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Primary and secondary human *in vitro* T-cell responses to soluble antigens are mediated by subsets bearing different CD45 isoforms

M. PLEBANSKI, M. SAUNDERS, S. S. BURTLES,* S. CROWE & D. C. HOOPER† *Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, Bristol, U.K., *Pre-clinical Research, Pharma Division, Sandoz Ltd, 4002 Basel, Switzerland, and †Department of Biology, Leidy Laboratory of Biology, University of Pennsylvania, U.S.A.*

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SUMMARY

A culture system has been developed which consistently supports *in vitro* proliferative responses to conventional soluble antigens by human CD4+ T cells from non-immunized donors. T cells exposed to an antigen in primary cultures could be restimulated *in vitro* in an antigen-specific manner to give secondary responses with greater magnitudes and a more rapid onset than the initial reaction. To characterize further the responding T-cell population in primary compared with secondary reactions, T cells were depleted of CD45RA+ or CD45RO+ cells and stimulated with recall and non-recall antigens. It was found that the soluble non-recall antigen keyhole limpet haemocyanin did not stimulate CD45RO+ T cells, yet induced strong proliferative responses from CD45RA+ T cells. Conversely, it was confirmed that human CD45RO+ T cells respond to the recall antigen-purified protein derivative from *Mycobacterium tuberculosis*. Cell mixing experiments indicated that CD45RO+ T cells are unlikely to have any suppressive effect on the reactivity of CD45RA+ cells to non-recall antigens. These data provide new support for the hypothesis that CD45RA+ represents the naive and CD45RO+ the memory phenotype of human CD4+ T cells.

INTRODUCTION

The CD45 membrane glycoprotein family is uniquely expressed on leucocytes and their progenitors. The CD45 gene has a region of three exons, A, B and C, that can be alternatively spliced to give rise to the different isoforms of this molecule (for review see ref. 1). It has been proposed that CD4+ T cells of the CD45RO+ phenotype (exons A, B and C excised) are memory T cells and that the CD45RA+ phenotype (exons A product detectable) is characteristic of naive CD4+ T cells.[2-5] One important finding which supports this hypothesis is that the T cells which respond strongly *in vitro* to recall antigens are those which bear CD45RO.[2-7] This hypothesis also predicts that CD45RA+ CD4+ T cells should respond mainly to non-recall antigens. The allogeneic primary mixed lymphocyte reaction (MLR) has not satisfied this prediction, insofar as the responding T cells in these cultures are of both CD45RA+ and CD45RO+ phenotypes.[7-9] However, there is still controversy about the nature of the target antigens in the primary MLR, and the possibility of recall determinants being present has not been excluded.[10] Hitherto it has proved difficult to obtain human primary *in vitro* responses of CD4+ T cells to other non-recall antigens, particularly conventional soluble antigens, that could be directly assessed by assays of proliferation or lymphokine production.

We have previously described a culture system which permits naive murine CD4+ T cells to respond to both particulate[11-13] and soluble antigens.[14-16] These primary *in vitro* responses require MHC class II-associated antigen and are characterized by proliferation and lymphokine production. CD4+ cells recovered from primary cultures show altered functional properties and an anamnestic reactivity consistent with antigen-specific immunization.[11-16]

The purpose of the present investigation was to develop similar cultures to obtain primary *in vitro* proliferative T-cell responses from non-immunized human donors against conventional soluble antigens. We have shown that, as in the murine system, the responses of the naive human T cells are distinct from those of primed cells, and that the primary culture elicits specifically immune T cells. This culture system was then used to assess the CD45 isoform phenotype of the cells responding to non-recall as compared with recall antigens.

Abbreviations: BGG, bovine gamma-globulin; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorter.

Correspondence: Magdalena Plebanski, Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, University Walk, Bristol, BS8 1TD, U.K.

