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Antigen-Experienced CD4 T Cells Display a Reduced Capacity for Clonal Expansion In Vivo That Is Imposed by Factors Present in the Immune Host[1]

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It is thought that protective immunity is mediated in part by Ag-experienced T cells that respond more quickly and vigorously than naive T cells. Using adoptive transfer of OVA-specific CD4 T cells from TCR transgenic mice as a model system, we show that Ag-experienced CD4 T cells accumulate in lymph nodes more rapidly than naive T cells after in vivo challenge with Ag. However, the magnitude of clonal expansion by Ag-experienced T cells was much less than that of naive T cells, particularly at early times after primary immunization. Ag-experienced CD4 T cells quickly reverted to the slower but more robust clonal expansion behavior of naive T cells after transfer into a naive environment. Conversely, the capacity for rapid clonal expansion was acquired by naive CD4 T cells after transfer into passively immunized recipients. These results indicate that rapid in vivo response by Ag-experienced T cells is facilitated by Ag-specific Abs, whereas the limited capacity for clonal expansion is imposed by some other factor in the immune environment, perhaps residual Ag. ***The Journal of Immunology*****, 2000, 164: 4551–4557.**

Initial exposure to a microbe or its Ags often induces a long-lived state of immunity that will protect the host from subsequent infection by that microbe (1). Protective immunity, also known as immunological memory, correlates with a secondary immune response that is faster, larger in magnitude, and more tailored to clearing the Ag than the primary immune response (2). Immunological memory is thought to be related to the clonal expansion and differentiation of initially naive precursors into Ag-experienced T and B cells.

The capacity of T cells to mediate immunological memory is associated with several changes in function (2). Naive T cells express high levels of molecules that facilitate migration from blood into lymph nodes, for example CD62 ligand (CD62L), and low levels of molecules that facilitate migration into nonlymphoid tissues, for example CD44. In contrast, Ag-experienced T cells express low levels of CD62L and high levels of CD44, at least at early times after initial activation. These patterns suggest that naive T cells circulate through the secondary lymphoid organs, whereas Ag-experienced T cells can circulate through nonlymphoid tissues, allowing the latter population to respond rapidly at the site of Ag entry. Naive and Ag-experienced T cells also differ with respect to lymphokine production. Naive cells produce primarily IL-2, which is thought to facilitate the clonal expansion of initially rare T cells that express the appropriate TCRs, whereas Ag-experienced cells synthesize less IL-2 and produce effector lymphokines such as IL-4 and IFN-γ, which control the microbicidal activities of other cells (3).

The means by which these functional changes are maintained in Ag-experienced T cells are poorly understood. In vivo activation of viral Ag-specific CD8 T cells produces cells with the function and cell surface phenotype of Ag-experienced cells that persist for long time periods in the absence of detectable Ag (4–6). This suggests that immunological memory is maintained because of an irreversible differentiation step in Ag-experienced T cells. On the other hand, some work with CD4 T cells suggests that the functional (7) and phenotypic (8) changes displayed by Ag-experienced cells at early times after exposure to Ag are only maintained as long as Ag persists in the host. Thus, the changes ascribed to Ag-experienced CD4 T cells may be reversible and may not even be intrinsic properties of the T cells themselves; rather, they may be imposed by extrinsic factors present in the environment.

We addressed the roles of intrinsic changes and environmental factors in the behavior of Ag-experienced cells using an adoptive transfer model in which CD4 T cells of known specificity from TCR transgenic mice were tracked in immune or naive recipients. Our results suggest that the clonal expansion potential of Ag-experienced CD4 T cells is influenced by extrinsic factors present in the immune environment.

Materials and Methods

Mice

DO11.10 (9) TCR transgenic mice were bred in a specific pathogen-free facility under National Institutes of Health guidelines and screened for the TCR transgenes as previously described (10). These mice have been bred onto the BALB/c background for >15 generations and are histocompatible with normal BALB/c mice. BALB/c mice were purchased from the National Cancer Institute (Frederick, MD) and housed in a conventional facility. DO11.10 SCID donors were obtained by crossing DO11.10 BALB/c mice for two generations with SCID BALB/c mice (purchased from The Jackson Laboratory, Bar Harbor, ME) and selecting offspring that expressed the DO11.10 TCR but lacked B cells in the peripheral blood. B cell-deficient (μMT) mice (11), provided by Dr. Steven L. Reiner (University of Chicago, Chicago, IL), were bred to BALB/c mice for five generations.

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Adoptive transfer and immunization

The behavior of naive T cells was studied by adoptive transfer (via i.v. injection) of lymph node and spleen cells from naive wild-type or SCID DO11.10 donors, containing 2.5×10^6 $CD4^+$, KJ1-26^+ cells, into unirradiated normal BALB/c mice (10). In cases where B cell-deficient mice were used as recipients, lymph node and spleen cells from the DO11.10 donors were exhaustively depleted of B cells using an anti-B220 mAb (RA3-3A1/6.1, American Type Culture Collection, Manassas, VA) and rabbit complement (Accurate Chemical and Scientific, Westbury, CT). The elimination of B cells from the cell preparation was confirmed before transfer by staining with FITC-labeled goat anti-mouse Ig (Caltag, South San Francisco, CA). Recipients were immunized 1 to several days after transfer by injection of 100 μg of chicken OVA (Sigma, St. Louis, MO) or 300 μg of OVA peptide 323–339 emulsified in CFA or IFA into two s.c. sites on the back or by i.v. injection of 300 μg of OVA peptide 323–339 plus 150 μg of LPS (serotype *Escherichia coli* 026:B6; Difco, Detroit, MI). In some experiments bromodeoxyuridine was offered to mice in the drinking water (0.8 mg/ml) ad libitum, beginning on the day of immunization. In other experiments recipients of naive DO11.10 T cells were passively immunized by i.p. injection every day for 4 days with 1.2 mg of purified IgG from OVA/CFA-primed or naive mice, ending on the day before immunization with OVA/IFA. The titers of anti-OVA IgG1 and IgG2a Abs in the passively immunized animals were similar to those of DO11.10 recipients immunized 3 wk previously with OVA/CFA. In some cases animals received a second set of s.c. injections of 100 μg of OVA or 300 μg of OVA peptide 323–339 in CFA or IFA at several different sites on the back, 9–50 days after the first injection.

