^+$, the resulting T cells were able to induce significantly higher GVH responses (Table 1). This increase in GVH activity represented a major change in function and was not simply due to culture conditions which might, for example, allow the preferential survival of cells capable of GVH induction. Viability after overnight culture of CD45RC$^-$ T cells was approximately 70%, whereas GVH activity was increased by up to threefold. In addition, the GVH response induced by CD4 T cells enriched for CD45RC (81% CD45RC$^+$) did not increase significantly after culture. Finally, when the CD45RC$^+$ overnight "converts" were removed, the T cells which remained CD45RC$^-$ continued to show poor GVH activity. In some cases, the GVH response of CD45RC$^+$ overnight "converts" was not as great as that of uncultured CD45RC$^+$ CD4 T cells. Part of the reason may be that, in these cultures (Table 1), only 60% of the CD45RC$^-$ T cells became CD45RC$^+$ within 20 h. Perhaps the time required for T cells to undergo a full functional switch may exceed the relatively short period allowed by the present protocol. Certainly CD45RC$^+$ "converts" obtained from nude recipients some months after injection of CD45RC$^-$ T cells displayed GVH responsiveness identical with that of freshly isolated CD45RC$^+$ T cells [13].

Using mAb against both the RA and RB exon products, three phenotypic subdivisions of human CD4 T cells were described [37]: (1) CD45RA$^+$RB$^+$, held to be naive T cells, (2) CD45RA$^-$RB$^+$, and (3) CD45RA$^-$RB$^-$ held to be subdivisions of memory T cells, on the assumption that CD45RA and CD45RO isoforms were reciprocally expressed. It was further assumed that RB in human was comparable to the RC exon product in the rat [37] in which case the equivalent subsets in the rat would be: (1) CD45RC$^+$ (presumed RO$^-$) and derived from recent thymic emigrants, truly naive, (2) CD45RC$^+$ "converts" (presumed RO$^+$) derived from CD45RC$^-$ T cells, and (3) CD45RC$^-$ (presumed RO$^+$) T cells, products of antigen encounter. It is not yet clear from our own work whether the CD45RC$^+$ "converts" differ from CD45RC$^+$ descendants of the thymus; we have, however, shown that both populations share the same functional capabilities detected by the GVH assay (Table 1, [13]). Work is in progress to further characterize CD45RC$^+$ "converts".

The view that CD45R isoforms mark naive and memory T cells is underpinned by the belief that CD45RA and CD45RO isoforms are reciprocally expressed [18, 19, 33, 38]. However, many studies have demonstrated co-expression of CD45RA and CD45RO on human T cells *in vitro*, in particular following T cell activation [18, 19, 33, 34]. It was argued that such cells were in transition (CD45RA to RO). This conclusion has been challenged. Recent studies using especially selected resting T cells showed that a large percentage of the CD45RA$^+$RO$^+$ T cells were, in fact, stable and not in transition [39]. Others found that a 205-kDa CD45RA isoform was up-regulated following stimulation of CD45RO clones [34, 40] and suggested that the CD45RA$^+$RO$^+$ phenotype in these circumstances was indicative of impending mitosis [40]. CD45RA$^+$RO$^+$ T cells were also observed *in vivo* [33, 41]; in a small number of apparently normal individuals, all CD45RO T cells co-expressed CD45RA [41]. The assumption that these exon products are always reciprocally expressed on separate subsets is no longer valid.

In view of the rapid *in vitro* switch in the rat, it is curious that a similar low-to-high molecular weight CD45R isoform switch has not been reported for human T cells. Perhaps this relates to detection in the human of the RA exon product rather than RC. There is evidence to suggest that exon usage between rodents and humans may differ [12, 42]. In mice, CD4$^+$ splenic T cells had no detectable CD45 mRNA transcripts for exons A or AB; LN T cells had only minor or trace levels of these exon transcripts [42]. Likewise, the existence of CD45RA on rat T cells has yet to be confirmed. Its absence is implied by the finding that binding of anti-CD45 mAb OX33 to rat lymphocytes (an mAb that does not stain rat T cells [2]) is blocked by soluble sCD45.ABC but not by sCD45.B, sCD45.C or sCD45.RO [12]. Despite possible differences in exon usage the striking cross-species similarity in function of CD45R T cell subsets suggests that the rat is not exceptional.

The precise role of different CD45R isoforms in T cell function is not clearly understood. The CD45 molecule has a key role in signal transduction during T cell activation, by

virtue of the tyrosine phosphatase activity of its highly conserved cytoplasmic domain [4]. The extracellular component of CD45 is also thought to associate with various cell surface proteins and recently the B cell antigen CD22β has been identified as a ligand for CD45RO [43]. How, if at all, these characteristics can explain the differential function of CD4 T cell subsets bearing different CD45R isoforms remains to be determined.

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