MATERIALS AND METHODS

Antigens

Keyhole limpet haemocyanin (KLH, Sigma Chemical Co., St Louis, MO), bovine serum albumin (BSA, Fraction V, Sigma), egg albumin (OVA, grade III, Sigma), bovine gamma-globulin

(BGG), purified in our laboratory from bovine serum,[8] and *Mycobacterium tuberculosis* purified protein derivative (PPD, Statens Seruminstitut, Denmark) were all dialysed against Dulbecco's phosphate-buffered saline (PBS) pH 7·2 before use. In some experiments, endotoxin was removed from antigen preparations by Detoxi-gel (Pierce). These treated antigen preparations were seen to elicit comparable responses to untreated antigens. Antigens were used between 20 and 100 μg per ml of culture.

Antibodies

The UCHL1 (anti-CD45RO) murine IgG2a monoclonal antibody (mAb)-producing cell line was a kind gift from Professor P. C. L. Beverley (London, U.K.).[3] The SN130 (anti-CD45RA) murine IgG1 mAb-producing cell line supernatant was a kind gift from Professor G. Janossy (London, U.K.).[4] Anti-CD3, anti-CD4, anti-CD8, anti-CD2 and anti-CD19 fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mAb were obtained from Dako Ltd, and Leucogate, B/T, Helper/Suppressor and control Simultest mAb reagents were from Becton-Dickinson. Sheep anti-human Fab (IgG) and fluorescein-conjugated goat anti-mouse IgG were produced in our laboratory.

Cells

Peripheral blood leucocytes (PBL) from healthy donors (Blood Bank, Southmead Hospital, Bristol, U.K.) or from known tuberculin-positive volunteers were separated from whole blood on a Ficoll–Hypaque gradient (Sigma). Viability was assessed by trypan blue dye exclusion (>99%). Unselected PBL were utilized as autologous antigen-presenting cells (APC). In some experiments, division of these cells was prevented by irradiation with 2000 rads from a caesium source (Gravatom Industries Ltd). T cells were purified by passage through a human immunoglobulin (Ig) sheep anti-human Fab (IgG) glass bead affinity column according to the method of Wigzell *et al.*[17] These cells were shown to be >90% T cells without contaminating B cells or monocytes by fluorescence-activated cell sorter (FACS) analysis (Epics-Coulter or FACScan, Becton-Dickinson).

CD45RO- and CD45RA-depleted T-cell populations

Purified T cells were incubated at 1×10^6/ml at 4° for 30 min in RPMI-1640 medium supplemented with 10% autologous human serum or with a UCHL1 or SN130 cell line supernatant with 10% autologous human serum at a previously determined optimal mAb-binding concentration. T cells which bound mAb were depleted by panning.[18] Both depleted cell preparations had <10% cells expressing the depleted CD45 isoform, as assessed by fluorescence microscopy and by FACS analysis (Epics-Coulter or FACScan, Becton-Dickinson).

T-cell culture and proliferation assay

Cells were cultured at $0{\cdot}6$–$1{\cdot}25 \times 10^6$/ml for T cells and $0{\cdot}6$–$1{\cdot}25 \times 10^6$/ml for PBL as APC in the presence or absence of antigen. Proliferative responses were seen to be similar over this range of cell concentrations. The medium used was alpha-minimum essential medium (αMEM Gibco) supplemented with 5% autologous heat-inactivated plasma, 4 mM L-glutamine, 20 mM HEPES buffer pH 7·2, 100 U/ml benzylpenicillin, 100 μg/ml streptomycin sulphate and 5×10^{-5} M 2-mercaptoethanol. All cultures were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°. After the periods of culture indicated (Days 5–11 if no time-course shown), proliferation was estimated using tritiated thymidine incorporation of triplicate samples as described previously.[14] Background proliferative responses refer to cultures where PBS was used to replace antigen. The statistical significance of differences between cell cultures was determined using Student's *t*-test.

Restimulations

After 9 days of primary culture, the cells were collected and washed three times in PBS. These cells were then cultured in αMEM medium (supplemented as above) at $0{\cdot}8 \times 10^6$/ml. Antigens were added at 100 μg/ml. Cells were incubated at 37° in a humidified 5% CO_2 atmosphere. Triplicate samples for the assay of tritiated thymidine incorporation were taken daily from Days 2 to 5 after restimulation.