A serial adoptive transfer protocol was used for study of Ag-experienced DO11.10 T cells in a naive environment. Naive DO11.10 T cells ($5–7.5 \times 10^6$/mouse) were injected i.v. into naive BALB/c recipients, which were then immunized with 100 μg of chicken OVA in CFA as described above. After 9–12 days, draining lymph node cells (cervical, brachial, axillary, and inguinal) were recovered, and the CD4 T cells were enriched by depletion of CD8 T cells with anti-CD8 mAb (clone 83.12.5, American Type Culture Collection) and rabbit complement (Accurate Chemical and Scientific) and of B cells and adherent cells using a Cellect T cell column (Biotex, Edmonton, Canada). The enriched CD4 T cells, which contained 3–5% Ag-experienced DO11.10 T cells, were then transferred to naive BALB/c recipients such that each recipient received $1.25–2.5 \times 10^6$ DO11.10 T cells. Recipient mice were then injected with OVA in CFA as described above.

In some cases lymph node cells from naive DO11.10 mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE;[3] Molecular Probes, Eugene, OR) before transfer, using a modification of a previously described technique (12). Briefly, cells were washed once in HBSS (Biofluids, Rockville, MD), adjusted to a final concentration of 5×10^7 cells/ml in HBSS, and incubated with 5 μM CFSE for 10 min in a 37°C water bath. An equal volume of cold HBSS containing 10% FCS was added to stop the labeling, and the cells were washed before adoptive transfer. The number of cell divisions that the CFSE-labeled DO11.10 T cells experienced after Ag activation in vivo was determined as previously described (13), based on the assumption that the CFSE signal is reduced by half at each cell division.

Detection of TCR transgenic T cells

DO11.10 T cells were identified in adoptive recipients by two-color flow cytometry as previously described (10). The analysis was performed on lymph nodes cells or enriched CD4 T cells that were prepared from lymph nodes as described above. Before addition of labeled mAb, cells were incubated on ice for 10 min with anti-FcR mAb in 10% rat and mouse serum to block FcRs. When unlabeled DO11.10 T cells were transferred, cells were stained with PE-labeled anti-CD4 mAb (PharMingen, San Diego, CA), biotinylated KJ1-26 mAb (which recognizes the DO11.10 TCR) (14), and FITC-labeled streptavidin (SA; Caltag). When CFSE-labeled DO11.10 T cells were transferred, cells were stained with CyChrome-labeled anti-CD4 mAb (PharMingen), biotinylated KJ1-26 mAb, and PE-labeled SA (Caltag). Thirty-thousand events were collected on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed using CellQuest software. DO11.10 T cells were identified as $CD4^+$, KJ1-26^+ events. The number of DO11.10 T cells present in the draining lymph nodes was determined by multiplying the total number of viable cells recovered by the percentage of $CD4^+$, KJ1-26^+ events obtained by flow cytometry.

The size of the DO11.10 T cells was estimated from the forward light scatter values of 1000–5000 $CD4^+$, KJ1-26^+ events.

The cell surface phenotype of DO11.10 T cells was determined by staining lymph node cells with allophycocyanin-labeled anti-CD4 mAb, biotin-labeled KJ1-26, peridinin chlorophyl protein (PerCP)-labeled SA (Becton Dickinson, San Jose, CA), and PE-labeled anti-CD45RB or anti-LFA-1 mAbs; with digoxygenin-labeled KJ1-26, sheep anti-digoxygenin Ab, PE-labeled donkey anti-sheep Ab, CyChrome-labeled anti-CD4 mAb, biotin-labeled anti-CD45RB mAb, and allophycocyanin-labeled SA; or with biotin-labeled KJ1-26, CyChrome-labeled anti-CD4 mAb, PerCP-labeled SA, and FITC-labeled anti-CD45RB mAb (all mAb except KJ1-26 were from PharMingen). Five hundred to 1000 $CD4^+$, KJ1-26^+ events were collected using a FACScaliber flow cytometer. The fluorescence intensity related to CD45RB or LFA-1 expression was measured in the appropriate channel.

Intracellular IL-2 was detected by flow cytometry as initially described by Openshaw et al. (15) with the modifications reported by Khoruts et al. (16). Naive recipients of DO11.10 T cells or recipients of DO11.10 T cells that were immunized with OVA plus LPS 17 days previously were injected i.v. with 300 μg of OVA peptide to stimulate IL-2 production in vivo. Spleen cells were stained with CyChrome-labeled anti-CD4 mAb and biotinylated KJ1-26, followed by FITC-labeled SA. The cells were then washed in PBS and fixed for 20 min at room temperature in PBS containing 2% formaldehyde, permeabilized with two washes in staining buffer containing 0.5% saponin (Sigma), and then incubated for 30 min at room temperature with PE-labeled anti-IL-2 mAb or a similarly labeled control mAb of the same isotype (PharMingen). The cells were then washed twice with saponin buffer and twice with PBS. The PE channel fluorescence of at least 1000 $CD4^+$, KJ1-26^+ events was measured on a FACScan flow cytometer.

