

Distinct Time Effects of Vaccination on Long-Term Proliferative and IFN- γ -producing T Cell Memory to Smallpox in Humans

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Abstract

Residual immunity to the smallpox virus raises key questions about the persistence of long-term immune memory in the absence of antigen, since vaccination ended in 1980. IFN- γ -producing effector-memory and proliferative memory T cells were compared in 79 vaccinees 13–25 yr after their last immunization and in unvaccinated individuals. Only 20% of the vaccinees displayed both immediate IFN- γ -producing effector-memory responses and proliferative memory responses at 6 d; 52.5% showed only proliferative responses; and 27.5% had no detectable vaccinia-specific responses at all. Both responses were mediated by CD4 and CD8 T cells. The vaccinia-specific IFN- γ -producing cells were composed mainly of CD4Pos CD45RANeg CD11aHi CD27Pos and CCR7Neg T cells. Their frequency was low but could be expanded in vitro within 7 d. Time since first immunization affected their persistence: they vanished 45 yr after priming, but proliferative responses remained detectable. The number of recalls did not affect the persistence of residual effector-memory T cells. Programmed revaccination boosted both IFN- γ and proliferative responses within 2 mo of recall, even in vaccinees with previously undetectable residual effector-memory cells. Such long-term maintenance of vaccinia-specific immune memory in the absence of smallpox virus modifies our understanding of the mechanism of persistence of long-term memory to poxviruses and challenges vaccination strategies.

Key words: smallpox • vaccine • central memory T cells • effector-memory T cells

Introduction

Residual immunity to smallpox virus is a burning question for public health and future vaccine development. It raises key questions about factors influencing the persistence of long-term immune memory in the absence of antigen, since smallpox was eradicated and vaccination stopped in 1980 (1). It is the ultimate goal of a vaccine to develop long-lived immunological protection against pathogens through the development of an abundant pool of memory cells (2, 3). T cell-mediated immune memory, widely studied in mouse viral infection models, requires clonal expansion of effector-memory T cells from naive T cells followed by the contraction and then stabilization of the size of the antigen-specific memory T cell population (4,

5). After this stabilization, their number is estimated to be at least 1,000-fold higher than the number of naive T cells (6). However, it is still unclear how long this immunity can last and whether the long-term persistence of memory T cells is determined at priming or if antigen boosts are needed to maintain this population. According to mouse models, initial vaccine priming determines the number of antigen-specific memory CD8 T cells (7–9). Their persistence depends on periodic reexposure to the nominal antigens (3, 10–14) or to cross-reactive heterologous viruses (15). In humans, the long-term persistence of immune memory to vaccines is still poorly understood.

The eradication of smallpox (16, 17) makes it possible to investigate the factors influencing the persistence of vaccinia-specific memory T cells in vaccinated individuals in the ab-

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Abbreviations used in this paper: BCG, Bacille de Calmette et Guérin; BrdU, bromodeoxyuridine; SFC, spot-forming cell.

sence of the smallpox and vaccinia viruses 25 yr after mass vaccination ended. Studies during the smallpox vaccination era, when both smallpox and vaccinia viruses were still circulating, suggested that protective immunity to smallpox declines 5–10 yr after vaccinia inoculation and accordingly set a vaccination schedule at 1, 11, and 21 yr (16, 18). Neutralizing antibody responses are considered to decline within 3–5 yr of vaccination (19), whereas T cell responses mediated by proliferating T cells and CTL precursors are thought to persist for up to 50 yr after immunization (20). A recent study (21) reports that 90% of vaccinated individuals still have vaccinia-specific IFN- γ -producing CD4 or CD8 cells. However, the frequency of these cells is very low in some vaccinees, close to those in unvaccinated controls. Given that the nominal antigens (smallpox and vaccinia viruses) no longer circulate, the frequency of short-term cytokine-producing effector–memory responses, which are supposed to depend on antigen presence, was indeed expected to be low. These cells, however, may not be representative of the entire vaccinia-specific memory response, which should include memory T cells capable of expansion and proliferation against virus challenge. Murine models show that proliferating or central memory T cells do indeed differ from the immediate effector–memory T cells: they display distinct phenotypes and functions and are more protective (3). However, the distinction between effector–memory and central memory demonstrated in humans (22) has never been explored in an appropriate model of long-term T cell memory and in the complete absence of nominal antigen.

To explore the persistence, intensity, and quality of T cell memory to vaccinia in the vaccinated population decades after vaccination ended and to better understand the mechanisms that make possible this long-term maintenance of memory T cells to vaccines in humans, we simultaneously investigated two aspects of vaccinia-specific T cells in a population of persons vaccinated long ago: their proliferation capacity and the frequency of those producing IFN- γ . Because monitoring vaccine-specific T cells necessitates a precise definition of memory T cell frequencies, we defined our threshold relative to frequencies in unvaccinated unexposed young individuals. Our results showed clearly that although proliferative memory responses to vaccinia persisted in 72% of the population and were not influenced by age or vaccine recalls, an IFN- γ response above the threshold of the unvaccinated unexposed controls was detected in only 20% of the subjects and declined after the age of 45 yr, independently of recalls. Recent revaccination boosted both IFN- γ and proliferative responses in all revaccinated individuals.

Materials and Methods

Donors. Fresh PBMCs were obtained from 79 volunteers vaccinated previously against smallpox (age range, 25–68; vaccination at the age of 1–43 yr) including 17 who were revaccinated after the first vaccination (age range 32–57), and from 10 unvaccinated young healthy donors (age range, 20–25), all with their written informed consent. The sex ratio was 60% women and

40% men. The people included in this study were volunteers for the French National Emergency Team against Smallpox.