RESULTS

Proliferative T-cell responses to soluble antigens

As the majority of blood donors in the U.K. have been immunized against the tuberculosis bacillus (Tb),[19] recall *in vitro* proliferative T-cell responses against PPD are readily obtained (Fig. 1b). We demonstrate here that human T cells also proliferate *in vitro* in response to conventional soluble antigens such as KLH (Fig. 1a), BGG, BSA and OVA (Fig. 2), without the need for a previous immunization with these antigens. These responses would appear to be independent of previous priming, insofar as characteristic primary responses were obtained in all individuals tested: 40/40 for KLH, 7/7 for BGG and 3/3 for both BSA and OVA. Moreover, in the case of KLH, other workers have demonstrated that, when used to stimulate B cells from healthy donors, a T-cell-dependent IgM response is observed, with no detectable IgG.[20] Furthermore, this antigen had been previously employed for primary human *in vitro* sensitiza-

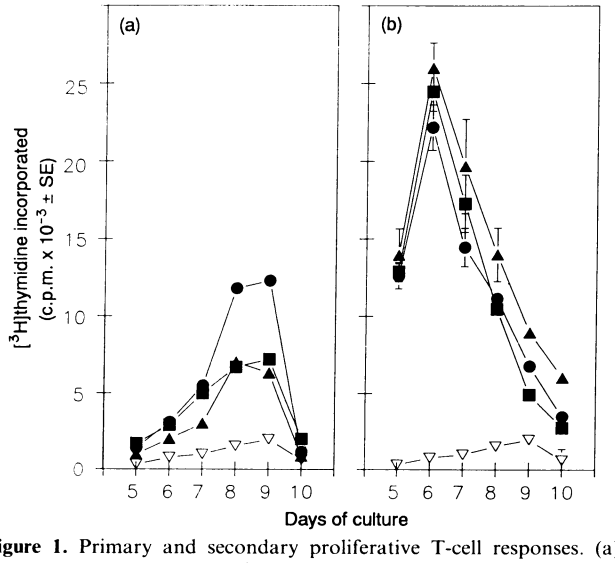

Figure 1. Primary and secondary proliferative T-cell responses. (a) Human T cells ($1{\cdot}25 \times 10^6$/ml) and irradiated autologous PBL ($0{\cdot}6 \times 10^6$/ml) were incubated from Day 0 without antigen (▽) or with 20 (▲), 50 (●) or 100 μg/ml (■) KLH. (b) The same cells were incubated without antigen (▽) or with 20 (▲), 50 (●) or 100 μg/ml (■) PPD. A representative experiment is shown ($n = 14$).

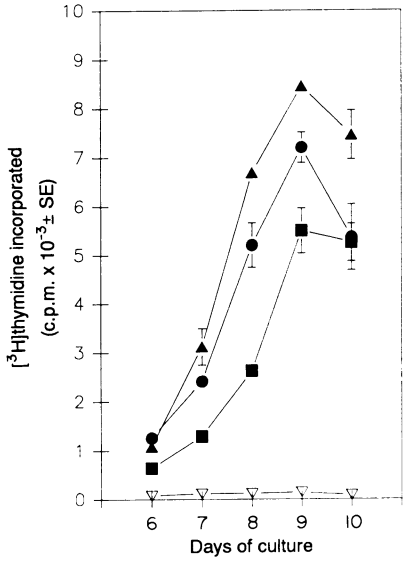

Figure 2. T-cell responses of non-immunized donors to conventional soluble antigens. T cells ($1{\cdot}25 \times 10^6$/ml) and irradiated autologous PBL ($1{\cdot}25 \times 10^6$/ml) were incubated from Day 0 without antigen (▽) or with 100 μg/ml BGG (▲), BSA (●) or OVA (■). A representative experiment is shown ($n = 3$).

