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Changes in CD45 Isoform Expression Vary according to the Duration of T-Cell Memory after Vaccination

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Healthy young (<40 years) and elderly (>60 years) adults were immunized with the 1992–1993 preparation of trivalent influenza vaccine, and changes in CD45 isoform expression on peripheral blood lymphocytes were measured in the pre- and postvaccination periods. Fluorescence-activated cell sorter analysis was used to study T-cell subsets in fresh peripheral blood lymphocytes (day 0) and after 6 days of culture with live influenza virus. We have reported previously that the interleukin-2 response to the stimulating strain of virus, A/Texas/16/89, did not decline until 26 weeks postvaccination. In ex vivo $CD4^+$ subsets, this interleukin-2 response was paralleled by a >10% increase in the proportion of cells expressing the $CD45RO^+$ phenotype following vaccination ($p < 0.0001$). In vitro stimulation had no effect on $CD4^+$ subsets prior to vaccination but, after vaccination, was associated with a >10% increase in $CD45RA^+RO^+$ cells ($P < 0.0001$). In addition, we have identified a change in the population of cells that express a CD45 isoform that is neither CD45RA nor CD45RO ($CD45RA^-RO^-$). At 26 weeks postvaccination, the proportion of $CD45RA^-RO^-$ cells in ex vivo $CD4^+$ peripheral blood mononuclear cells increased by ~15% from that measured at the earlier postvaccination time points ($P < 0.0001$). In vitro stimulation with influenza virus resulted in a further 20% increase in the proportion of $CD45RA^-RO^-$ cells ($P < 0.0001$). The $CD45RA^-RO^-$ phenotype may identify a population of cells undergoing apoptosis (programmed cell death) that limits the duration of helper T-cell ($CD4^+$) memory after vaccination.

Antigen presentation to the immune system activates a complex series of interactions through cell surface receptors and ligands. Memory B and T lymphocytes generated during this process remain in the lymphoid circulation to provide a more rapid and effective response to subsequent exposure to that antigen. Vaccines are developed to present either an attenuated or inactivated form of a particular pathogen to the immune system. Effective vaccines improve the defense mechanisms of the body by increasing antigen-specific antibody titers but also stimulate a number of cellular changes in the responding B and T lymphocytes. Immunity, thus generated, provides protection against future disease caused by that microbe.

The expression of many receptors and ligands on the surface of lymphocytes changes as a result of antigenic challenge of the immune system. Differentiation of antigen-specific lymphocyte subsets are marked by changes in the cell surface glycoproteins that underlie lymphocyte interactions. Monoclonal antibodies (MAbs) to different cell surface markers allow the phenotypic analysis of subsets of $CD4^+$ and $CD8^+$ T cells that respond to antigenic stimulation. CD45 (leukocyte common antigen) is expressed as different isoforms on the surface of peripheral blood B and T lymphocytes. CD45RA (molecular weight, 200,000 to 220,000) and CD45RO (molecular weight, 180,000) are the best characterized of the CD45 isoforms expressed on $CD4^+$ and $CD8^+$ T cells (18, 24). CD45RA has been associated with a naive T-cell population, while CD45RO appears to be a marker of memory T cells.

On activation, memory T cells produce a variety of cytokines (interleukin-2 [IL-2], IL-3, IL-4, IL-6, and gamma interferon), while naive T cells produce mainly IL-2 (3–5, 10, 21, 22). IL-2 produced by either T-cell subset stimulates antigen-specific T-cell proliferation. Memory T cells, therefore, provide much more effective help for B cells and antibody production and are the responding population for recall antigens (1, 17). Lymphocytes expressing CD45RA are naive T cells which, when activated, switch to expressing the smaller CD45RO molecule as the memory phenotype (1, 6, 23). IL-2 production has been linked to CD45 regulation (19), and IL-2 is produced by activated $CD45RA^+$ cells before undergoing a transition to $CD45RO^+$ cells (2). $CD45RA^+RO^+$ has been identified as the phenotype of cells undergoing this transition because of the early expression of the CD45RO isoform and the relative delay in the loss of CD45RA from the cell surface during stimulation (7, 21). Since $CD45RO^+$ cells also produce IL-2 (12), this cytokine can be used as a functional marker of the activation of helper T cells ($CD4^+$) that express CD45RA or CD45RO.

Changes in CD45 isoform expression occur as a result of influenza vaccination and during the subsequent response of lymphocytes to a live influenza virus challenge. We have previously shown that IL-2 production in virus-stimulated peripheral blood mononuclear cell (PBMC) cultures increases after vaccination of PBMC donors. Depending on the virus in the vaccine, the duration of increased in vitro IL-2 production is either less than 6 weeks or greater than 12 weeks (13, 14). We have postulated that this represents two forms of T-cell memory and have explored this further by studying changes in T-cell subsets expressing different CD45 isoforms. When T-cell memory for IL-2 production is of a short duration (less than 6 weeks), we have found that, in vivo, there is a progressive increase in peripheral blood T cells expressing the high-M_r CD45 isoform ($CD45RA^+RO^-$) rather than the expected increase in those cells expressing the classical memory phenotype ($CD45RO^+$). In vitro stimulation with influenza virus caused an increase in IL-2 production at the 6-week but not the

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12-week postvaccination time point. At 6 weeks, the increased in vitro IL-2 production was paralleled by a significant increase in T cells expressing a CD45 isoform that is neither RA nor RO ($CD45RA^-RO^-$) (16).

The purpose of this study was to measure changes in vivo and in vitro in CD45 isoform expression that follow vaccination with two different types of influenza vaccine, whole-virus vaccine (WVV), which contains inactivated whole virus, and split-virus vaccine (SVV), which contains disrupted virus particles. We found that the vaccine strain rather than the type of vaccine was the major determinant of the duration of the IL-2 response to vaccination. One of the strains of virus contained in the vaccine (A/Texas/16/89) was shown to stimulate increased levels of IL-2 in PBMC cultures prepared at different time points in the 12-week postvaccination period. Herein we report the in vivo and in vitro changes in CD45 isoform expression associated with this prolonged IL-2 response (>12 weeks) to vaccination in healthy young and elderly adults. The increase in $CD45RA^-RO^-$ T-cells was again observed and further characterized.

MATERIALS AND METHODS

Study participants. Thirty healthy elderly individuals (14 males and 16 females) were recruited for the study at two centers, Edmonton and Vancouver (15 individuals per center). All were carefully screened for the absence of chronic disease, were on no medications, and had normal histories, physical examinations, and laboratory investigations. Laboratory investigations included a complete blood count, renal and liver profiles, fasting blood glucose, and an electrocardiogram. The elderly study participants ranged in age from 62 to 85 years, with a mean age of 75 years (median age, 72 years). The control group consisted of 30 healthy young adults (14 males and 16 females) from the two centers (15 per center) ranging in age from 22 to 39 years; young controls were university students, medical residents, or laboratory personnel.