ELISpot Assays. The ELISpot assay was run after careful determination of the optimal antigen doses and reproducibility of the assays in vaccinated individuals as positive controls and naive unvaccinated unexposed donors as negative controls. Briefly, PBMC were infected for 1 h with the live vaccinia Copenhagen wild-type strain (obtained from M.P. Kieny, Transgène, Strasbourg, France) at three infectious doses (1, 2.5, and 5 pfu/cell) and tested in triplicate experiments in an ELISpot assay as described previously (24). Repeated experiments performed on the same samples gave similar results (100% concordance for spot-forming cells (SFCs)/million PBMCs with a median coefficient of variation of 9%) for 18 vaccinees and for 9 unvaccinated donors. We then selected 1 pfu/cell as the optimal dose, as did Frey et al. (26). Cells were washed and added to 96-well ELISpot plates (Millipore) coated with anti-human IFN- γ antibody (Diaclone). Negative controls were uninfected cells with medium alone. Positive controls were uninfected PBMCs stimulated with purified PHA (1 μ g/ml; GIBCO BRL). Cells were cultured in triplicate wells at 1×10^5 PBMCs at 37°C for 18 h. Spots were detected with an automated microscope (Carl Zeiss MicroImaging, Inc.) and counted as positive if there were at least 50 vaccinia-specific SFCs/million PBMCs (after subtracting the background obtained with unstimulated cells). This threshold for positivity was established by taking into account: (a) the variability of the background obtained with cells alone (3 ± 5 SFCs/million PBMCs, range, 0–75); (b) the SFCs range in the 10 naive unvaccinated individuals used as negative controls: median 1.5 SFCs/million PBMCs (range, 0–40 SFCs/million PBMCs); (c) the laboratory reproducibility of the assay on a given sample; and (d) the fluctuation over time of individual vaccinia-specific frequencies at a month's interval (tested in five individuals; concordance of 100% and coefficient of variation, 29%). This threshold of 50 SFCs/million PBMCs yielded 100% negative results for the naive unvaccinated unexposed donors.

In some experiments, freshly isolated PBMCs were depleted of CD8⁺ cells with magnetic beads, according to the manufacturer's instructions (Dyna). The CD4⁺ enrichment was verified by flow cytometric analysis (>90% CD3⁺CD4⁺ cells).

Proliferation Assays. The T cell proliferation assay was run after a careful determination of the optimal antigen doses and reproducibility of the assays in vaccinated individuals as positive controls and naive unvaccinated unexposed donors as negative controls. Briefly, triplicate wells containing cells (10^5) were cultured in RPMI medium supplemented with 10% AB human serum for 7 d, with 0.1 pfu/cell Copenhagen vaccinia strain for 7 d in the 3H-thymidine assay. A prior comparison of three infectious doses (0.1, 0.25, and 0.5 pfu/cell) had been performed with similar results on blood from both vaccinated and unvaccinated donors. 3H-thymidine was added during the last 18 h of culture, and cells were harvested and counted on a Microbeta-plate β -counter (Beckman Coulter). Positive responses were defined by a positive stimulation index of 3 and a minimum 3H-thymidine incorporation of 3,000 cpm as described (38). In the bromodeoxyuridine (BrdU) assay, performed with a 1 pfu/cell infectious dose, BrdU was added at 100 ng/ml at the beginning of cultures tested at days 3 and 7. Cells were washed and analyzed by flow cytometric intracytoplasmic staining as described in the next paragraph.

Flow Cytometry Analyses. Fresh PBMCs were stimulated for 1–7 d with autologous adherent 1 pfu/cell vaccinia-infected monocytes. Membrane staining of stimulated T cells used anti-CD8, anti-CD4, FITC, or Cy-Chrome-conjugated anti-CD45RA, FITC- or PE-conjugated CD27, PE-conjugated anti-CCR7 or PE-con-

jugated CD11a (Becton Dickinson) monoclonal antibodies. Intracellular IFN- γ , IL-2, or BrdU detection used anti-IFN- γ , IL-2-PE or APC, or anti-BrdU-FITC-conjugated Ab staining (Becton Dickinson). Briefly, cells were fixed with PFA 4% for 20 min, washed in PBS-FCS 2-0.1% saponin buffer and incubated for 20 min at 37°C with DNase (2.5 μ g/ml) in DNase Buffer (Sigma-Aldrich). Cells were washed in PBS-FCS 2-0.1% saponin buffer and incubated with the labeled Abs. Flow cytometric analyses were performed on a FACScalibur™ with CellQuest™ Pro software (Becton Dickinson) on \sim 3,000,000 events.

Statistical Analyses. Data handling, analysis, and graphic representation used Prism 2.01 (GraphPad Software) with a χ^2 test. Statistical significance was set at $P < 0.05$.

Results

Quantification of Residual Effector-Memory and Proliferative Responses to Vaccinia in Long-Term Vaccinees. We first studied the residual cell-mediated immune responses to the vaccinia virus in 89 healthy volunteers: 79 vaccinees, aged 25–68 yr, who had received one to five vaccinations from 1943 through 1989, and 10 unvaccinated unexposed individuals, younger than 25 yr. We stimulated PBMCs in vitro with the live vaccinia virus to activate all donor cells. We chose live virus because the limited number of CD8 epitopes defined are restricted to the HLA-A2 type (23). We counted the number of rapidly mobilized, “immediate” vaccinia-specific effector-memory T cells during this short-term PBMC stimulation in an 18-h IFN- γ ELISpot assay (Fig. 1 a). Because cell frequencies were expected to be low, the specificity and sensitivity of the ELISpot assay were carefully established first (as described in Materials and Methods). The median IFN- γ -producing cell frequency for the 10 unvaccinated donors was 1 SFCs/million PBMCs above background (range, 0–40) (Fig. 1 a). Therefore, positive response to vaccinia was defined as >50 SFCs/million PBMCs over background, and this threshold, commonly used for monitoring T cell responses to new vaccines (24, 25), was used to assess the assay specificity. Only 20% ($n = 16$) of the vaccinees showed vaccinia-specific effector-memory cells above this threshold (median for responders, 98 SFCs/million PBMCs; range, 53–843). Similarly, we defined positive proliferative responses (Fig. 1 b) in comparison with responses in unvaccinated donors. In contrast to the low percentage of vaccinees with IFN- γ -producing cell responses, vaccinia-specific proliferative responses were detected in vitro after 6 d of virus exposure in the PMBCs of 72.5% of the vaccinees (50 of 69 tested) (Fig. 1 a). The simultaneous evaluation of both functions showed that: (a) when detectable (20% of cases), the rapidly mobilized effector-memory-type lymphocytes producing IFN- γ were always associated with a proliferative memory response to vaccinia; (b) 52.5% of the vaccinees displayed only an expandable pool of memory T cells (without IFN- γ response); and (c) 27.5% displayed neither effector-memory or proliferating memory T cell responses as defined above (Fig. 1 b). Simultaneous analysis of proliferative and IFN- γ -producing cells against vaccinia and smallpox thus showed that rapidly mobilized effector-memory responses and the expandable