Bromodeoxyuridine was detected in DO11.10 cells by flow cytometry as described by Pape et al. (17).

Results

In vivo production of Ag-experienced T cells

OVA peptide I-A^d-specific CD4 T cells from the DO11.10 TCR transgenic mouse line were transferred into normal recipients, which were then immunized with OVA to produce a uniform population of Ag-experienced DO11.10 T cells. Although most CD4 T cells from the normal DO11.10 strain express the DO11.10 TCR and a naive phenotype ($CD45RB^{high}$), these mice contain a small population of CD4 T cells that express the DO11.10 TCR and a memory/activated phenotype ($CD45RB^{low}$). These T cells express an endogenous TCR and presumably became Ag-experienced cells via recognition of an environmental Ag (18). To eliminate any contribution of these cells, DO11.10 BALB/c SCID donors were used for the initial experiments because they contain only naive CD4 T cells that express the DO11.10 TCR.

Lymph node and spleen cells from DO11.10 BALB/c SCID donors were labeled with the cytoplasmic fluorochrome CFSE before transfer, so that their cell division history could be monitored in the recipient. Recipient mice were immunized via s.c. injection of OVA in CFA. Within 1 day of transfer, KJ1-26^+ DO11.10 T cells were identified in unimmunized recipients at low, but consistent, levels in the secondary lymphoid organs of recipient mice (Fig. 1*A*). These T cells uniformly expressed the high level of CD45RB characteristic of naive T cells (Fig. 1*B*), lacked the $CD45RB^{low}$ population of Ag-experienced CD4 T cells found in normal mice (Fig. 1*B*), and retained large amounts of CFSE, indicating that they had not entered the cell cycle (Fig. 1*D*). In contrast, most of the DO11.10 T cells that were present in the draining lymph nodes 3 days after injection of OVA/CFA were blasts (Fig. 1*G*) and contained low amounts of CFSE (Fig. 1, *D* and *F*) as would be expected if these cells had divided many times. This possibility was supported by the finding that the number of DO11.10 T cells present in the lymph nodes that drained the OVA/CFA injection site increased dramatically, such that 20-fold more cells were present on day 5 after Ag injection than were present before immunization (Fig. 1*E*). The number of DO11.10 T cells in

[3] Abbreviations used in this paper: CFSE, carboxyfluorescein diacetate succinimidyl ester; PerCP, peridinin chlorophyl protein; SA, streptavidin: CD62L, CD62 ligand.

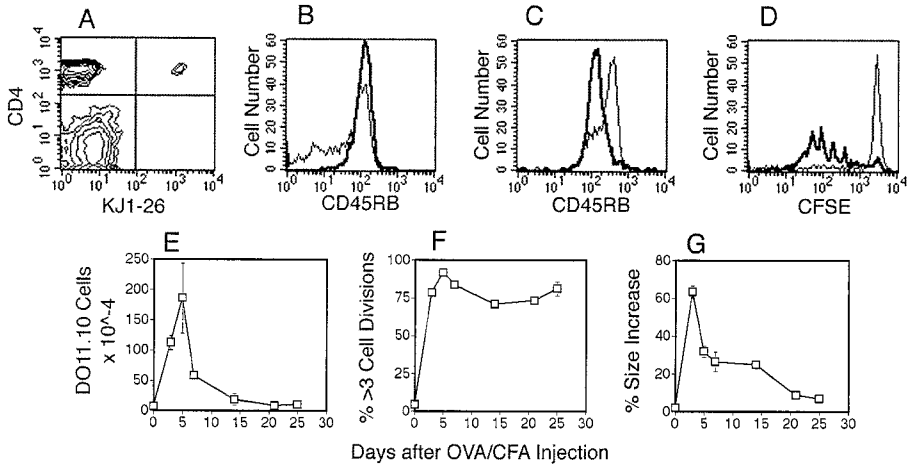

FIGURE 1. Ag-experienced T cells have divided in the past and changed their surface phenotype. A contour plot of expression of CD4 and the DO11.10 TCR (recognized by the KJ1-26 mAb) on lymph node T cells from a BALB/c recipient of DO11.10 T cells is shown in *A*. $CD4^+$, KJ1-26^+ cells, which appear of the *upper right quadrant* of *A*, were not detected among the lymph node cells of BALB/c mice that did not receive DO11.10 T cells (10, 19). The levels of CD45RB on polyclonal BALB/c (thin line) or DO11.10 CD4 T cells (thick line) from mice that were not immunized (*B*) or were immunized with OVA/CFA 21 days earlier (*C*) are shown. CD45RB was detected with different fluorochromes that gave differing degrees of fluorescent signal in *B* (FITC) and *C* (allophycocyanin). This accounts for the difference in the shape of the histograms for polyclonal BALB/c CD4 cells, which served as internal markers for the high and low levels of CD45RB expression. Despite the difference in intensity between the fluorochromes used to detect CD45RB, it is apparent that the naive DO11.10 cells overlaid the $CD45RB^{high}$ BALB/c cells in *B*, and the Ag-experienced DO11.10 cells overlaid the $CD45RB^{low}$ BALB/c cells in *C*. *D*, CFSE levels in DO11.10 T cells from the draining lymph nodes of unimmunized recipients (thin line) or mice that were injected 3 days earlier with OVA/CFA (thick line). The mean number of DO11.10 T cells in the draining lymph nodes, the percentage of these cells that divided more than three times based on CFSE dilution, and the sizes of these cells at the indicated times after injection of OVA/CFA are shown in *E–G*, respectively. The error bars indicate the range of duplicate measurements.