Vaccination. All volunteers gave informed consent to participate in the study. All study participants were vaccinated with 0.5 ml of the 1992–1993 preparation of trivalent influenza vaccine containing 15 μg each of A/Texas/16/89 (H1N1), A/Beijing/353/89 (H3N2), and B/Panama/45/90 (Fluzone; Connaught Laboratories, Inc., Willowdale, Ontario, Canada). Each of the young and elderly groups were randomized to receive either the SVV or the WVV preparation of the vaccine; 15 young and 14 elderly participants received SVV, and the other 15 young and 16 elderly volunteers received WVV. Venous blood samples were collected prevaccination and at 3, 7, and 12 weeks postvaccination. At 6 months postvaccination, 23 of the 30 Edmonton participants (11 young and 12 elderly adults) were also studied. The principal investigator, J.M., did not participate in patient selection or blood collection for the study and was blinded to the age and vaccination history of the individual participants until the completion of the study.

PBMC purification and stimulation. PBMCs were isolated from 30 ml of heparinized venous whole blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient purification and resuspended in 1.5 ml of AIM V medium (Gibco Laboratories, Inc., Grand Island, N.Y.) at a concentration of 1.5×10^6 cells per ml. For the day 0 results, 10% fetal calf serum (FCS) was added to the purified PBMCs, and the cells were stored overnight at 4°C and labelled the following day for fluorescence-activated cell sorter (FACS) analysis. A second set of PBMCs was prepared in 1.5 ml of AIM V medium at 1.5×10^6/ml, added to 24-well trays (Costar, Toronto, Canada), and stimulated with a live influenza virus preparation of A/Texas/16/89 (generous gift of Connaught Laboratories), the H1N1 strain contained in the administered vaccine. Virus was added at 0.1 μg of hemagglutinin per ml, and cultures were incubated at 37°C in a 5% CO_2 incubator. As a control, similar PBMC cultures were prepared without the addition of virus. PBMC cultures were harvested and prepared for FACS analysis. MAbs coupled directly to a fluorochrome included immunoglobulin G1 (IgG1)-phycoerythrin (PE), IgG2-fluorescein isothiocyanate (IgG2-FITC), Leu18-FITC (CD45RA), Leu2-PE (CD8), Leu3-PE (CD4), and Leu4-PE (CD3), all obtained from Becton Dickinson (San Jose, Calif.). For indirect staining to detect CD45RO (UCHL1 from P. Beverley) (24), biotinylated goat anti-mouse immunoglobulin (BioCan Scientific, Mississauga, Ontario, Canada) and streptavidin-TANDEM (Southern Biotech, Birmingham, Ala.) were purchased.

Three-color immunofluorescence. Expression of surface antigens CD4, CD8, and CD45RA was measured by a direct staining procedure. An indirect staining technique was used to detect UCHL1 (CD45RO) as described previously (20). PBMCs (~3×10^5 per well) were suspended in phosphate-buffered saline (PBS) containing 1% FCS and 0.2% sodium azide (NaAz). MAbs were added in the appropriate concentration. Cells were incubated for 30 min at 4°C, centrifuged at $600 \times g$ for 3 min, and washed twice with PBS-FCS-NaAz. Goat anti-mouse biotin (50 μl) was added to the resuspended cells, and the mixture was incubated for 30 min at 4°C. After washing twice, cells were resuspended in PBS-FCS-NaAz containing 1 μg of IgG from mouse serum (BioCan Scientific) per ml for blocking and incubated at room temperature for 10 min. Cells were centrifuged and resuspended in streptavidin-TANDEM. The other two MAbs coupled to PE and FITC were added directly, and 25 μl of PBS-FCS-NaAz was added. After a 20-min incubation at 4°C, cells were washed twice and fixed in 1% formalin for flow cytometric analysis.

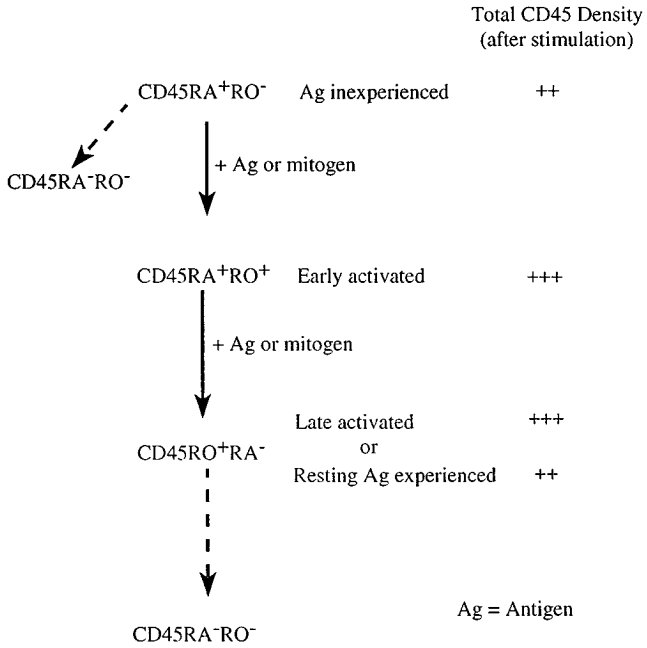

FIG. 1. Diagrammatic representation of the transitions in CD45 isoform expression on T cells. $CD4^+$ and $CD8^+$ T cells which have had little or no previous exposure to antigen (i.e., naive) express the $CD45RA^+RO^-$ phenotype. Antigenic stimulation results in T-cell activation and the concomitant expression of CD45RO. As a result of the rapid expression of CD45RO and the relative delay in the removal of CD45RA from the T-cell surface, both isoforms are expressed. The strength of and/or number of exposures to the antigenic stimulus that are required to complete the transition to the $CD45RO^+RA^-$ phenotype is not well defined. The $CD45RO^+RA^-$ phenotype has nevertheless been identified as the T cell with functional memory either in a resting or an activated state. Total CD45 density (i.e., of all isoforms) is shown for each of the phenotypes. From previous work and the current study, we propose a functional correlate of the $CD45RA^-RO^-$ phenotype.

Analysis of three-color immunofluorescence. Samples were analyzed on a FACScan (Becton Dickinson) with a single laser used to detect FITC (green), PE (orange), and TANDEM (red). Dead cells and erythrocytes were excluded by gating on forward-angle light scatter. Files of 20,000 cells were collected per sample. Each experiment included isotype-matched control antibodies to establish the specificity of antibody binding on an identically gated set of cells. Three-color data were analyzed by electronic gating on $CD4^+$ or $CD8^+$ cells and then analysis of $CD45RA^+$ and $CD45RO^+$ cells within each of the $CD4^+$ and $CD8^+$ populations. Distinguishing between dimly and brightly positive cells and in comparison with identically gated isotype control staining, CD4-PE was between channels 450 and 675, and CD8-PE was between channels 675 and 999. For CD45 isoforms CD45RA-FITC and CD45RO indirectly coupled to TANDEM, positive cells were between channels 264 and 1012 on the vertical (CD45RA) and horizontal (CD45RO) axes. $CD4^+$ or $CD8^+$ cells that did not express either of the two CD45 isoform MAbs were scattered throughout the $CD45RA^-RO^-$ quadrant, i.e., these cells were not an overlapping population from the $CD45RA^+RO^+$ quadrant.