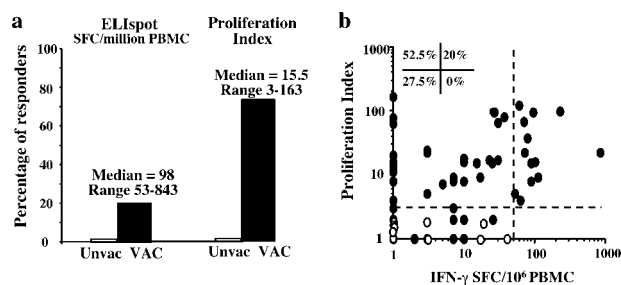


Figure 1. Residual T cell-mediated immune responses to vaccinia virus in smallpox vaccinees. We examined fresh PBMCs from healthy individuals including 79 long-term vaccinees (VAC) (age, 25–68 yr; median, 39) and 10 unvaccinated unexposed volunteers (UNVAC) (age, 19–24 yr; median, 23). (a) Results from IFN- γ ELISpot assays after stimulation with the live Copenhagen vaccinia strain are represented as the percentage of healthy responders who are long-term vaccinees (black bars) (median for responders, 98; range, 53–843 SFCs/million PBMCs) and unvaccinated volunteers (white bar). Results from 3H-thymidine proliferation assays after 7 d stimulation with the live Copenhagen vaccinia strain are represented as the percentage of healthy responders who are long-term vaccinees (black bars) (median proliferation index, 15.5; range, 3–163); unvaccinated volunteers (white bars). (b) Reciprocal distributions of ELISpot and proliferation assays are represented for long-term vaccinees (●) and unvaccinated unexposed individuals (○). Positive responses were defined after analysis of responses obtained in unvaccinated control individuals: proliferative stimulation index ≥ 3 and IFN- γ -producing cells ≥ 50 SFCs/million PBMCs after subtraction of the background (shown by dashed lines). The percentage of vaccinees in each quadrant is indicated.

pool of memory T cells had clearly distinct patterns of long-term maintenance in the absence of antigen.

Characterization of Long-Term Memory T Cells Specific for Vaccinia. To assess the cell populations involved in these vaccinia-specific responses, we first assayed IFN- γ production and proliferation after CD8 cell depletion (Fig. 2, a and b). Residual vaccinia-specific effector and proliferative responses were still observed in most vaccinated individuals; this suggests that CD4 T cells were the major component of these persistent effector-memory T cell responses. That absolute numbers of vaccinia-specific SFCs in the

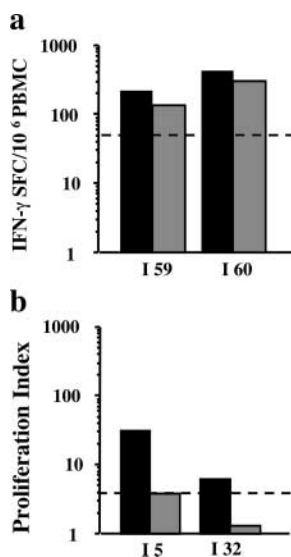


Figure 2. Vaccinia-specific CD4 and CD8 memory cells. CD8-depleted PBMCs (grey bars) compared with total PBMCs (black bars) were tested by ELISpot assay (a) and proliferation assay (b). Results are shown for two representative individuals.

CD4-enriched PBMC fractions did not increase suggests that IFN- γ -producing CD8 cells were also involved.

We next analyzed the nature of the vaccinia-specific proliferative responses after in vitro cell expansion during culture with the live virus. At day 3, the BrdU incorporation assay confirmed for the 10 vaccinees tested that both CD4 (mean \pm SEM; $0.46 \pm 0.25\%$) and CD8 (mean \pm SEM; $0.51 \pm 0.37\%$) vaccinia-specific T cells were expandable (Fig. 3 a). IL-2-producing CD4 T cells were also detected in two cases and correlated with the intensity of the vaccinia-specific proliferative responses detected in the proliferation assay (Fig. 3 a). However, IL-2 production was undetectable in most cases (unpublished data). The vaccinia-specific cell expansion assay at 7 d showed amplification of both CD8 and CD4 cells producing IFN- γ ; these represented 7 and 10% of the T cells, respectively (Fig. 3 b). These results showed that both CD4 and CD8 T cells participate in vaccinia-specific memory responses, with the relative proportions differing among

a

	% BrdU+ cells (day 3) *		Proliferation Index (day 7)	IL-2 (48 h) **
	CD4+	CD8+		
Vaccinated				
I-2	0.14	0.24	16	0
I-5	0.15	0.26	122	80
I-11	0.77	1.44	15	0
I-12	0.60	0.36	80	40
I-33	0.57	0.27	16	ND
unvaccinated				
N6	0	0	1	0
N8	0	0	1	0

* background has been subtracted.

** IL-2-producing cells/million CD4+ cells, background has been subtracted

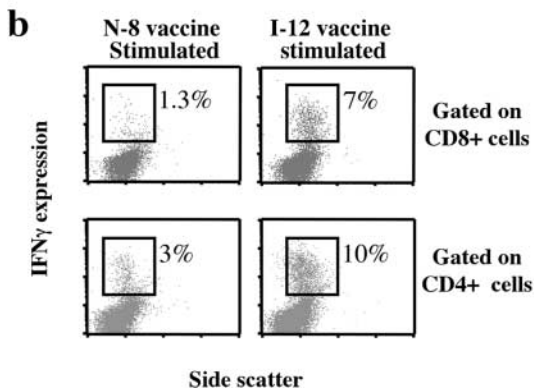


Figure 3. Vaccinia-specific CD4 and CD8 amplification leads to an increase in the frequency of effector-memory cells. (a) Percentage of BrdU+ cells in CD8 and CD4 T cells of five vaccinated individuals and two unvaccinated individuals after fresh PBMCs infected with either 1 pfu/cell vaccinia strain or medium alone were cultured for 3 d in the presence of BrdU (background subtracted). The number of IL-2-producing cells in the CD4 population was estimated by flow cytometric analysis at 48 h after antigenic stimulation (0–80 IL-2-producing cells/million CD4+ cells). (b) Representative flow cytometric analyses show IFN- γ production in vaccinia-specific T cells expanded for 7 d and restimulated on day 7 by monocytes infected with vaccinia for an additional 18 h at 1 pfu/cell (unvaccinated and long-term vaccinated individuals).

individuals. They also indicated that in vitro stimulation of residual vaccinia-specific CD4 and CD8 T cells increased the frequency of effector-memory cells capable of producing IFN- γ .