the draining lymph nodes then fell dramatically by day 7 and slowly thereafter to a level on day 25 that was about twice the starting level. The majority of DO11.10 T cells that remained in the lymph nodes 21–25 days after immunization were no longer blasts (Fig. 1*G*), showed evidence of at least four cell divisions (Fig. 1*F*), and expressed the lower levels of CD45RB (Fig. 1*C*) characteristic of Ag-experienced T cells. Identical results were obtained when normal DO11.10 mice were used as donors (data not shown). Thus, the small population of pre-existing Ag-experienced cells present in normal DO11.10 donors had no detectable effect on the clonal expansion and contraction of naive DO11.10 T cells or on the production of OVA-experienced DO11.10 T cells during the primary response to OVA. For this reason, normal DO11.10 donors were used for subsequent experiments. Similar results were obtained when OVA/LPS or OVA/IFA were used as the s.c. immunogens instead of OVA/CFA (10, 19).

Clonal expansion by Ag-experienced T cells

The clonal expansion of Ag-experienced DO11.10 T cells during a secondary response was tested by challenging recipient mice with OVA at various times after primary immunization. In the experiment shown in Fig. 2*A*, recipients were immunized with OVA peptide/CFA, and as expected, the initially naive DO11.10 T cells increased 30-fold in the draining lymph nodes on day 5 and then fell to a lower level on day 12 that was still significantly greater than the starting level. When these recipients were injected

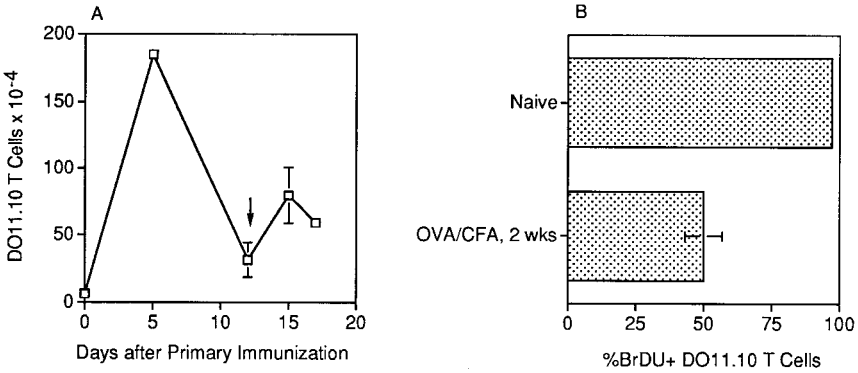

FIGURE 2. Clonal expansion by Ag-specific CD4 T cells during the primary and secondary responses. *A*, Recipients of naive DO11.10 T cells were injected with 300 μg of OVA peptide 323–339 in CFA. Twelve days later, these mice were injected again with 100 μg of OVA peptide in IFA (indicated by the arrow). The mean number $\pm$ range of DO11.10 T cells present in the draining lymph nodes at the indicated times ($n = 2$ for each group and at each time point) were measured by flow cytometry. Similar results were obtained in five independent experiments. *B*, Naive recipients of DO11.10 T cells or DO11.10 recipients that were injected with OVA/CFA 2 wk previously were injected s.c. with OVA/IFA. Recipients were offered bromodeoxyuridine beginning on the day of OVA/IFA challenge. The mean percentage $\pm$ SEM of DO11.10 T cells that incorporated bromodeoxyuridine into DNA 5 days after OVA/IFA challenge are shown. Less than 5% of the DO11.10 T cells incorporated bromodeoxyuridine in naive or OVA/CFA-primed mice that were not challenged with OVA/IFA (17) (data not shown).

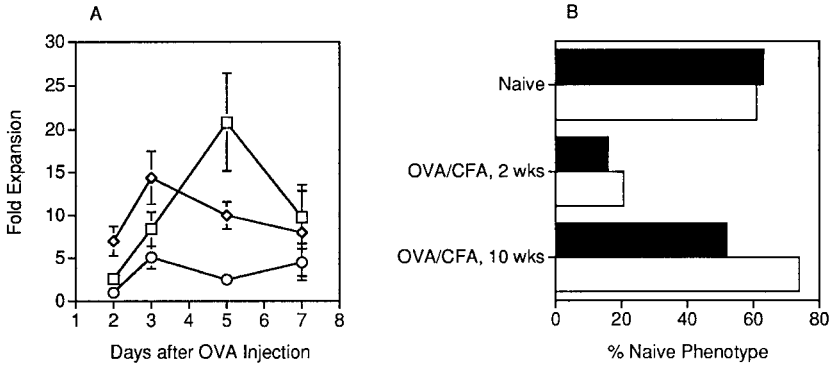

FIGURE 3. Clonal expansion by Ag-experienced T cells at early and late times after primary immunization. *A*, Recipients of naive DO11.10 T cells were injected with 100 μg of OVA in CFA. Two weeks (circles) or 10 wk (diamonds) later, these mice or a naive control group (squares) were injected with 100 μg of OVA in IFA. The number of DO11.10 T cells present in the draining lymph nodes was measured by flow cytometry at the indicated times after OVA/IFA injection. These values were divided by the number of DO11.10 T cells present in each group on the day of OVA/IFA injection to calculate the fold expansion. The mean fold expansion ± SEM are shown (n = 3–4 for each group and at each time point). *B*, The percentage of $CD4^+$, $KJ1\text{-}26^+$ cells expressing the naive phenotype (high levels of CD45RB (■) or low levels of LFA-1 (□) in each group at the time of OVA/IFA injection.