Fluorescein-labelled, virus-stimulated PBMCs were also analyzed by FACS, without or with fixation prior to labelling. The proportions of the CD45 subsets were unchanged from those obtained with fixation after labelling. In addition, the use of these MAbs has been studied extensively, and no effect of fixation has been found (7–9, 17).

Ethics committee approval. The protocol was reviewed and approved by the Ethics Review Committee for Human Experimentation of the Faculty of Medicine, University of Alberta; the Research Committee of the Grey Nuns Hospital, Edmonton; and the Clinical Screening Committee for Research and Other Studies Involving Human Subjects, University of British Columbia.

Statistical analysis. Changes in the proportions of CD45 subsets in day 0 and

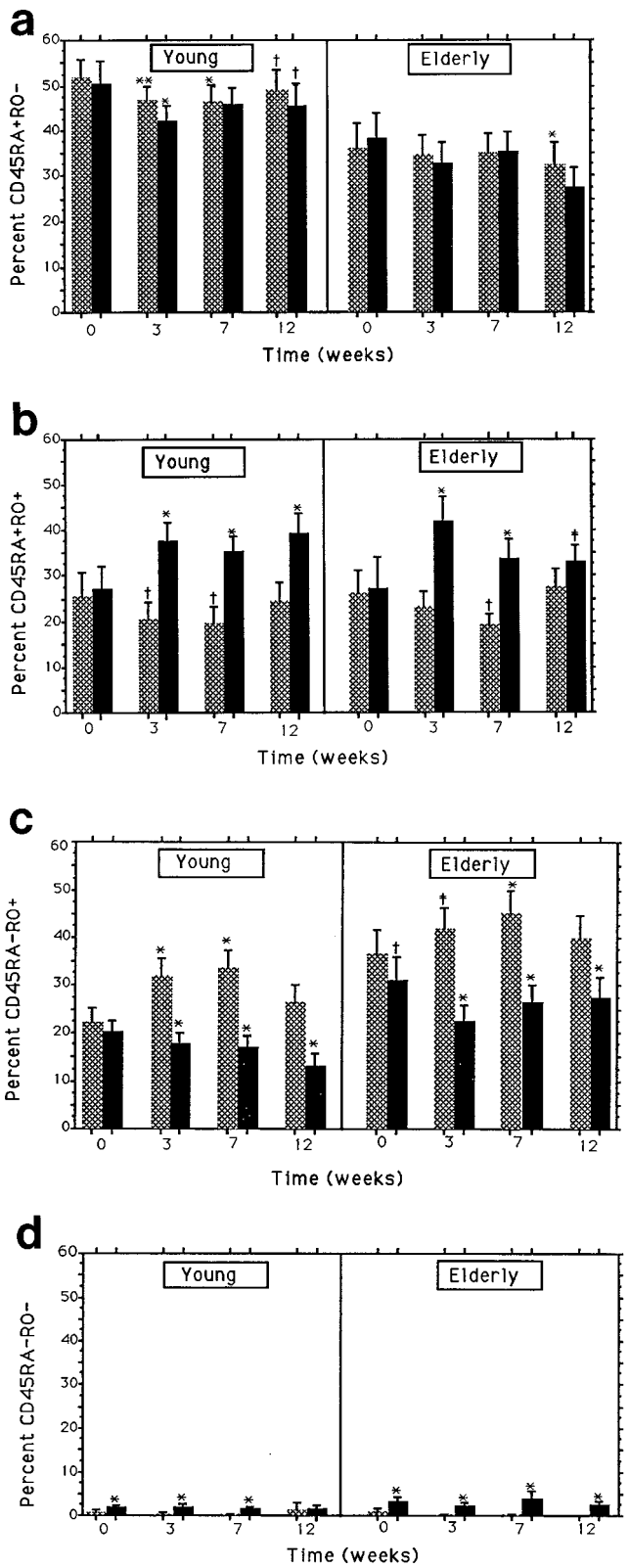

FIG. 2. Effect of in vitro virus stimulation compared with in vivo effect of influenza vaccination on different CD45 populations. Results of $CD4^+$ T cells labelled on day 0 (cross-hatched bars) and day 6 (solid bars) are shown for CD45 subsets $CD45RA^+RO^-$ (a), $CD45RA^+RO^+$ (b), $CD45RA^-RO^+$ (c), and $CD45RA^-RO^-$ (d). Day 0 results represent ex vivo PBMCs. Day 6 results represent the changes in CD45 isoforms on $CD4^+$ lymphocytes after 6 days of virus stimulation (A/Texas/16/89) of PBMCs. The average proportions of each of the CD45 subsets for the young and elderly groups are illustrated. Error bars represent 95% confidence intervals. Symbols over the day 0 bars represent the statistical significance of the difference between the prevaccination and each of the postvaccination results for that particular CD45 subset. Symbols over the day 6 bars represent the statistical significance of the difference between day 0 and day 6 results at each of the time points. Where there are no symbols, the difference was not statistically significant. Symbols of statistical significance: †, $P < 0.05$; ‡, $P < 0.01$; **, $P < 0.001$; *, $P < 0.0001$.

day 6 PBMC cultures were analyzed by the Student *t* test for paired data. Differences in the proportions of the CD45 subsets between the two age groups were determined by the Student *t* test for unpaired data.

RESULTS

Cultures of PBMCs prepared prevaccination and 3, 7, and 12 weeks postvaccination were analyzed on day 0 (not stimulated with virus) and after 6 days in culture with live influenza virus. Control cultures were also analyzed. PBMC cultures were stimulated with the same H1N1 strain of influenza virus as that in the vaccine administered to study participants (A/Texas/16/89). To maintain the appropriate immunoregulatory balance, we chose to culture unfractionated PBMCs and use multicolor flow cytometry to distinguish the different T-cell subsets. Since we had found previously that all $CD3^+$ cells were either $CD4^+$ or $CD8^+$, CD3 expression was not analyzed in this study. PBMC cultures were set up at 1.5×10^6 cells per ml, and this concentration decreased by ~25% before increasing to the initial concentration by day 6 in the presence of virus in the culture. We have previously found that $CD4^+/CD8^+$ ratios do not change with aging (16). Results were, therefore, calculated as the percentage of $CD4^+$ or $CD8^+$ cells expressing each of the four possible combinations of CD45RA and CD45RO in each of the young and elderly groups. From the literature, the model presented in Fig. 1 represents the changes in CD45 isoform expression that result from antigenic or mitogenic stimulation (1, 6, 7, 9). The transition from the expression of CD45RA to CD45RO as a result of antigenic exposure is outlined; a possible functional correlate of the $CD45RA^-RO^-$ phenotype is shown and is further developed from this study.