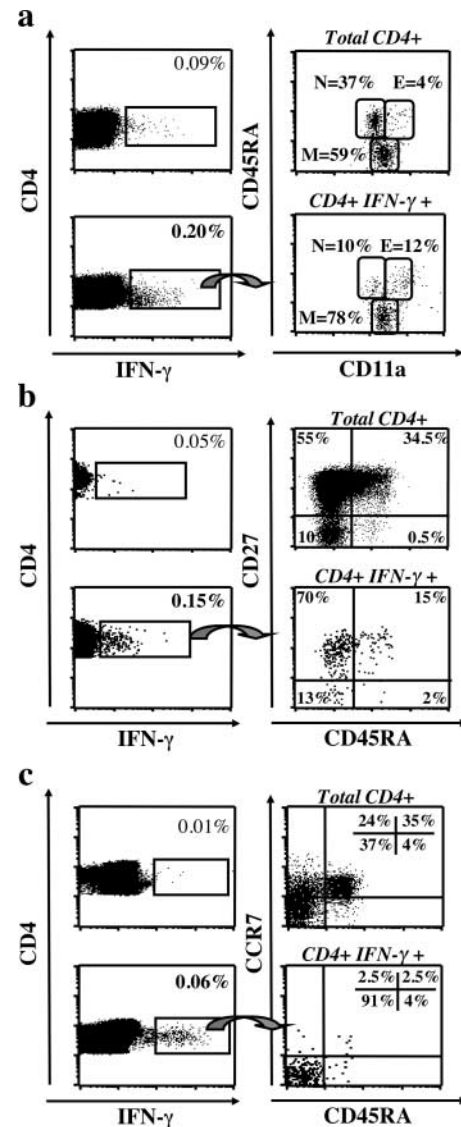


Figure 4. Phenotypic analyses of vaccinia-specific memory T cells. Intracytoplasmic IFN- γ production by flow-cytometry assays of CD4 T cells after 18 h stimulation with vaccinia virus in selected volunteers with high frequencies of vaccinia-specific cells (>200 SFC/million PBMCs) (a–c, left panels top quadrant, unstimulated cells; bottom quadrant, vaccinia-stimulated cells). (a) CD45RA and CD11a expression gated on total CD4Pos cells or CD4PosIFN- γ Pos cells (in two individuals, I5 and I12; 78 and 70% of vaccinia-specific IFN- γ -producing CD4 cells were CD45RANegCD11aPos). N, naive T cells; E, effector T cells; M, memory T cells. Flow cytometry analyses for CCR7 expression showed that N, CCR7Pos, E, CCR7Neg, and M, CCR7Pos/Neg (not depicted). (b) CD45RA and CD27 expression are gated on either total CD4Pos cells or CD4PosIFN- γ Pos cells (in five individuals: I1, I2, I5, I7, and I9 (60–70% of vaccinia-specific CD4 cells were CD45RANegCD27Pos)). (c) CD45RA and CCR7 expression are gated on either total CD4Pos cells or CD4PosIFN- γ Pos cells. Experiments were performed with two individuals: I1 and I2 (80–91% of vaccinia-specific CD4 cells were CD45RANegCCR7Neg).

We further investigated the phenotypic characteristics of the vaccinia-specific T cells to assess in particular the effector-memory late/early cell surface markers proposed for humans (3). Using two distinct definitions, we found that most of the vaccinia-specific IFN- γ -producing residual vaccinia-specific T cells, assessed ex vivo, displayed characteristics of memory CD4 T cells. The vaccinia-specific IFN- γ -producing CD8 cells were below detectable levels. Specifically, most vaccinia-specific CD4⁺ cells were CD45RA^{Neg}CD11a^{Hi} (70–78%) or CD45RA^{Neg}CD27^{Pos} (60–70%), whereas a few met the definition of effector T cells, i.e., CD45RA^{Pos}CD11a^{hi} (12–17%) or CD45RA^{Pos}CD27^{Neg} (10–13%) (Fig. 4). In addition, most vaccinia-specific IFN- γ -producing CD4⁺ cells were defined as CD45RA^{Neg}CCR7^{Neg} (80–91%), whereas only a few were CD45RA^{Neg}CCR7⁺ (5–10%) (Fig. 4 c). Since the proliferation experiments were assessed after 3–7 d expansion, the vaccinia-specific T cells differentiated in vitro during the expansion process. Therefore, the original phenotype cannot be assessed. These results suggest that most of the residual T cells capable of immediate IFN- γ production in response to vaccinia decades after vaccination display the characteristics of effector-memory T cells.

Effector-Memory but not Proliferative Responses Vanish 45 yr after Antigen Priming. Because murine models of lymphocytic choriomeningitis virus infection indicate that the quality and quantity of T cell priming during the first encounter with antigen might determine the size of the memory T cell pool (3), we investigated whether a higher frequency of memory responses in vaccinees was associated with a shorter delay from priming. We distinguished three groups according to time since priming: 25–35, 36–45, and >45 yr, with 20, 32, and 27 vaccinees per group, respectively (Fig. 5, a

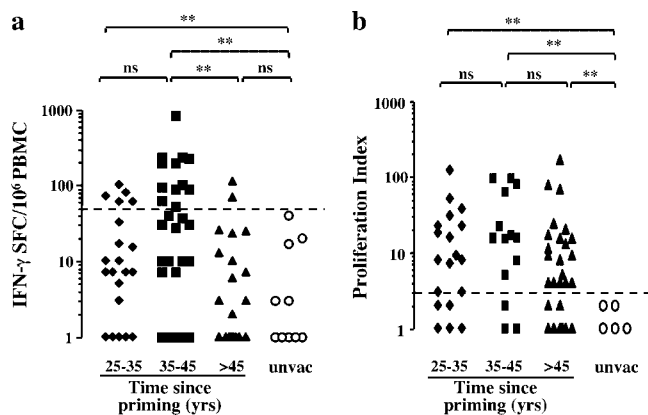


Figure 5. Vaccinia-specific effector-memory response vanishes 45 yr after priming. (a and b) Distribution of vaccinia-specific responses in vaccinated individuals according to time since priming for ELISpot assay (a) and proliferation assay (b). Three groups were distinguished according to time since priming, 25–35, 36–45, and >45 yr, and were compared with unvaccinated unexposed donors. A precise, documented vaccination history was available for only 44 individuals as follows: one ($n = 12$), two ($n = 23$), three ($n = 4$), four ($n = 4$), or five ($n = 1$) immunizations. For all graphs, statistical analysis used the χ^2 test. Statistical significance was set at $P < 0.05$ (**). ns, not significant.