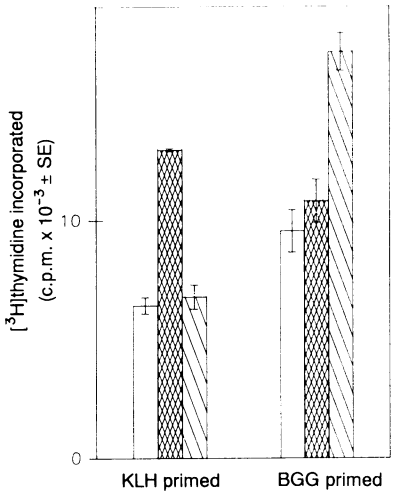

Figure 4. Specificity of the non-recall antigen-induced proliferation. T cells sensitized with either KLH or BGG were restimulated on Day 9 with either KLH (▩) or BGG (▧) or the negative control PBS (□). Antigens were used at 100 μg/ml. Peak proliferative responses are shown. A representative experiment is shown ($n = 3$).

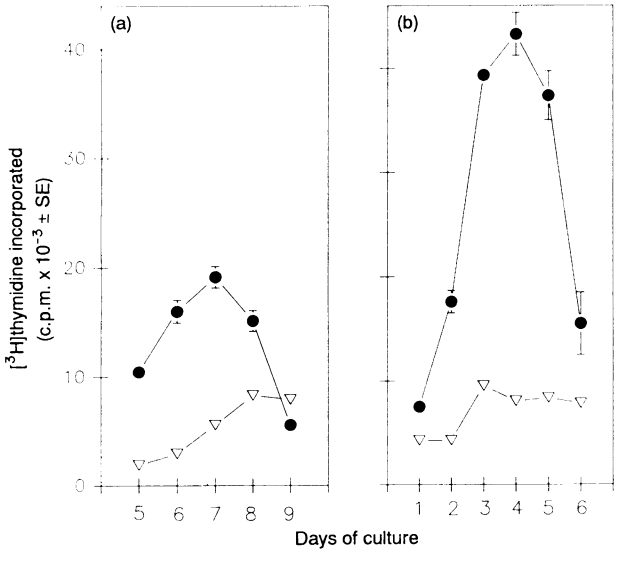

Figure 3. *In vitro* primary response (a) and *in vitro* restimulated T-cell response (b) to KLH. (a) T cells and viable PBL each at $1{\cdot}25 \times 10^6$/ml were cultured alone (▽) or with 100 μg/ml KLH (●). (b) The latter were washed on Day 9 and put back into culture at $0{\cdot}8 \times 10^6$ cells/ml in fresh medium with (●) or without (▽) KLH at 100 μg/ml. A representative experiment is shown ($n = 3$)

tion.[21,22] These observations led to our choice of KLH as the main soluble antigen for the further characterization of primary responses in humans.

When primary and secondary responses were compared within each experiment, the former were seen to be characteristically smaller and to peak 2–3 days later than recall responses. A representative experiment with KLH (non-recall) and PPD (recall) is shown in Fig. 1. This observation was consistent at the various antigen concentrations tested (20–100 μg/ml). Known tuberculin-positive donors showed the secondary pattern of response to PPD seen in Fig. 1b, whereas a characteristic primary response to PPD (smaller and slower to develop) was obtained if the donor was tuberculin negative (M. Plebanski, unpublished). All T-cell responses were dependent upon the presence of antigen-presenting cells (APC), as T cells alone did not proliferate in response to antigen in their absence (data not shown). In experiments comparing viable, irradiated and mitomycin C-treated APC it was seen that all supported similar T-cell reactions (M. Plebanski, unpublished). To explore further the identity of the proliferating T cells in primary cultures, FACS analysis was performed of cell cultures with KLH, as compared with cells from control non-stimulated cultures. Two days after the KLH-induced peak proliferation an average threefold increase in the number of $CD4^+$ T cells and no increase in $CD8^+$ T cells was observed ($n = 4$ experiments).