SVV and WVV produce similar responses that are not affected by age. We found no difference in the response to the two vaccines administered (SVV or WVV) with respect to changes in CD45 isoform expression. The results were, therefore, combined in the data analysis. Figure 2 shows the results for $CD4^+$ lymphocytes expressing different CD45 isoforms. Day 0 results represent the effect of vaccination (ex vivo), while day 6 results demonstrate the effect of live virus challenge in PBMC cultures (in vivo). Results for both young and elderly groups are represented in the graphs. As we and others have found previously (16, 17, 25), the elderly group, at baseline, had a significantly lower proportion of $CD45RA^+RO^-$ (naive) T cells and a reciprocally higher proportion of $CD45RA^-RO^+$ (memory) T cells (both $CD4^+$ and $CD8^+$). In spite of these age-related changes in the proportion of the different CD45 phenotypes, PBMCs from the elderly group showed similar changes in the proportions of the different CD45 subsets as a result of vaccination (day 0) and virus stimulation (day 6). The response to vaccination in both $CD4^+$ and $CD8^+$ T cells appeared to be similar in the two age groups, as will be described in more detail below.

Vaccination results in an increase in the proportion of $CD4^+$ T cells expressing the CD45 memory phenotype. The day 0 (ex vivo) results in Fig. 2 show that there was a progressive and statistically significant increase in the proportion of helper ($CD4^+$) T cells (T_h) expressing the memory cell

phenotype $CD45RA^-RO^+$ after vaccination. This was associated with a reciprocal decline in $CD45RA^+RO^+$ T_h and, to a lesser extent, $CD45RA^+RO^-$ T_h. Comparing pre- with postvaccination results in the young adult group, the proportion of $CD45RA^-RO^+$ T_h was increased to a maximum at 7 weeks postvaccination and then declined. The reciprocal decrease in other CD45 subsets was distributed between the $CD45RA^+RO^+$ and $CD45RA^+RO^-$ populations. The changes in these two subsets were of variable statistical significance, probably because of the smaller changes in proportions. Similar changes were observed in the elderly group, although the decrease in the $CD45RA^+RO^-$ population was not significant in this group. The statistical significance of these changes is shown in Fig. 2. Considering that only a small fraction of T cells would be influenza specific, these changes represent a major response in this small subset of T cells.

Live virus stimulation in vitro produces an increase in the proportion of $CD45RA^+RO^+$ cells. Day 6 results are shown with the day 0 results to demonstrate the effect of in vitro virus stimulation (day 6) superimposed on the in vivo (day 0) effect of influenza vaccination on different CD45 populations. Prior to vaccination, culture with live virus produced no change in the proportion of the different CD45 subsets of $CD4^+$ lymphocytes. At each of the time points postvaccination, live virus stimulated an increase in the proportion of $CD45RA^+RO^+$ cells in the young and elderly groups. In these cultures, the increase in the proportion of $CD45RA^+RO^+$ cells was accompanied by a reciprocal decrease in the proportion of $CD45RO^+RA^-$ cells in both age groups. Although the elderly group had higher proportions of T_h expressing a memory phenotype ($CD45RA^-RO^+$) and lower percentages of T_h expressing a naive phenotype ($CD45RA^+RO^-$) as a baseline measurement, the net change in the proportion of each of the CD45 populations was similar to that of the young control group.

PBMCs obtained from a small subset of the study population (four young and four elderly adults) were prepared as controls at 3, 7, and 12 weeks postvaccination to confirm that the changes observed in vitro were an effect of virus stimulation. PBMCs cultured in the absence of virus showed ~50% of cells dead or lysed by day 6. The patterns of CD45 isoform expression on unstimulated $CD4^+$ T cells were either unchanged or changed in a direction (increased or decreased) opposite to that effected by virus (results not shown).

Dot plots of raw data characterize the changes in the density of different CD45 isoforms expressed on each of the $CD4^+$ T-cell subsets. Representative dot plots generated from the FACS analysis are shown for $CD4^+$ T cells from both age groups in Fig. 3. These dot plots were generated from one young and one elderly study participant at representative time points in the study; the 7-week time point is representative of the data obtained during the first 12 weeks after vaccination. The graphs illustrate the changes in the proportions of the respective CD45 subsets. Day 0 results show fewer $CD45RA^+$ cells after vaccination and an increase in $CD45RO^+$ cells. In vitro stimulation results in an increase in the $CD45RA^+RO^+$ subset that is much more dramatic following vaccination. At 26 weeks postvaccination, the dot plots obtained are much different as a result of a marked increase in the proportion of $CD45RA^-RO^-$ T cells. The quantification and characterization of the $CD45RA^-RO^-$ subset are described below.

Expression of the $CD45RA^-RO^-$ phenotype increases at 26 weeks postvaccination. PBMCs were rechallenged with virus in vitro at 26 weeks postvaccination for 23 of the 30 participants from one of the study centers (11 young and 12 elderly). The responses of the two age subgroups were not different from

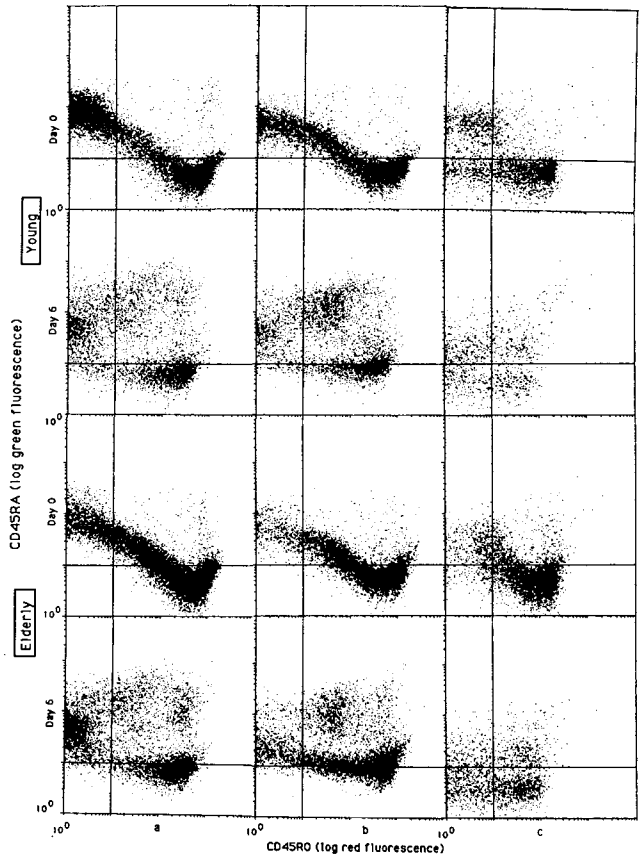

FIG. 3. Dot plots of the results obtained by FACS analysis of CD45RA and CD45RO isoforms expressed on $CD4^+$ T cells. Datum points above and below the horizontal lines on the graphs are $CD45RA^+$ and $CD45RA^-$, respectively. The vertical lines within the graphs delineate $CD45RO^+$ (to the right) from $CD45RO^-$ (to the left) T cells. Data representative of the changes in CD45 isoform expression are shown for each of the two age groups as obtained from serial PBMC isolates from one young and one elderly study participant. Day 0 (unstimulated) and day 6 (virus-stimulated) results are shown for three time points, namely, prevaccination (a), 7 weeks postvaccination (b), and 26 weeks postvaccination (c). These results are representative of the variation in the expression of each of the combinations of CD45RA and CD45RO during the 26-week study period. The average proportion of T-cells falling into each quadrant is given in Fig. 2.

their respective groups in the first 12 weeks after vaccination. Changes in the CD45 isoform expression by $CD4^+$ T cells at 26 weeks postvaccination are shown in Fig. 4. In vivo (day 0), the proportion of $CD45RA^-RO^+$ (memory) cells remained significantly elevated compared with prevaccination levels and there was a reciprocal decrease in the proportions of $CD45RA^+RO^+$ and $CD45RA^+RO^-$ cells. The most notable change was the significant increase in the proportion of $CD4^+$ cells that were $CD45RA^-RO^-$. In the initial 12 weeks after vaccination, the $CD45RA^-RO^-$ population represented only a few percent of $CD4^+$ cells at the day 0 and day 6 time points. At 26 weeks postvaccination, the proportion of this population among freshly isolated (day 0) $CD4^+$ T cells increased to 15% in both young and elderly groups.