and b). The proportion of IFN- γ -producing effector-memory T cells was highest in the 36–45 yr group (12 out of 32 or 37.5%), and lower (5 out of 21 or 24%), but not significantly so, among the group with the least time since priming (25–35 yr) (Fig. 5 a). Vaccinia-specific effector-memory responses tended to vanish in the group primed more than 45 yr earlier (2 out of 27 or 7.5%): they did not differ from the frequencies observed in the unvaccinated group and were significantly lower than in the 25–35 and 36–45 yr groups ($P = 0.0068$ and 0.019 , respectively) (Fig. 5 a). In contrast, the proliferative vaccinia-specific memory T cells remained stable whatever the time since priming with 73, 73, and 70% of responders in each group (Fig. 5 b). Thus, more than 45 yr after priming the intensity of IFN- γ -producing effector-memory response tended to revert to that observed in unvaccinated individuals.

To investigate whether this decay in antivaccinia immune responses might simply reflect aging, we also evaluated the frequencies of IFN- γ -producing cells against control vaccine antigens, such as tuberculin, in 10 of these vaccinees aged 25–63 yr and exposed to Bacille de Calmette et Guérin (BCG) in their childhood. Tuberculin-specific IFN- γ -producing cells were detectable in all of the vaccinees aged 45–63 yr (range, 90–150 SFCs/million PBMCs) and in 70% of those who were younger (age, 25–45 yr; range, 50–343 SFCs/million PBMCs). Of note, BCG-

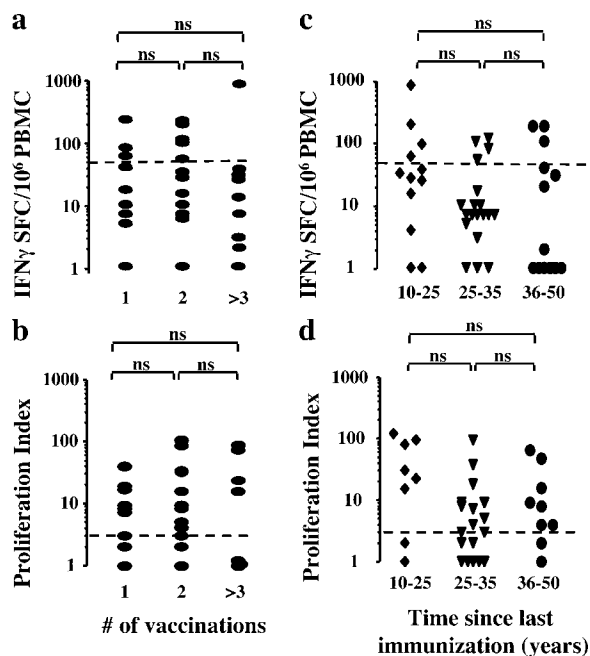


Figure 6. Lack of influence of vaccinia recalls and time since last immunization on the long-term persistence of IFN- γ -producing effector-memory T cells. Distribution of vaccinia-specific responses in 44 vaccinated individuals with a known vaccination history, according to the number of recalls they received up to 13 yr ago for both ELISpot (a) and proliferation (b) assays and according to time since last immunization for ELISpot (c) and proliferation (d) assays. The same three groups described in Fig. 3 were distinguished according to time since priming: 10–25, 25–45, and >45 yr. For all graphs, statistical analysis was performed as in Fig. 5.

vaccinated individuals are still exposed to cross-reactive mycobacteria, whereas smallpox-vaccinated individuals are not. These data suggest that the loss of vaccinia-specific immune memory does not simply reflect aging.

It is a tenet of vaccinology that booster inoculations help to maintain high levels of long-term effector–memory immune responses. Smallpox vaccination schedules typically imposed vaccinia priming at 1 yr and recall injections at 11 and 21 yr (16, 18). Detailed information about prior vaccination was available for only 44 of the 79 vaccinees. Thus, we classified them according to the number of vaccinations they received: one ($n = 12$), two ($n = 23$), three ($n = 4$), four ($n = 4$), or five ($n = 1$) immunizations. All had their first vaccination at the age of 1 yr. We found that the number of immunizations did not affect either the rapid effector–memory response (Fig. 6 a) or the vaccinia-specific memory T cell proliferation (Fig. 6 b).

Because the delay from priming influenced the persistence of memory responses to vaccinia virus, we also looked at the influence of the last vaccination recall injection on both types of memory responses. Figure 6, c and d, depicts three groups according to their last antigen exposure: 10–25, 25–35, and 35–50 yr ago. No correlation was observed between the time since the last vaccinia virus exposure and the detectability of memory responses assessed by either IFN- γ ELISpot or proliferation assays. Thus, more than 25 yr after the last recall similar vaccinia-specific long-term memory T cell responses persisted in all subjects who received one or more immunizations, regardless of the time since the last exposure to vaccinia virus.

Revaccination with Vaccinia Virus Boosts Previously Undetectable Long-Term Effector–Memory Responses. The observations above—that neither the number of immunizations nor

the delay since the last immunization influence persistence of long-term memory—raised two questions. Could effector–memory responses, when undetectable, be reinduced *in vivo* after revaccination? Would the discrepancy observed between effector–memory and proliferative responses in long-term vaccinees influence response after a recent antigen recall? Vaccinia-specific assays were performed 1 and 2 mo. after revaccination of 17 of the 79 vaccinees tested above. We found intense effector–memory and proliferative vaccinia-specific responses in all but one of them (median for positive responders, 154 SFCs/million PBMCs; range, 68–593) (Fig. 7 a). The median frequency of effector–memory T cells was twice as high in recently revaccinated individuals as in long-term vaccinees, 2 mo. after revaccination. CD8 depletion experiments showed that both CD4 and CD8 contributed to vaccinia-specific effector (Fig. 7 b) and proliferative (Fig. 7 c) responses in these recently revaccinated individuals. Furthermore, revaccination of those subjects who had no detectable IFN- γ -producing cells on initial testing induced potent effector–memory and proliferative responses within 2 mo. of antigen recall (Fig. 7, a, d, and e). This is in accordance with our finding that *in vitro* proliferation of virus-specific T cells led to the expansion of IFN- γ -producing cells, and it should be compared with a recent report that vaccinia-specific responses are detectable in all new vaccinees, 6 mo. after priming (26).

