Virus-induced polyclonal T cell activation is followed by apoptosis: partitioning of CD8$^+$ T cells based on α4 integrin expression

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Abstract

Systemic infection with lymphocytic choriomeningitis virus (LCMV) is accompanied by marked splenomegaly, primarily reflecting the accumulation of CD8$^+$ T cells with an activated phenotype (e.g. VLA-4$^{hi}$). Analysis of DNA content using 7-aminoactinomycin-D revealed that as many as 30% of CD8$^+$ T cells are cycling around day 6 post-infection and that virtually all cycling cells express a high level of VLA-4. In accord with the relatively stable CD4$^+$ cell number, only few cycling CD4$^+$ cells were observed. Following virus control, splenic lymphocyte numbers decreased gradually and during this period many apoptotic cells were detected in the white pulp using terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end labeling. Flow cytometric analysis of DNA content revealed a high frequency of cells with subnormal levels of DNA in the CD8$^+$VLA-4$^{hi}$ subset, whereas the frequency was low for other lymphocyte subsets studied (CD4$^+$, CD8$^+$VLA-4$^{lo}$ and B cells). In addition, numbers of CD8$^+$VLA-4$^{hi}$ cells constitute ~30% of splenocytes at the peak of the response and undergo preferential decrease during normalization of splenocyte numbers. Together these findings indicate that LCMV-induced activation of T cells is followed by apoptosis of many of the triggered cells. Those CD8$^+$VLA-4$^{hi}$ cells which do persist in LCMV immune mice are more sensitive to treatment with the cell-cycle-specific drug hydroxyurea than are phenotypically naive T cells. Our results therefore indicate that LCMV infection induces polyclonal activation of CD8$^+$ cells which is followed by apoptosis of most of the triggered cells while a smaller subset persists as a primed population which include cycling cells.

Introduction

Systemic virus infections induce T cells to become activated and start proliferating (1). This results in a marked increase in the frequency of virus-specific CTLp that in the course of more severe infections may be as great as 500- to 1000-fold (2,3). Often also marked bystander activation is observed, causing an enlargement of involved lymphoid organs and significant lymphocytosis (1,4–8). Generally, this increase in the number of lymphocytes is accounted for by a preferential increase in CD8$^+$ T cells with a preactivated/memory phenotype, whereas the expansion of the CD4$^+$ T cell subset tends to be more limited (4,8–10). After resolution of the acute infection, T cell numbers gradually return to normal, while at the same time significant numbers of virus-primed cells are selected to persist as memory cells (3,11). Precisely how this selection occurs is not presently known, but based on observations in vitro, indicating that previously activated cells are likely to undergo programmed cell death (12), it has been suggested that the majority of activated T cells are destined to undergo apoptosis, unless rescued by some extrinsic event. Recently this hypothesis has received essential experimental support through observations in mice injected with the bacterial superantigen staphylococcal enterotoxin B (SEB). In this model system it has been found that only cells initially proliferating in response to SEB eventually undergo apoptosis, whereas no apoptosis was detected in cells that had not been activated (13). Thus these results strongly support a close association between activation/proliferation, on the one hand, and apoptosis, on the other (14). However, it is not known whether the same rules apply for immune responses to more conventional pathogens such as viruses, although
there is evidence suggesting that this might be the case at least in some viral infections (2,12,15); however, observations from other viral model systems challenge this view (11). Therefore, to address this question, we applied two new experimental approaches for detection of apoptosis, i.e. staining with 7-aminoactinomycin-D (7-AAD) (16) and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) technique (17), to studying T cell turnover in the already well characterized murine lymphocytic choriomeningitis virus (LCMV) infection model. This infection is associated with marked proliferation of CD8+ T cells leading to a 4- to 10-fold increase in their numbers and accumulation of phenotypically characteristic (e.g. VLA-4hi) virus-primed cells which at the peak of the response constitute ~70-85% of the CD8+ T cells present in the spleen (8,9). Moreover, sorting experiment have clearly shown that most if not all LCMV-activated CD8+ effector cells are to be found within this population (8,18; J. P. Christensen et al., submitted for publication). Thus, this model system is ideally suited to study the mechanisms underlying the increase and subsequent decline in virus-induced effector T cells. Using the above-mentioned techniques we show here that LCMV infection initially induces a conversion of some CD8+ T cells from a VLA-4lo phenotype to VLA-4hi phenotype, which then proliferate extensively. More important, our results also reveal that part of the cells with this phenotype eventually undergo apoptosis in vivo, while another part persists as a population with increased cycling relative to naive-type cells.