In vitro restimulation of primary T-cell cultures is antigen specific and presents the characteristics of a secondary response

When cells taken from a primary proliferative response to KLH (Fig. 3a) were restimulated on Day 9 by addition of the same antigen, a more rapid and higher response was obtained (Fig. 3b). These characteristics are reminiscent of a secondary proliferation of primed T cells, such as those seen to PPD (Fig. 1b). To test whether the primary proliferative response was antigen specific, cells primed with an antigen were challenged with the same or an unrelated antigen. Figure 4 shows that cells from a primary KLH reaction gave a proliferative response when restimulated with KLH, but did not respond to the unrelated antigen BGG. The converse was also true. Similar results were obtained using 50 μg/ml antigen for both the priming and the restimulation (data not shown).

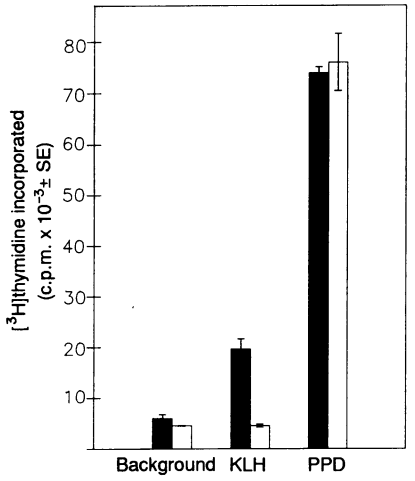

Figure 5. Proliferative responses to soluble antigens of CD45RA$^+$-depleted T cells. Unselected T cells (■) or CD45RA$^+$-depleted T cells (□) were incubated at $0{\cdot}8 \times 10^6$ cells/ml with irradiated PBL at $0{\cdot}6 \times 10^6$ cells/ml alone (left) or with 50 μg/ml KLH or PPD. Maximal proliferative responses are shown. A representative experiment is shown ($n=8$).

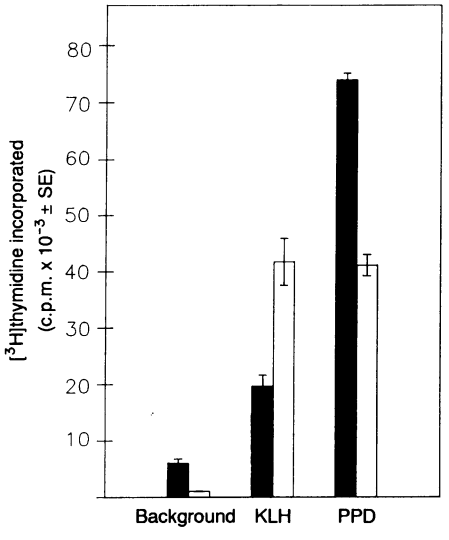

Figure 6. Proliferative responses to soluble antigens of CD45RO$^+$-depleted T cells. Unselected T cells (■) or CD45RO$^+$-depleted T cells (□) at $0{\cdot}8 \times 10^6$ cells/ml and irradiated PBL at $0{\cdot}6 \times 10^6$ cells/ml were incubated alone (left) or with 50 μg/ml KLH or PPD. Maximal proliferative responses are shown. A representative experiment is shown ($n=14$). The cells for this experiment were from the same donor as those used in Fig. 5.

CD45RA$^+$-depleted T cells do not respond to KLH but continue to respond in recall responses to PPD

If the responding cells in primary cultures are CD45RA$^+$ (as proposed for naive CD4$^+$ T cells), then decreasing the number of CD45RA$^+$ cells should decrease the response. As can be seen from the representative experiment shown in Fig. 5, primary responses to KLH were reduced down to background levels by CD45RA$^+$ depletion. In contrast, a secondary response to PPD was not affected (Fig. 5). Statistical analysis of the cumulative data from all experiments ($n=8$) showed that the proliferation of CD45RA$^+$-depleted T cells in the presence of KLH was not significantly different from their background proliferation. Maximal values of proliferation in all cultures with KLH were achieved on Day 9, on average. PPD-induced proliferation was slightly increased (10% ± 2 SE) (not significant, NS) using CD45RA$^+$-depleted T cells compared with unselected T cells, and peaked on average on Day 6 using either T-cell preparation. Background responses of depleted T cells were slightly smaller (9% ± 14 SE) (NS) than the undepleted cell backgrounds and were maximal on average on Day 9.