Discussion

This study investigated the remnants of T cell immunity to vaccinia and smallpox in a population of vaccinees aged 25–68 yr who had been exposed to one or more vaccinia challenges two decades earlier and compared it with this

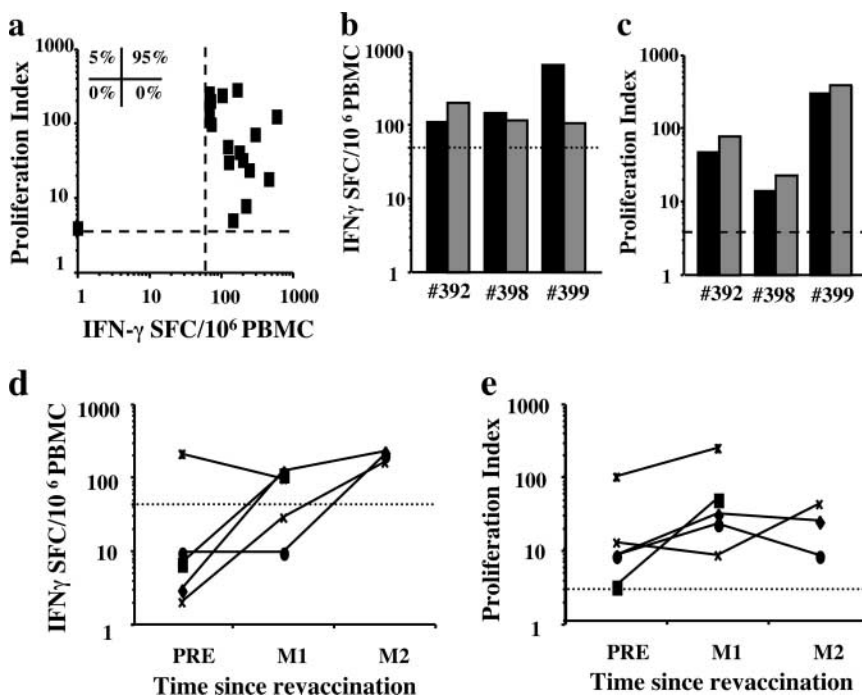


Figure 7. Recent vaccinia recall allows a complete immune response recovery in vaccinees independently of the time since priming. (a) Reciprocal distributions of ELISpot and proliferation assays for 17 recently revaccinated individuals (age, 32–57 yr; median, 41) tested 2 mo after revaccination. Positive responses were defined according to the same criteria: a stimulation index ≥ 3 and a frequency of IFN- γ -producing cells ≥ 50 SFC/million PBMCs after subtraction of background (shown by dashed lines). (b and c) CD8-depleted PBMCs of recently revaccinated individuals (grey bars) compared with total PBMCs (black bars) were tested by ELISpot (b) and proliferation (c) assays. Graphs represent changes in IFN- γ production (d) and proliferative responses (e) of five representative recently revaccinated individuals: PRE (before revaccination, 1–2 mo) M1 and M2 (after revaccination).

immunity in unvaccinated unexposed young volunteers. We showed that vaccinia-specific effector-memory T cells capable of immediate IFN- γ production and memory T cells with proliferative potential have distinct patterns of maintenance. First, immediate effector-memory T cells persisted (above the levels in unvaccinated unexposed naive donors) in a much smaller percentage of long-term smallpox vaccinees than did proliferative memory T cell responses to vaccinia; the latter were correlated to the detectability of IL-2-producing cells and remained detectable in 72.5% of cases. Both memory functions were simultaneously observed in the 20% of the vaccinees with effector-memory responses, whereas 27.5% of the population surveyed did not have memory T cell responses to vaccinia any higher than the unvaccinated unexposed controls.

The discrepancy observed between immediate and proliferative/IL-2-producing memory responses may be explained by various factors including the relative sensitivity of the assays and the intrinsic properties of the cells involved. The *ex vivo* ELISpot assay allows direct measurement of the *in vivo* frequency of vaccinia-specific cells but has a lower sensitivity than the proliferation assays, which include several *in vitro* cycles of cell proliferation. The threshold of the ELISpot assay was carefully established to provide 100% negative results in naive and unexposed unvaccinated donors. It resulted in positive IFN- γ responses in all but one recently revaccinated individuals. The threshold of 50 SFCs/million PBMCs used here is the same as that used for monitoring T cells specific for other viruses, such as HIV, during vaccine trials (24, 25). However, it may explain some of the discrepancies between our results and a recent study that detected extremely low frequencies of vaccinia-specific T cells producing IFN- γ and/or TNF- α , as low as 10/million PBMCs in 90% of a similar population of vaccinees—frequencies not clearly distinguishable from the naive background (21).

However, beyond these technical distinctions proliferative and immediate IFN- γ production are two distinguishable T cell functions: the so-called central memory T cells capable of producing IL-2 and of proliferating and involved in clonal expansion of vaccinia-specific T cells as opposed to the effector-memory T cells producing IFN- γ , which have lower proliferative and survival abilities and are involved in antigen-specific effector functions (27). This dichotomy has been reported and discussed in humans during chronic viral infection (27, 28). We show here for the first time the phenotypic characteristics of long-term memory T cells persisting in humans in the complete absence of nominal antigens. The individuals who responded to vaccinia with immediate IFN- γ production maintain memory cells that belong mostly to the CD4^{Pos}CD45RANeg11aHi27PosCCR7Neg subset. Thus, the majority of residual vaccinia-specific CD4 T cells producing IFN- γ did not express CCR7 and meet the definition of effector-memory T cells (22), even when tested more than 25 yr after vaccination.