with OVA again on day 12, the now Ag-experienced DO11.10 T cells increased in the draining lymph nodes 3 days later to a maximal level that was only 3-fold higher than the number present on the day of secondary challenge. Large numbers of DO11.10 T cells were not present in the thoracic duct lymph (assessed by cannulation of the thoracic duct as described in Ref. 20), at the s.c. challenge site, or in the spleen at any time after secondary challenge (R. Merica, unpublished observation), indicating that rapid migration out of the lymph nodes was not responsible for the poor accumulation of Ag-experienced DO11.10 T cells. On the contrary, poor proliferation was involved because DO11.10 T cells in mice that received primary immunization 2 wk earlier, incorporated less bromodeoxyuridine into DNA after secondary challenge than naive DO11.10 T cells did after primary exposure to OVA (Fig. 2*B*). Therefore, although the Ag-experienced DO11.10 T cells accumulated maximally in the lymph nodes more rapidly after Ag challenge than naive DO11.10 T cells, the magnitude of their proliferation was much smaller.

The results of a second experiment of this type are shown in Fig. 3. The fold increase in DO11.10 T cells present in the draining lymph nodes after OVA injection is shown to correct for the different numbers of DO11.10 T cells present at the time of primary (for naive cells) or secondary (for Ag-experienced cells) immunization. Again, naive DO11.10 T cells underwent a 20-fold expansion in the draining lymph nodes that peaked 5 days after Ag injection. In contrast, DO11.10 T cells that experienced primary immunization 2 wk before secondary challenge increased only 5-fold, with maximal accumulation occurring on day 3. Ag-experienced DO11.10 cells accumulated to a greater extent after secondary challenge if 10 wk passed between the time of the first and second injections of OVA, although again the peak of clonal expansion was lower than that achieved by naive cells and occurred on day 3 instead of day 5 (Fig. 3*A*). Notably, DO11.10 T cells that experienced Ag 2 wk earlier possessed the memory/activated phenotype ($CD45RB^{low}$, $LFA\text{-}1^{high}$), whereas the DO11.10 T cells that experienced Ag 10 wk earlier could not be distinguished from naive T cells based on surface phenotype (Fig. 3*B*). Therefore, the improvement in clonal expansion potential by Ag-experienced DO11.10 T cells between 2 and 10 wk correlated with reversion to a naive cell surface phenotype.

Despite exhibiting a small degree of clonal expansion, DO11.10 T cells that were exposed to Ag 2 wk earlier were very sensitive to restimulation by Ag in vivo, requiring less Ag to achieve a maximal response on day 3 than naive DO11.10 T cells required to

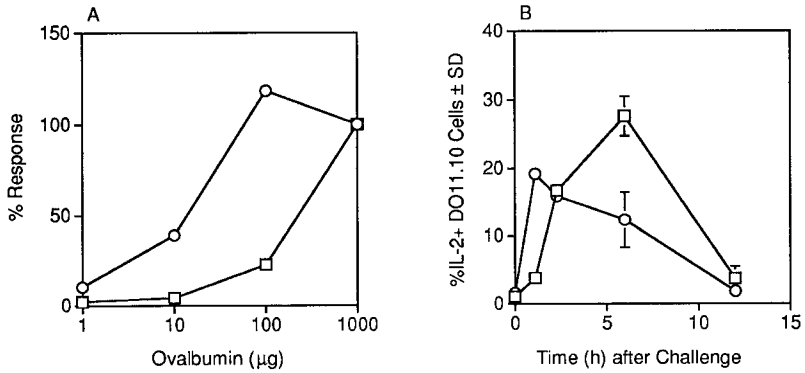

FIGURE 4. Ag sensitivity and IL-2 production kinetics of Ag-experienced CD4 T cells. *A*, Recipients of naive DO11.10 T cells were injected with 100 μg of OVA/CFA (○) or CFA alone (□). Two weeks later, the mice were injected with the indicated amounts of OVA in IFA. The percent maximal response was calculated by dividing the number of DO11.10 T cells present in the draining lymph nodes at a given Ag dose at the time of maximal clonal expansion (day 5 for the naive group (□), day 3 for the Ag-experienced group (○)) by the number present when 1 mg of OVA was injected into the naive group, and multiplying by 100. *B*, Recipients of naive DO11.10 T cells were not immunized (□) or were injected with OVA/LPS (○) to produce Ag-experienced T cells. Eighteen days after adoptive transfer and 17 days after OVA/LPS injection, recipients were challenged with an i.v. injection of 100 μg of OVA peptide. At the indicated times after challenge, the percentage of $CD4^+$, $KJ1\text{-}26^+$, IL-2-producing DO11.10 T cells was assessed by flow cytometry. The mean ± range (n = 2 at each time point) are shown.