CD45$^+$-enriched T cells respond strongly to KLH and show low reactivity to PPD

If the responding cells in primary cultures are CD45RA$^+$ T cells, by enriching this population a corresponding increase in responses to non-recall antigens should be observed. Primary responses to KLH were increased by using CD45RA$^+$-enriched responder T cells (Fig. 6). In contrast, secondary responses to PPD were decreased (Fig. 6). Statistical analysis of the maximal responses from all depletion experiments was performed ($n=14$). A significant increase was seen in KLH-induced responses (91% ± 46 SE) ($P<0{\cdot}001$) by the CD45RA$^+$-enriched

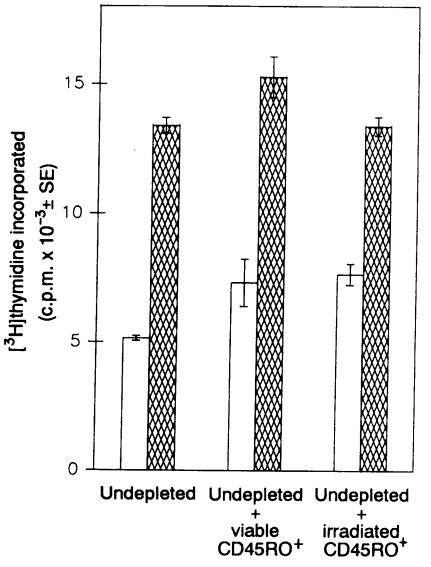

Figure 7. Effect of CD45RO$^+$-enriched T cells on a primary T-cell response to KLH. On Day 0, cultures of undepleted T cells ($0{\cdot}6 \times 10^6$ cells/ml) and irradiated PBL ($0{\cdot}6 \times 10^6$ cells/ml) (left) or these same cultures with added CD45RO$^+$-enriched viable or irradiated T cells ($0{\cdot}6 \times 10^6$/ml), were set up without antigen (□) or with 50 μg/ml KLH (▩). Peak proliferative responses are shown. A representative experiment is shown ($n=3$).

T cells as compared with undepleted T cells. This enhanced proliferative response also peaked on Day 9, on average. In contrast, responses to the recall antigen PPD were significantly decreased (41% ± 6 SE) ($P < 0.001$). Interestingly, these decreased responses had the delayed kinetics (average peak proliferation on Day 9) characteristic of primary responses. Background responses were increased (32% ± 21 SE) (NS), and were maximal on average on Day 9.

CD45RO$^+$ T cells do not have a suppressive role in KLH-induced proliferative responses

As noted above, CD45RA$^+$ cells respond more vigorously to non-recall antigens than undepleted T cells. Although unlikely, there was a possibility that this improved responsiveness was due to the removal of CD45RO$^+$ cells with suppressive activity in primary reactions. To test for this, CD45RO$^+$-enriched T cells were added into a primary T-cell culture with KLH. As can be seen in Fig. 7, the presence of these cells during culture, either viable or irradiated, did not suppress the response of undepleted T cells to KLH.

DISCUSSION

The investigation of antigen-specific *in vitro* T-cell proliferative responses has been restricted largely to those elicited by recall antigens or MHC determinants. Here we describe primary antigen-specific proliferation of human CD4$^+$ T cells from non-immunized donors to soluble conventional antigens, and present evidence to suggest that these responses have been elicited from the CD45RA$^+$ T-cell subpopulation. Certain features of the *in vitro* assay employed in these studies are likely to have facilitated the detection of the primary responses. These include the use of autologous serum, cell cultures left undisturbed during the early phase of the response, and the use of relatively high concentrations of responder T cells, which may have helped to detect the proliferation induced from low numbers of antigen-specific reactive CD45RA$^+$ naive T-cell clones.