In vitro stimulation at 26 weeks postvaccination dramatically increases the $CD45RA^-RO^-$ subset. In vitro stimulation for 6 days resulted in a further increase in $CD45RA^-RO^-$ cells, to average proportions of 35 and 40% in young and elderly groups, respectively. The in vitro increase in $CD45RA^-RO^-$ $CD4^+$ cells was paralleled by a reciprocal decline in

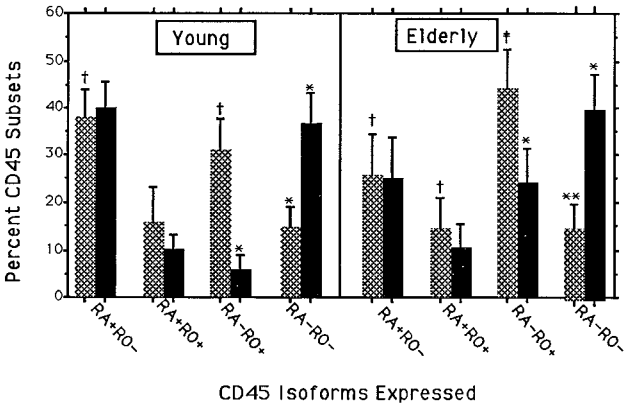

FIG. 4. Graph of changes in the CD45 isoform expression by $CD4^+$ T cells obtained at 26 weeks postvaccination in the subset of 23 participants studied. Day 0 (cross-hatched bars) and day 6 (solid bars) are shown for each of the $CD4^+$ CD45 populations. Error bars represent 95% confidence intervals. Symbols over the postvaccination day 0 bars represent the statistical significance of the difference when compared with day 0 results obtained prevaccination for each of the CD45 subsets. Symbols over day 6 results represent the statistical significance of the difference when compared with day 0 results for that particular CD45 subset. Symbols represent the same *P* values as described in the legend to Fig. 2.

$CD4^+$ cells expressing the $CD45RA^-RO^+$ (memory) phenotype in both young and elderly groups. Other subsets defined by CD45 isoforms did not change significantly with in vitro stimulation. The large increase in the proportion of $CD45RA^-RO^-$ cells observed in vivo, which was further increased after in vitro stimulation, was observed in repeated experiments between 26 and 28 weeks postvaccination. However, the proportion of these cells in freshly prepared PBMC cultures from study participants gradually declined to baseline levels by approximately 30 weeks postvaccination, suggesting that the appearance of an increased proportion of $CD45RA^-RO^-$ T cells was of limited duration (results not shown).

IL-2 does not replace the effect of virus stimulation in culture. At 26 weeks postvaccination, PBMC cultures were prepared without virus but were supplemented with 20 U of IL-2 per ml as a control for nonspecific changes in cell populations that could result from IL-2 produced in virus-stimulated cultures. In contrast to unstimulated cultures, there was very little cell death in the cultures to which IL-2 was added. Over 6 days of culture, the patterns of CD45 isoform expression in IL-2-treated PBMCs remained similar to those measured on day 0 and exhibited none of the changes observed after viral stimulation (results not shown).

$CD45RA^-RO^-$ cells express an alternate CD45 isoform. To further characterize the $CD45RA^-RO^-$ population at 26 weeks postvaccination, $CD4^+$ cells were labelled additionally with a pan-CD45 MAb which binds to all CD45 isoforms, including CD45RA and -RO. The dot plot in Fig. 5 illustrates the distribution of cells in each of the four CD45RA and -RO subsets measured in the study. For this particular example, 14% of the $CD4^+$ cells were in the $CD45RA^-RO^-$ quadrant. The next two histograms show the range of fluorescence obtained for each of the MAbs to CD45RA and CD45RO. For these two MAbs, the distribution of fluorescence is throughout the range such that the cells can be divided into negative and positive for binding to each of the CD45RA and CD45RO MAbs. The last histogram shows that in spite of the relatively large number of $CD4^+$ cells which were $CD45RA^-RO^-$, staining with a pan-CD45 MAb showed that virtually all of the $CD4^+$ cells demonstrated high-intensity staining. These data indicate that all cells, including the $CD45RA^-RO^-$ subset, express at least one CD45 isoform. The $CD45RA^-RO^-$ cells must, therefore, express an alternate CD45 isoform not detected by our MAbs to CD45RA or CD45RO.

A technique of back gating was used to examine changes in the size (or granularity) of the $CD45RA^-RO^-$ subset. By a computer manipulation, $CD4^+$ cells expressing either the CD45RA or -RO isoform were separated from those expressing neither isoform ($CD45RA^-RO^-$). Figure 6 shows that there is less side scatter in the $CD45RA^-RO^-$ population than in the aggregate of all other CD45 subsets, indicating that the $CD45RA^-RO^-$ cells are smaller in size. Similar, although not as dramatic, differences were found in the forward-scatter histogram (data not shown). The decreased size of $CD45RA^-RO^-$ subsets is consistent with apoptotic changes in these cells.

In vitro stimulation of $CD8^+$ cells produces changes in CD45 subsets both before and after vaccination. The effects of vaccination (day 0) and subsequent in vitro stimulation (day 6) on $CD8^+$ (cytotoxic or suppressor) lymphocytes are shown in Fig. 7. Compared with $CD4^+$ T cells, $CD8^+$ T cells showed a somewhat different in vivo response to influenza vaccination.