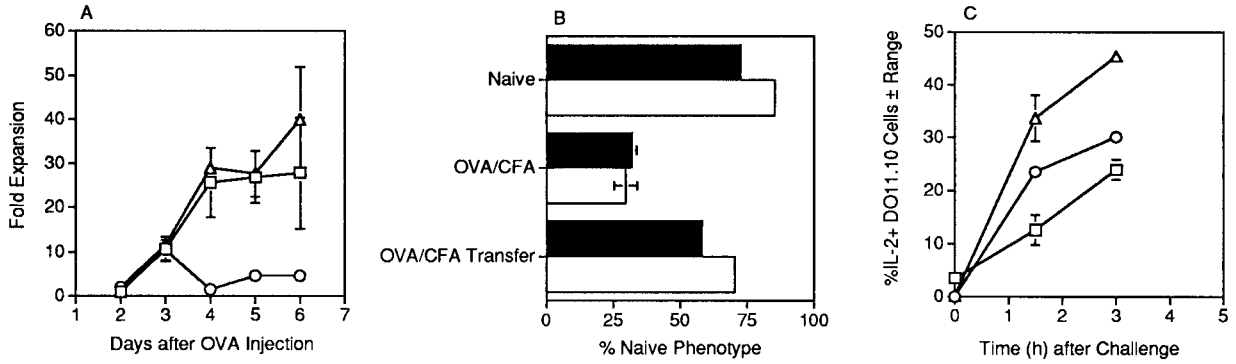

FIGURE 5. Ag-experienced T cells revert to the clonal expansion behavior of naive T cells after transfer into a naive environment. Three types of adoptive transfer mice were prepared: a naive group in which naive DO11.10 T cells were transferred into naive recipients (□), an Ag-experienced group consisting of DO11.10 T cell recipients that had been injected with OVA/CFA 3 wk previously (○), and an Ag-experienced transfer group in which draining lymph node cells (containing 1.25–2.5 × 10^6 DO11.10 Ag-experienced T cells) from DO11.10 recipients that had been injected with OVA/CFA 2 wk previously were parked in naive recipients for 1 wk (△). *A*, The mean fold expansion values over a prechallenge baseline ± SEM (n = 3–16 for each group and at each time point) of DO11.10 T cells from each group, present in the draining lymph nodes at the indicated times after s.c. challenge with OVA/CFA. *B*, The percentages of DO11.10 T cells expressing high levels of CD45RB (■) or low levels of LFA-1 (□) in the three groups on the day of rechallenge. *C*, The mean percentage of IL-2^+, $CD4^+$, KJ1-26^+ cells ± range (n = 2 for each group and at each time point) from each group, present in the spleen or lymph nodes at the indicated times after i.v. challenge with 100 μg of OVA peptide.

achieve a maximal response on day 5 (Fig. 4*A*). In addition, Ag-experienced DO11.10 T cells produced IL-2 more rapidly in response to in vivo Ag challenge than did naive DO11.10 T cells. As shown in Fig. 4*B*, naive DO11.10 T cells produced IL-2 in the lymph nodes within 3 h of i.v. injection of OVA peptide, with maximal production occurring at 6 h. Approximately 25% of the DO11.10 T cells contained intracellular IL-2 at the peak of production. Naive DO11.10 T cells stopped producing IL-2 by 12 h after OVA peptide injection. In contrast, DO11.10 T cells in mice immunized 17 days earlier achieved peak production of IL-2 by 1.5 h after i.v. challenge with OVA peptide, and production declined rapidly thereafter. Therefore, although Ag-experienced DO11.10 T cells displayed a reduced capacity for clonal expansion, these cells were very sensitive to Ag stimulation and were rapid lymphokine producers in vivo.

Clonal expansion potential by Ag-experienced T cells in a naive environment

It was possible that a factor(s) present in the immune environment was responsible for the reduced clonal expansion potential of Ag-experienced DO11.10 T cells at early times after primary immunization. If this supposition was correct, then Ag-experienced T cells would have been expected to recover the capacity for robust clonal expansion after transfer into naive recipients. This was tested using a serial adoptive transfer method. Naive DO11.10 T cells were transferred into naive recipients, which were immunized with OVA/CFA. Several weeks later, purified CD4 T cells (containing ~10^6 Ag-experienced DO11.10 T cells) from these mice were transferred into a new set of naive recipients that had never been exposed to OVA. One week after cell transfer, the recipient mice were challenged with OVA. As shown in Fig. 5*A*, the Ag-experienced DO11.10 cells that resided in a naive environment for 1 wk displayed the robust clonal expansion pattern of naive DO11.10 T cells. The recovery of clonal expansion potential in a naive environment was accompanied by reversion of the Ag-experienced DO11.10 cells to a naive cell surface phenotype (Fig. 5*B*). However, the Ag-experienced DO11.10 cells that resided for 1 wk in naive hosts did not behave like naive T cells with respect to IL-2 production. Like Ag-experienced DO11.10 T cells that remained in immune hosts, the Ag-experienced DO11.10 T cells that resided in naive hosts produced IL-2 more quickly than naive cells during the first 1.5 h after in vivo challenge with OVA peptide (Fig. 5*C*). In addition, a larger fraction of the Ag-experienced DO11.10 T cells that resided in naive hosts produced IL-2 than did Ag-experienced DO11.10 T cells that remained in the immune environment. Therefore, Ag-experienced CD4 T cells that resided in a naive environment for 1 wk reverted to the surface phenotype and robust clonal expansion behavior of naive T cells, but retained the capacity to produce IL-2 rapidly.