Both CD4 and CD8 T cells participated in the maintenance of memory to smallpox in long-term vaccinees. However, the relative contributions of each varied among

individuals independently of age or delay since priming or boosting. In accordance with recent studies (21), we found a predominance of CD4 memory T cells in long-term vaccinees. Our findings suggest that CD4 and CD8 memory T cells may differ in their antigen dependence.

When analyzing the factors influencing persistence of memory to smallpox/vaccinia, we found that delay from priming had a major impact on the maintenance of immune responses. More specifically, the antivaccinia proliferative T cell responses were maintained over the years after antigen exposure, but vaccinia-specific effector-memory T cells vanished 45 yr after the first vaccinia inoculations. This loss of vaccinia-specific effector-memory T cells does not simply reflect aging because tuberculin responses, generated by the BCG vaccine during childhood, were maintained in the same individuals. Memory to these two vaccines might differ because BCG-vaccinated individuals are exposed to cross-reactive mycobacteria, whereas smallpox-vaccinated individuals are not, although some cross-reactivity has been reported with other viruses (*molluscum contagiosum*, lymphocytic choriomeningitis virus) that might participate, to some extent, in the maintenance of vaccinia-specific memory (15). The amount of antigen received during the first immunization might nonetheless determine the size of the vaccinia-specific memory pool (7–9). Although we assumed that all vaccinated individuals had received similar vaccinia doses, the actual number of virus particles penetrating the skin is known to vary widely between vaccinees, and diluting the vaccine reduces the rate of successful vaccination (29).

In addition, we showed that the number of vaccination recalls did not significantly influence the long-term maintenance of effector-memory cells. Given that both variola and vaccinia viruses are thought to be completely cleared from the organism after acute infection or inoculation, vaccinia-specific memory appears to persist despite the absence of both viruses for the last 25 yr. According to an emerging consensus, murine long-term CD8 memory does not require antigen persistence (14, 30–32). Similarly, murine CD4 T cell memory specific for the readily eliminated Sendai virus is maintained for more than 2 yr (29, 33). The observation that periodic reexposures to the vaccine do not increase the size of the residual effector-memory pool when measured decades after the last immunization suggests that priming exerts a stronger influence than subsequent exposures on its long-term persistence. Nonetheless, effector-memory T cells can still be reexpanded by reimmunization *in vivo*, as they were on day 3 of *in vitro* exposure; this expansion resulted in an increased frequency of effector-memory T cells at day 7. Both vaccinia-specific proliferative and immediate effector-memory T cell responses were detectable at high levels in all recently revaccinated subjects a month or two afterwards, even those for whom effector-memory cells were undetectable before revaccination and those older than 45 yr. These results show the immune system's capacity to mobilize effector-memory T cells rapidly in response to vaccine reexposure.

Preliminary reports in accordance with our findings but on a much smaller scale offer little evidence that the effector–memory–type component of vaccinia-specific memory decreases in vaccinees older than 39 (26). A recent large study also reports the persistence of residual immunity to vaccinia in previously vaccinated individuals (21). This immunity, mediated by cytokine-producing effector–memory T cells, decays with age and is not influenced by vaccine recalls (21). The authors did not study the proliferative capacity of residual vaccinia-specific cells. Prior studies also suggest that memory T cells for vaccinia are still expandable 20 yr after the last immunization (20), whereas neutralizing antibody responses decline 5 yr after vaccination (19). We found that PMBCs of individuals who had no detectable IFN- γ -producing T cells before revaccination showed an increase in effector–memory cells within 2 mo of vaccinia recall.

This large-scale study of long-term immune memory to vaccinia in humans leads us to propose a new model of immune memory to live viral vaccines: the proliferative capacity of specific memory CD4 and CD8 T cells (so-called central memory) is maintained in the absence of antigen and does not decay with age, at least up to 67 yr after priming; in contrast the pool of vaccine-specific effector–memory T cells decays with time in the absence of antigen but can expand in vivo upon reexposure to the virus but only for a limited period.

In conclusion, the end of smallpox vaccination provides a unique model of long-term persistence of T cell memory in humans in the absence of nominal antigen. It allows us to distinguish two types of vaccinia-specific memory T cells, according to their proliferative and IFN- γ -producing capacities (34, 35). As suggested, proliferative central memory T cells have a greater capacity to persist in vivo and should help mediate a stronger protective immunity (35) than effector–memory T cells, although the question cannot be explored in this model in the absence of circulating smallpox. Therefore, evaluating the frequency of effector–memory cells by immediate cytokine production capacity might not reflect the current status of residual immune memory to smallpox. At a time when the risks of bioterrorism, the recent monkeypox virus outbreaks in humans (36), and the future intensive usage of poxviruses as vectors for new vaccines against HIV, malaria, or tuberculosis (37) call attention to the need for better knowledge of residual immunity to poxviruses in the worldwide human population, our findings offer new clues to the mechanism of persistence of long-term memory to these viruses and challenge current vaccine strategies.