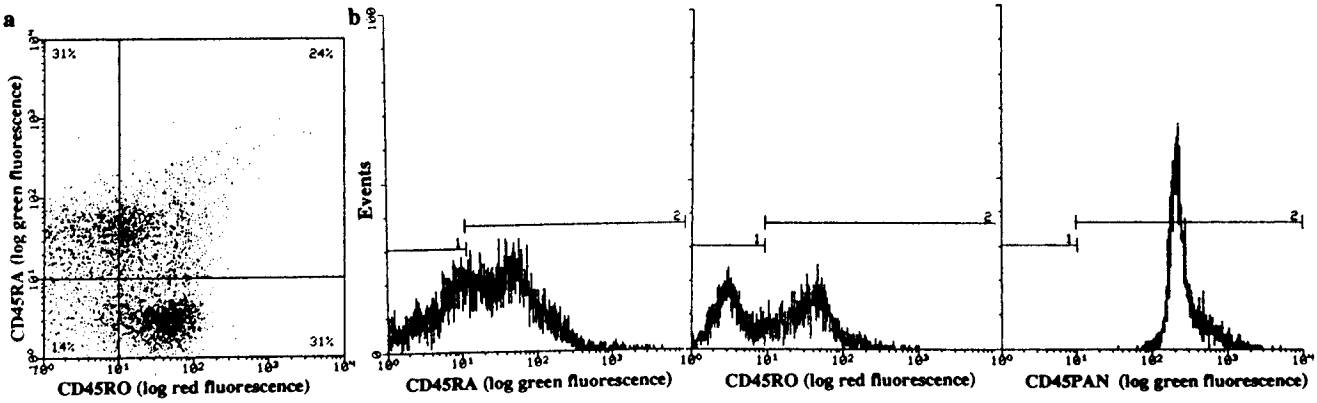

FIG. 5. Graphs characterizing the expression of the different CD45 isoforms on $CD4^+$ T cells at 26 weeks postvaccination. The dot plot (a) represents the distribution of cells, with horizontal and vertical lines delineating the different CD45 isoforms as described in the legend to Fig. 3. The percentages of cells within the different quadrants are noted in the respective corners. The next three graphs (b) are histograms showing the density of binding to $CD4^+$ T cells for each of the MAbs, namely, CD45RA, CD45RO, and pan-CD45. Higher levels of fluorescence indicate the presence of high-density MAb binding, and conversely, low levels of fluorescence represent a negative cell population with respect to the expression of the particular CD45 isoform. Whereas both $CD45RA^+$ and $-RA^-$ and $CD45RO^+$ and $-RO^-$ subsets are demonstrated, there were essentially no $CD4^+$ cells that did not stain with the pan-CD45 marker.

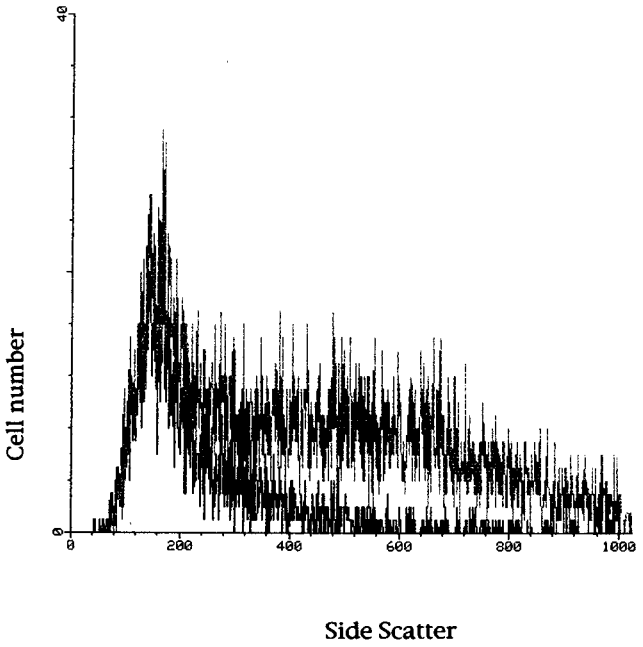

FIG. 6. Graph of a histogram of side scatter (representing granule density) of $CD4^+$ cells obtained 26 weeks postvaccination. The graph illustrates the difference in cell size between the cell populations expressing CD45RA or -RO (indicated by the upper histogram) and the cell population expressing neither CD45RA nor CD45RO (indicated by the lower histogram). This graph shows that the $CD45RA^-RO^-$ cells are smaller than the cells which are $CD45RA^+$ and/or $CD45RO^+$, suggesting that the former cell population is apoptotic. Similar but less dramatic changes in forward scatter consistent with the side-scatter changes were observed between the two populations.

With $CD8^+$ cells, an increase in the proportions of both $CD45RA^+RO^-$ (naive) and $CD45RA^-RO^+$ (memory) T cells was accompanied by a reciprocal decline in $CD45RA^+RO^+$ cells in both young and elderly groups. By comparison, in the same samples, only the $CD4^+$ cells expressing the $CD45RO^+RA^-$ phenotype increased. Similar to $CD4^+$ cells, in vitro stimulation of $CD8^+$ cells was associated with an increase in $CD45RA^+RO^+$ cells, but the reciprocal decline was in the $CD45RA^-RO^+$ (memory) population as well as the $CD45RA^+RO^-$ (antigen-inexperienced) subset. As seen for $CD4^+$ cells, $CD8^+$ cells from the elderly group showed a response similar to that of the young adult group with respect to the changes in the proportions of the different $CD8^+$ CD45 subsets, both after vaccination (in vivo) and with live virus stimulation (in vitro).

Dot plots of raw data characterize the changes in the density of different CD45 isoforms expressed on the $CD8^+$ T-cell surface. Figure 8 shows the representative dot plots of the changes in the proportions of each of the four $CD8^+$ subsets. Again, the data from one study participant from each age group at two time points in the study are shown. The 7-week postvaccination results are representative of the data obtained in the first 12 weeks following vaccination. Day 0 (ex vivo) results show the increase in $CD45RO^+$ and the decrease in $CD45RA^+$ cells following vaccination. With in vitro stimulation, there are an increase in the $CD45RA^+RO^+$ population and mainly a decrease in the $CD45RA^-RO^+$ subset.

DISCUSSION

In this study, we have demonstrated the changes in the proportions of T-cell subsets expressing different CD45 isoforms in response to influenza vaccination and the further changes in those proportions effected by stimulation with virus in vitro. In vitro stimulation with live virus had no significant effect on CD45 isoform expression in the $CD4^+$ population prior to vaccination. Over the 12-week postvaccination period, a similar method of in vitro stimulation resulted in a significant decrease in the proportion of $CD4^+$ cells expressing the $CD45RA^-RO^+$ (memory) population and a reciprocal increase in the $CD45RA^+RO^+$ phenotype which appears to be a transitional stage in the activation of T cells. Although these results may be related to the reexpression of CD45RA on $CD45RA^-RO^+$ cells, we favor the explanation that $CD45RA^-RO^+$ cells which lack specificity for the virus are dying in culture, while the $CD45RA^+RO^+$ cells which are likely to be virus-specific T-cells expand in response to viral stimulation. In this study, the T cells that responded to viral antigen could not be distinguished from the total T-cell population. Our observations, therefore, represent a combination of the effect of stimulating some T cells with virus (i.e., T cells that would grow and/or differentiate) and culturing other T cells without their specific antigenic stimulation (i.e., T cells that would die in culture).