It was possible that the robust clonal expansion behavior of Ag-experienced T cells that were parked in naive hosts was not explained by reversion, but, rather, by the fact that truly naive T cells that did not experience Ag during primary immunization survived preferentially in the naive hosts. The observation that Ag-experienced DO11.10 T cells that resided in naive hosts retained a rapid IL-2 potential suggested that this was not the case. Furthermore, the Ag-experienced DO11.10 T cells that resided in naive hosts for 12 days showed evidence of approximately four cell divisions (Fig. 6*C*) like Ag-experienced cells that remained in immune hosts (Fig. 6*B*), not less than one cell division like naive cells (Fig. 6*A*). Thus, although the DO11.10 T cells from immunized mice that were parked in naive hosts possessed a naive surface phenotype and clonal expansion potential, these cells clearly experienced Ag in the past based on their cell division history.

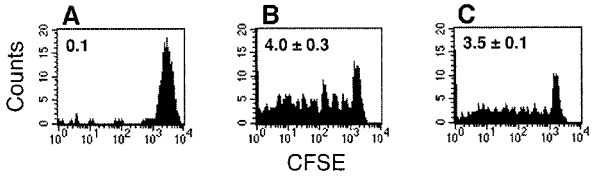

FIGURE 6. Cell division history of Ag-experienced CD4 T cells after transfer into naive hosts. CFSE histograms are shown for the following groups: naive DO11.10 T cells that were labeled with CFSE, transferred into naive recipients, and analyzed 23 days later (*A*); naive DO11.10 T cells that were labeled with CFSE, transferred into naive recipients that were then injected with OVA/CFA, and analyzed 23 days later (*B*), and naive DO11.10 T cells that were labeled with CFSE, transferred into naive recipients that were then injected with OVA/CFA, transferred from these mice into naive recipients 11 days later, and analyzed 12 days after that (C). The mean number of cell divisions ± range (n = 2) are shown in each histogram.

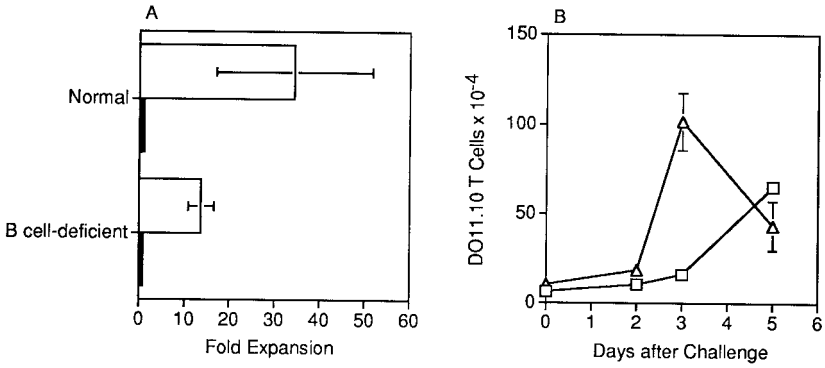

FIGURE 7. Effect of Ab on the clonal expansion of CD4 T cells. *A*, DO11.10 T cells were transferred into normal or B cell-deficient BALB/c mice, half of which were injected s.c. with OVA/CFA. Five weeks later recipients were challenged with OVA/CFA, and 5 days later the fold expansion of the DO11.10 T cells was determined as described in Fig. 3. □, The fold expansion ± SEM (n = 2–5 mice/group) of naive DO11.10 T cells in the two types of recipients; ■, the fold expansion of the DO11.10 from 5-wk-immune recipients. *B*, Naive BALB/c mice were injected with IgG from naive mice (□) or mice that were immunized with OVA/CFA (△). The recipients then received naive DO11.10 T cells and were challenged the next day with OVA/IFA. The mean ± SEM number of DO11.10 T cells present in the draining lymph nodes at the indicated times after immunization are shown.

Role of Ag-specific Ab in the clonal expansion of T cells

Ag-specific Ab could have been the factor in immune hosts that limited the clonal expansion potential of Ag-experienced T cells by rapidly clearing the OVA after challenge injection (21). However, DO11.10 T cells that were immunized with OVA in normal or B cell-deficient hosts underwent limited clonal expansion compared with naive DO11.10 cells, 3 (data not shown) or 5 days after secondary challenge with OVA (Fig. 7*A*). Thus, Ag-specific Abs cannot account for the factor in immune mice that limits the clonal expansion potential of Ag-experienced T cells. This conclusion was supported by passive immunization experiments. Naive DO11.10 T cells were transferred into recipient mice that had been injected with anti-OVA Abs and were then immunized with OVA/CFA. As shown in Fig. 7*B*, the clonal expansion of naive DO11.10 T cells was not inhibited in recipients that had been passively immunized with anti-OVA Abs. On the contrary, the OVA-specific Abs increased the tempo of the expansion of the DO11.10 T cells, such that a maximal level was reached on day 3 instead of day 5.