Soluble KLH stimulated APC-dependent human T-cell proliferation. The observed increase in the CD4$^+$/CD8$^+$ T-cell ratio in cultures stimulated with KLH suggested that CD4$^+$ T cells were the main proliferating cell type. The characteristics of primary and secondary human T-cell responses were similar to the ones reported in the murine model.[11,16] The slower onset of primary compared with secondary responses could be the result of a low number of T cells specific for any particular antigen in the naive cell compartment and/or qualitative differences between naive and memory cells. In support of the latter, lower levels of surface adhesion and activation molecules have been observed on CD45RA$^+$ (proposed naive cells) than on CD45RO$^+$ T cells (proposed memory cells),[6,23] and priming has been suggested to lead to the association of the T-cell receptor complex with the low molecular weight CD45R isoforms.[24] Moreover, in the present study, it was observed that CD45RA$^+$-enriched T cells responded to KLH with an almost doubled magnitude, but with no temporal shift to an earlier maximal response.

Cells recovered from a primary reaction and restimulated with the same antigen gave larger and more rapid responses, suggesting an expansion in antigen-specific clones and, possibly, the generation *in vitro* of primed cells. In support of the latter, antigen-specific *in vitro* secondary responses have been observed previously after a primary culture in which there had been no expansion in antigen-reactive clones.[21,22] We also found that unrelated antigens were unable to induce a secondary reaction, which implies that the dividing cells were antigen specific. The clones elicited in the recall response in our cultures could be of either high or low affinity. Preliminary experiments indicate that it is still possible to obtain significant recall responses with low doses of antigen (5–20 μg/ml), suggesting that at least some high-affinity clones may be generated.

It has been proposed that naive cells express CD45RA whereas CD45RO$^+$ cells are memory cells. Our survey of reactivity to KLH and PPD of CD45RA- and CD45RO-bearing cells supports this concept. Tuberculin-positive donors gave early and high secondary responses to PPD. PPD elicited the greatest secondary responses in cultures of CD45RO$^+$-enriched T cells. In contrast, responses to the non-recall antigen KLH were optimal in cultures of CD45RA$^+$-enriched T cells. Additionally, primary *in vitro* responses to another soluble antigen, BGG, were also optimal when CD45RA$^+$-enriched T cells from non-immunized donors were used as responder cells (M. Plebanski, unpublished). It should be noted that our results do not exclude the possibility that CD45RO$^+$ T cells generated in culture from CD45RA$^+$ T cells could proliferate in response to KLH or BGG.

CD45RA$^+$-enriched T-cell proliferation to PPD shows the characteristics of a primary response, whereas CD45RO$^+$-enriched T cells respond to PPD with the kinetics and magnitude characteristic of a secondary response. Possibly PPD elicits a mixed primary and secondary T-cell response in primed donors, which is then dissociated by separately stimulating CD45RA$^+$- and CD45RO$^+$-enriched T-cell preparations. It could be argued that the delayed CD45RA$^+$-enriched T-cell response is due to the stimulation of contaminating CD45RO$^+$ T cells. However, donors exhibiting a response to PPD with primary characteristics, such as those seen with a tuberculin-negative donor, had PPD reactivity limited to CD45RA$^+$ T cells (M. Plebanski, unpublished). Therefore, PPD is not intrinsically incapable of stimulating CD45RA$^+$ cells, and it is further suggested that the state of immunity of the donor determines the responding subpopulations.

It has recently been suggested that CD45RA$^+$ and CD45RO$^+$ cells represent reversible non-activated and activated T-cell states, rather than naive and memory stages, respectively.[25,26] Our results could therefore also be interpreted as being caused by differences in the continuous *in vivo* stimulation, possibly cross-reactive,[27] of KLH- and BGG-compared with PPD-reactive cell clones. However, in view of our observation that characteristic primary PPD responses are also mediated by CD45RA$^+$ T cells, more complex stimulatory interactions would have to be envisaged. Additionally, in the case of KLH, our results and those of others support the idea that non-immunized donors are truly non-primed.[20–22] Therefore, we believe this alternative view is not easily supported by our observations. In contrast, the simplest explanation for our results is the presence of CD45RA on naive and CD45RO on memory T cells.

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