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References

- Henderson, D.A. 1999. Lessons from the eradication campaigns. *Vaccine*. 17:S53–S55.
- Ahmed, R. 1996. Tickling memory T cells. *Science*. 272:1904.
- Kaech, S.M., E.J. Wherry, and R. Ahmed. 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nat. Rev. Immunol.* 2:251–262.
- Sprent, J., and C.D. Surh. 2001. Generation and maintenance of memory T cells. *Curr. Opin. Immunol.* 13:248–254.
- Zinkernagel, R.M. 2000. On immunological memory. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 355:369–371.
- Blattman, J.N., R. Antia, D.J. Sourdive, X. Wang, S.M. Kaech, K. Murali-Krishna, J.D. Altman, and R. Ahmed. 2002. Estimating the precursor frequency of naive antigen-specific CD8 T cells. *J. Exp. Med.* 195:657–664.
- Doherty, P.C., S. Hou, and R.A. Tripp. 1994. CD8+ T-cell memory to viruses. *Curr. Opin. Immunol.* 6:545–552.
- Oehen, S., H. Waldner, T.M. Kundig, H. Hengartner, and R.M. Zinkernagel. 1992. Antivirally protective cytotoxic T cell memory to lymphocytic choriomeningitis virus is governed by persisting antigen. *J. Exp. Med.* 176:1273–1281.
- Sourdive, D.J., K. Murali-Krishna, J.D. Altman, A.J. Zajac, J.K. Whitmire, C. Pannetier, P. Kourilsky, B. Evavold, A. Sette, and R. Ahmed. 1998. Conserved T cell receptor repertoire in primary and memory CD8 T cell responses to an acute viral infection. *J. Exp. Med.* 188:71–82.
- Varga, S.M., L.K. Selin, and R.M. Welsh. 2001. Independent regulation of lymphocytic choriomeningitis virus-specific T cell memory pools: relative stability of CD4 memory under conditions of CD8 memory T cell loss. *J. Immunol.* 166:1554–1561.
- Homann, D., L. Teyton, and M.B. Oldstone. 2001. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat. Med.* 7:913–919.
- Dutton, R.W., L.M. Bradley, and S.L. Swain. 1998. T cell memory. *Annu. Rev. Immunol.* 16:201–223.
- Sprent, J., and C.D. Surh. 2002. T cell memory. *Annu. Rev. Immunol.* 20:551–579.
- Hou, S., L. Hyland, K.W. Ryan, A. Portner, and P.C. Doherty. 1994. Virus-specific CD8+ T-cell memory determined by clonal burst size. *Nature*. 369:652–654.
- Kim, S.K., M.A. Brehm, R.M. Welsh, and L.K. Selin. 2002. Dynamics of memory T cell proliferation under conditions of heterologous immunity and bystander stimulation. *J. Immunol.* 169:90–98.
- Walsh, M. 2002. Smallpox: the disease and strategies for its control. *Nurs. Times*. 98:26–27.
- Henderson, D.A. 2002. Countering the posteradication threat of smallpox and polio. *Clin. Infect. Dis.* 34:79–83.
- Cohen, J. 2001. Bioterrorism. Smallpox vaccinations: how much protection remains? *Science*. 294:985.

19. el-Ad, B., Y. Roth, A. Winder, Z. Tochner, T. Lublin-Tennenbaum, E. Katz, and T. Schwartz. 1990. The persistence of neutralizing antibodies after revaccination against smallpox. *J. Infect. Dis.* 161:446–448.
20. Demkowicz, W.E., Jr., R.A. Littau, J. Wang, and F.A. Ennis. 1996. Human cytotoxic T-cell memory: long-lived responses to vaccinia virus. *J. Virol.* 70:2627–2631.
21. Hammarlund, E., M.W. Lewis, S.G. Hansen, L.I. Strelow, J.A. Nelson, G.J. Sexton, J.M. Hanifin, and M.K. Slifka. 2003. Duration of antiviral immunity after smallpox vaccination. *Nat Med.* 9:1131–1137.
22. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 401:708–712.
23. Terajima, M., J. Cruz, G. Raines, E.D. Kilpatrick, J.S. Kennedy, A.L. Rothman, and F.A. Ennis. 2003. Quantitation of CD8⁺ T cell responses to newly identified HLA-A*0201-restricted T cell epitopes conserved among vaccinia and variola (smallpox) viruses. *J. Exp. Med.* 197:927–932.
24. Sun, Y., E. Iglesias, A. Samri, G. Kamkamidze, T. Decoville, G. Carcelain, and B. Autran. 2003. A systematic comparison of methods to measure HIV-1 specific CD8 T cells. *J. Immunol. Methods.* 272:23–34.
25. Cao, J., J. McNevin, U. Malhotra, and M.J. McElrath. 2003. Evolution of CD8⁺ T cell immunity and viral escape following acute HIV-1 infection. *J. Immunol.* 171:3837–3846.
26. Frey, S.E., F.K. Newman, J. Cruz, W.B. Shelton, J.M. Tennant, T. Polach, A.L. Rothman, J.S. Kennedy, M. Wolff, R.B. Belshe, and F.A. Ennis. 2002. Dose-related effects of smallpox vaccine. *N. Engl. J. Med.* 346:1275–1280.
27. Harari, A., S. Petitpierre, F. Vallelian, and G. Pantaleo. 2003. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood.* 103:966–972.
28. Younes, S.A., B. Yassine-Diab, A.R. Dumont, M.R. Boulassel, Z. Grossman, J.P. Routy, and R.P. Sekaly. 2003. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4⁺ T cells endowed with proliferative capacity. *J. Exp. Med.* 198:1909–1922.
29. Topham, D.J., and P.C. Doherty. 1998. Longitudinal analysis of the acute Sendai virus-specific CD4⁺ T cell response and memory. *J. Immunol.* 161:4530–4535.
30. Murali-Krishna, K., L.L. Lau, S. Sambhara, F. Lemonnier, J. Altman, and R. Ahmed. 1999. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science.* 286:1377–1381.
31. Garcia, S., J. DiSanto, and B. Stockinger. 1999. Following the development of a CD4 T cell response in vivo: from activation to memory formation. *Immunity.* 11:163–171.
32. Swain, S.L., J.N. Agrewala, D.M. Brown, and E. Roman. 2002. Regulation of memory CD4 T cells: generation, localization and persistence. *Adv. Exp. Med. Biol.* 512:113–120.
33. Doherty, P.C., D.J. Topham, R.A. Tripp, R.D. Cardin, J.W. Brooks, and P.G. Stevenson. 1997. Effector CD4⁺ and CD8⁺ T-cell mechanisms in the control of respiratory virus infections. *Immunol. Rev.* 159:105–117.
34. Veiga-Fernandes, H., U. Walter, C. Bourgeois, A. McLean, and B. Rocha. 2000. Response of naive and memory CD8⁺ T cells to antigen stimulation in vivo. *Nat. Immunol.* 1:47–53.
35. Wherry, E.J., V. Teichgraber, T.C. Becker, D. Masopust, S.M. Kaech, R. Antia, U.H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol.* 4:225–234.
36. Stephenson, J. 2003. Monkeypox outbreak a reminder of emerging infections vulnerabilities. *JAMA.* 290:23–24.
37. McMichael, A.J., and T. Hanke. 2003. HIV vaccines 1983–2003. *Nat. Med.* 9:874–880.
38. Autran, B., G. Carcelain, T.S. Li, C. Blanc, D. Mathez, R. Tubiana, C. Katlama, P. Debre, and J. Leibowitch. 1997. Positive effects of combined antiretroviral therapy on CD4⁺ T cell homeostasis and function in advanced HIV disease. *Science.* 277:112–116.