Discussion

We noted that the clonal expansion potential of Ag-experienced CD4 T cells in the lymph nodes in response to secondary challenge with Ag was lower than that observed for naive T cells during the primary response. This phenomenon is also apparent in the work of McHeyzer-Williams and colleagues (22, 23), who showed that moth cytochrome-specific naive T cells in normal mice expand 300-fold during the primary response, whereas Ag-experienced T cells of the same specificity expanded 30-fold in response to secondary challenge. Similarly, Kerksiek et al. (24) and Kedl et al. (25) showed that certain Ag-specific CD8 T cells undergo extensive clonal expansion after primary, but not secondary, stimulation. On the other hand, Busch et al. (26) reported that the number of peptide-class I MHC-specific CD8 T cells present at the peak of the secondary response exceeded the number present at the peak of the primary response (26). However, because it was not possible to assess the frequency of naive Ag-specific T cells present in the normal repertoire before primary immunization, it is still possible that the fold expansion of naive T cells in the primary response exceeded that of Ag-experienced T cells in the secondary response. It is also noteworthy that the Ag-experienced CD8 T cells had reverted to high level expression of CD62L by the time the secondary challenge was performed (26). Therefore, it is possible that the Ag-experienced T cells had reverted to the more robust clonal expansion behavior of naive T cells.

The poor accumulation of Ag-experienced T cells in the draining lymph nodes at early times after immunization could not be explained by rapid migration of these cells from the lymph nodes to the spleen or site of secondary immunization. Ag clearance by Ag-specific Ab was also ruled out as a cause of the reduced response, because Ag-experienced CD4 T cells accumulated poorly during a secondary response in B cell-deficient, and thus Ab-deficient, recipients. Because the Ag-experienced CD4 T cells displayed limited DNA synthesis in the lymph nodes in response to secondary immunization, it is more likely that poor proliferation was responsible for their reduced accumulation. This was not easily explained by poor production of T cell growth factors, because Ag-experienced CD4 T cells produced IL-2 quickly and in nearly the same quantity as naive T cells. It is therefore more likely that the blunted clonal expansion is related to a block in proliferation in response to T cell growth factors. Th1 clones that are exposed to high concentrations of Ag produce IL-2 but do not proliferate despite expression of the IL-2R (27). Therefore, it is conceivable that Ag-experienced CD4 T cells are chronically exposed to residual Ag derived from the primary immunization, and that this situation promotes rapid T cell growth factor production, but prevents the T cells from proliferating maximally in response to the growth factor. The finding that Ag-experienced T cells undergo robust clonal expansion when challenged in naive hosts or in immune hosts long after primary immunization supports the possibility that exposure to residual Ag is the factor that limits the proliferation of Ag-experienced CD4 T cells.

The increased potential for clonal expansion by Ag-experienced CD4 T cells long after primary immunization or after transfer into naive recipients coincided with reversion to a naive cell surface phenotype. Reversion of Ag-experienced CD4 T cells to a naive surface phenotype has been observed in other studies (28–30). Garcia et al. (31) recently reported that Ag-experienced CD4 T cells retained the capacity to produce IFN-γ long after APC capable of presenting residual Ag disappeared. This result together with our finding that Ag-experienced DO11.10 T cells retained the capacity for rapid IL-2 production after transfer into the naive environment demonstrates that not all functions of Ag-experienced CD4 T cells revert to the naive state in the naive environment.

The Ag-experienced CD4 T cells that regained the capacity for robust clonal expansion 10 wk after primary immunization still

displayed a peak response on day 3 instead of day 5 like truly naive T cells. This response pattern was similar to that displayed by naive T cells that were exposed to Ag in passively immunized recipients. Thus, 10 wk after immunization, Ag-experienced T cells may be inherently capable of the slower clonal expansion rate of naive T cells, but may respond more quickly than naive T cells because Ag-specific Abs bind the challenge Ag and efficiently direct it to APC. Ab-mediated facilitation of Ag presentation may also contribute to the capacity of Ag-experienced cells to respond to lower Ag doses than naive T cells. Ag-Ab complexes have been reported to enhance Ag presentation by binding to Fc or complement receptors (21, 32–34) on APC.

Our results are consistent with the following model of Ag-experienced T cell function. Naive CD4 T cells are stimulated by peptide-MHC-bearing APC in the lymphoid tissue to proliferate and help B cells produce Abs. Many of the activated T cells then die, leaving behind a population of Ag-experienced T cells. These cells are chronically or periodically stimulated by APC bearing small numbers of peptide-MHC molecules derived from the immunogen. This chronic signaling causes the Ag-experienced T cells to express the memory/activated phenotype and exist in a state characterized by rapid lymphokine production potential but poor proliferative capacity. If the host is challenged with Ag during this period, the Ag is rapidly bound by Ab and targeted to complement or Fc receptor-bearing APC that activate the Ag-experienced T cells to produce IL-2 rapidly. The combined effects of Ab-facilitated Ag presentation and rapid lymphokine production lead to rapid clonal expansion of the Ag-experienced T cells. However, the poor capacity of these cells to proliferate in response to growth factors limits the magnitude of the expansion. As residual peptide-MHC molecules derived from the primary immunization disappear, the Ag-experienced T cells revert to the naive surface phenotype and slower response status of naive T cells. If the host is challenged with Ag during this period, Ag-specific Ab, which remains elevated in immune hosts for life, will direct the Ag to APC efficiently and enhance the activation of the Ag-experienced T cells that retain rapid lymphokine production potential but whose proliferation is not longer suppressed by residual Ag. If this scenario is correct, then, as proposed by Bell and colleagues (35), CD4 T cell-mediated protection after the primary immunogen has disappeared may be due to the concerted action of Ag-specific Ab and an expanded population of Ag-experienced T cells that share some functional properties with naive cells.

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