We conducted a parallel study of the IL-2 response to vaccination by measuring IL-2 production in virus-stimulated PBMC supernatants (15). In the IL-2 study, the duration of increased in vitro IL-2 production was different for the two strains of influenza A contained in the vaccine. As we have found previously, the IL-2 response to A/Beijing/353/89 (H3N2 strain in the vaccine) was of a relatively short duration (<7 weeks). We have shown previously that this short-term memory for influenza vaccination was associated with an increase in the naive ($CD45RA^+RO^-$) $CD4^+$ T-cell subset and no increase in the memory ($CD45RA^-RO^+$) cell population (16). By comparison, the IL-2 response to A/Texas/16/89 (H1N1 strain in the vaccine) was much more prolonged (>12 weeks) (15). Changes in the proportions of lymphocytes expressing different CD45 isoforms as a result of vaccination and subsequent in vitro stimulation with A/Texas/16/89 are reported here. During the postvaccination period when in vitro IL-2 production was elevated, there were a progressive increase in the proportion of T cells with the memory phenotype $CD45RA^-RO^+$ and reciprocal changes in other CD45 subsets. The changes in proportions of the different subsets are in some cases relatively small, but consistent changes were observed. These small changes are predictable, considering that only a small fraction of T cells would be influenza specific and, thus, represent a major response in this small subset of T cells. In contrast to the previous result with A/Beijing/353/89 where a limited IL-2 response was associated with an increase in the naive ($CD45RA^+RO^-$) T-cell population, a prolonged IL-2 response to A/Texas/16/89 paralleled an increase in the classical memory ($CD45RA^-RO^+$) T-cell population. It appears that there may be different CD45 subsets in which T-cell memory can be stored. The lack of a clear definition between naive and memory subsets is further supported by the work of Tough and Sprent (25). The results presented suggest that changes in IL-2 production and patterns of CD45 isoform expression on $CD4^+$ T cells are functional and phenotypic correlates of the memory that is generated as a result of influenza vaccination.

At 26 weeks postvaccination, there was a significant increase in the proportion of $CD45RA^-RO^-$ T cells measured ex vivo (day 0). This observation contrasted with the changes observed over the 12-week postvaccination period in which $CD45RA^-RO^-$ cells were a very minor component of the total T-cell population. In vitro stimulation at 26 weeks resulted in a

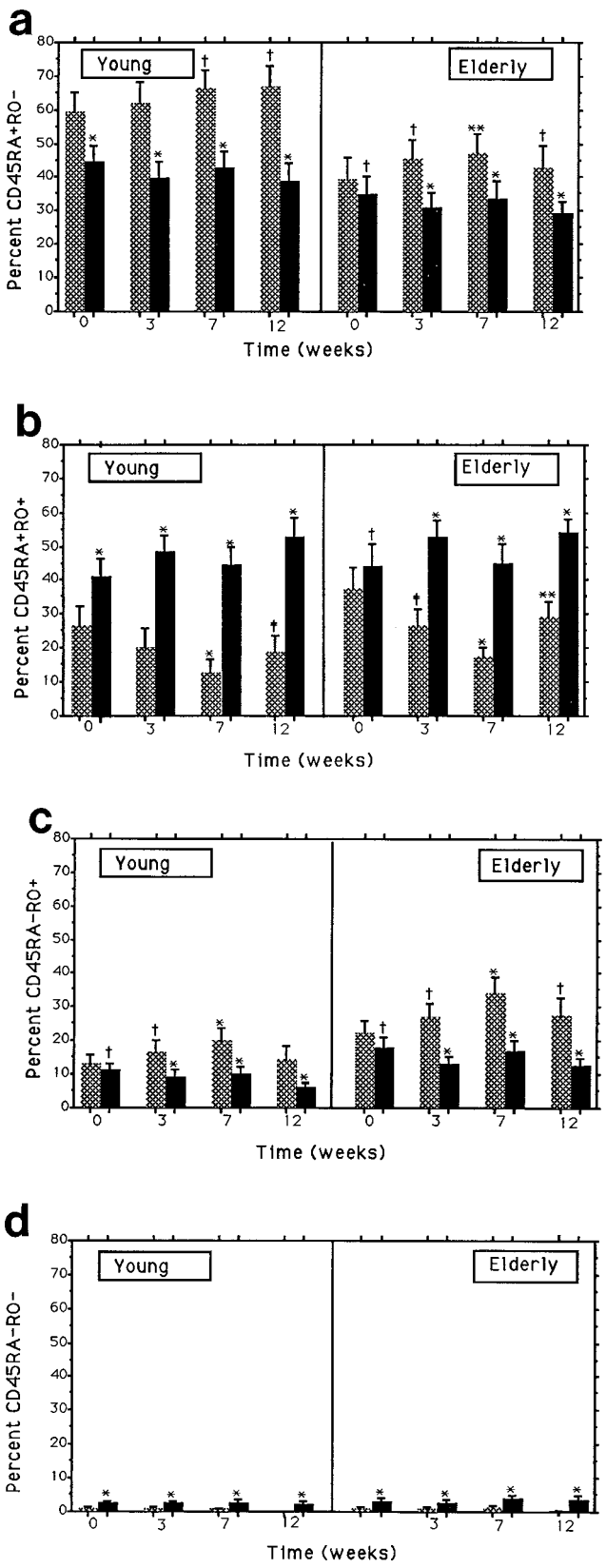

FIG. 7. Effects of vaccination and subsequent in vitro stimulation on $CD8^+$ lymphocytes. Results of $CD8^+$ T cells labelled on day 0 (solid circles) and day 6 (striped circles) are shown for CD45 subsets $CD45RA^+RO^-$ (a), $CD45RA^+RO^+$ (b), $CD45RA^-RO^-$ (c), and $CD45RO^+RA^-$ (d). Day 0 results represent ex vivo PBMCs. Day 6 results represent the changes in CD45 isoforms on $CD8^+$ lymphocytes after 6 days of virus stimulation (A/Texas/16/89) of PBMCs. The average proportions of each of the CD45 subsets for the young and elderly groups are illustrated. Error bars represent 95% confidence intervals. Statistical significances of the day 0 and day 6 results are represented by the symbols described in the legend to Fig. 2a.

further large increase in the proportion of $CD45RA^-RO^-$ cells, which was paralleled by a relative decline in the memory cell population ($CD45RA^-RO^+$). In this time period, when patients may begin to lose their immunity to influenza, the $CD45RA^-RO^-$ population was increased, and there was a reciprocal decline in the $CD45RA^-RO^+$ cell population which has been shown to produce IL-2 (3–5). This observation was paralleled by a decrease in in vitro IL-2 production to levels significantly below those observed prevaccination (15). Among ex vivo human thymocytes, the majority of cells undergoing apoptosis (programmed cell death) were $CD45RA^-RO^-$ (19a). Our data showing that the $CD45RA^-RO^-$ cells are smaller in size are consistent with this subset being an apoptotic cell population. If the increased proportion of $CD45RA^-RO^-$ cells observed at 26 weeks postvaccination represents virus-specific $CD45RA^-RO^+$ cells that have become apoptotic, rather than stimulated by influenza virus to produce IL-2, this may explain the very low IL-2 levels observed at 26 weeks postvaccination. This may be a mechanism to maintain homeostasis where most of the influenza-specific activated memory T cells ($CD45RA^-RO^+$) undergo apoptosis and a small residual population of resting memory T cells is established.

The change in CD45 isoform expression in response to vaccination and after in vitro stimulation is somewhat different in the cytotoxic or suppressor T-cell subset ($CD8^+$) as compared with the $CD4^+$ population. In vivo, there is an increase in the proportion of cells expressing either the $CD45RA^+RO^-$ or the $CD45RA^-RO^+$ phenotype, whereas the $CD4^+$ cells showed an increase only in the latter cell population. In prevaccination PBMCs, in vitro stimulation of $CD8^+$ cells with virus produced significant changes in CD45 isoform expression. This is in contrast to $CD4^+$ cells where, prior to vaccination, virus stimulation in vitro had no effect on CD45 subsets. The prevaccination changes on $CD8^+$ cells were mainly a decline in the $CD45RA^+RO^-$ subset of $CD8^+$ cells and a reciprocal increase in the $CD45RA^+RO^+$ subset. We speculate that this may reflect some long-lasting memory for previous exposure to influenza that resides in the $CD8^+$ $CD45RA^+RO^-$ subset. Such memory would be predicted to require $CD4^+$ helper T cells for its functional expression. Thus, the apparent loss of memory in $CD4^+$ T cells may underlie the loss in vaccine-mediated protection by the following year. These in vitro changes observed prior to vaccination were further augmented in the postvaccination period. In vitro stimulation resulted in a decline in $CD8^+$ cells expressing either the $CD45RA^+RO^-$ or $CD45RA^-RO^+$ phenotype with a concomitant increase in the proportion of $CD45RA^+RO^+$ cells. Little is known about the functional correlates of CD45 isoform expression on $CD8^+$ lymphocytes, which will be a focus of our future experiments.

Age-related changes in CD45 isoform expression on T cells that are due presumably to exposure to different antigens over a lifetime appear to result in a gradual transition from $CD45RA^+$ to $CD45RO^+$ T cells during the aging process. Healthy elderly adults have significantly fewer $CD45RA^+RO^-$ T cells and increased numbers of $CD45RA^-RO^+$ cells. This might suggest that the reserve capacity of aging T cells to respond to new antigens diminishes. However, our data suggest that at least with respect to influenza, there are sufficient

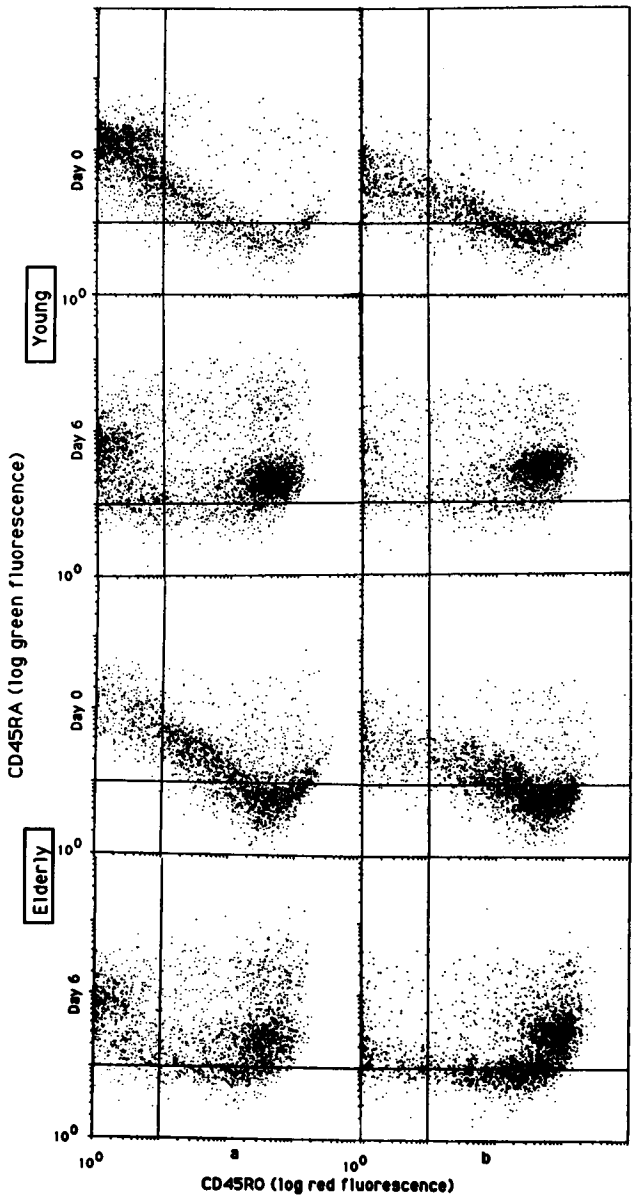

FIG. 8. Dot plot of the changes in density of different CD45 isoforms obtained for $CD8^+$ cells. The horizontal and vertical lines delineate the respective CD45RA and CD45RO subsets as described in the legend to Fig. 3. Data representing the changes in CD45 isoform expression are shown for each of the two age groups as obtained from serial PBMC isolates from young and elderly participants. Data obtained on day 0 and day 6 at two of the time points, prevaccination (a) and 7 weeks postvaccination (b), are representative of the variation in the expression of different $CD8^+$ CD45 subsets over the 12-week postvaccination period. The average proportion of T cells falling into each quadrant is detailed in Fig. 7.

numbers of cells in each of the CD45 T-cell subsets of aged individuals to mediate a response to influenza vaccination that is similar to that of young adults. This observation is supported by our IL-2 data which show that prior to vaccination, influenza virus often stimulates lower in vitro IL-2 production from T cells of elderly versus young adults. Vaccination results in an increase in in vitro IL-2 production from T cells of elderly adults that is, therefore, equivalent to that of young adults. It is important to emphasize that the healthy elderly adults studied have been screened carefully to exclude all individuals with chronic diseases and represent that subset of the elderly population who are "successfully aging". From our studies on this highly selected elderly group, it appears that aging alone results in an increase in the proportion of T cells expressing the $CD45RA^-RO^+$ phenotype. If aging is associated with an increase in the number of committed memory T cells, the reciprocal decline in naive T cells that can respond to new antigens might impair the response to the constantly changing antigens of influenza. However, in healthy elderly persons, it appears that there remains a reserve of T cells which can be stimulated to respond to influenza antigen in a manner similar to the T-cell response of young adults.

To conclude, this study shows that vaccination produces an increase in T cells expressing a memory phenotype as determined by analysis of different CD45 isoforms. In vitro IL-2 production parallels these changes in CD45 isoform expression at each time measured in the study (15). The appearance in the late postvaccination period of $CD4^+$ and $CD8^+$ populations which have lost both CD45RA and CD45RO but still express CD45 common determinants suggests that alternative isoforms, perhaps CD45 p190, characterize the $CD45RA^-RO^-$ population. The decline in T-cell memory for IL-2 production is marked by the appearance of a cell population that may identify T cells undergoing programmed cell death in a transition from active to resting T-cell memory. In addition, memory appears to be persistent in $CD8^+$ cells but transient for $CD4^+$ cells, perhaps providing another explanation for the need for annual vaccination and the loss of protection from these vaccines.

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