**Methods**

**Mice**

BALB/cA mice were obtained from Bomholtgaard (Ry, Denmark). Female mice, 7-10 weeks old, were used in all experiments and the animals were always allowed to aclimatize to the local environment for at least 1 week before use. Animals were housed under controlled conditions that included testing of sentinels for unwanted infections; no such infections were detected.

**Virus**

LCMV of the Traub strain, produced and stored as previously described (19), was used throughout the experiments. Virus titrations were carried out by intracerebral infection of 10-fold dilutions into young adult Swiss mice, and titers were calculated, and two-color immunofluorescence was used to determine the fraction of CD4+ and CD8+ T cells.

**Hydroxyurea (HU) treatment**

HU (Sigma, St Louis, MO) is a cell-cycle-specific drug that eliminates cells in the S phase of the mitotic cycle, while no immediate effects are registered on cells in the G0 or G1 phase (20,21). Mice were given a daily dose of 2x25 mg i.p. 6 h apart; previous experiments have shown that this dose given on day 6 post-infection almost completely blocks clonal expansion of CD8+ T cells in acutely infected mice (18).

**mAb for flow cytometry**

The following mAb were all purchased from PharMingen (San Diego, CA) as rat anti-mouse antibodies: FITC-conjugated anti-CD49d (common α-chain of LPAM-1 and VLA-4) (IgG2b), FITC- and phycoerythrin (PE)-conjugated anti-CD8α (IgG2a), PE-conjugated anti-CD4 (IgG2a), and biotinylated anti-L-selectin (MEL-14) (IgG2a). In addition, FITC-conjugated rat anti-B220 (CD45R, B cell marker) (IgG2a) from Caltag (San Francisco, CA) was used.

**Flow cytometry**

Staining for immunofluorescence was performed by incubating 1x10^6 cells in individual wells of a 96-well round-bottom microtiter plate for 5–10 min in a FACS medium containing 10% rat serum, 1% BSA and 0.1% NaN3 in PBS. Cells were then incubated with relevant antibodies in the dark for 30 min at 4°C. After incubation, cells were washed three times with 0.1% NaN3 in PBS and fixed with 1% cold paraformaldehyde in PBS (8,18).

For analysis of DNA content, cells were either stained using propidium iodide as previously described (21) or with 7-AAD (Sigma) as follows (16): after staining for cell surface markers, cells were washed in FACS medium and then in PBS con-
Cell cycle status of CD4+ and CD8+ cells as a function of time after infection with LCMV. BALB/cA mice were infected with $10^3$ LD$_{50}$ of LCMV i.v., and on the indicated days splenocytes were surface stained with FITC-anti-CD8 and PE-anti-CD4, then permeabilized and stained with 7-AAD. Gates were set for CD4+ and CD8+ cells respectively, and the fraction of cells with >2N content of DNA is indicated.

Fractionation of cells

Splenocytes were sorted using a Becton Dickinson FACStar Plus (8, 18). Post-sort analyses were performed to determine sort purity; the purity of all populations were >90%.

Tissue preparation

Spleens to be used for the TUNEL technique were fixed for 4 h in 4% buffered (0.1 M phosphate buffer) formaldehyde and embedded in paraffin after dehydration. Sections (4–5 μm thick) were transferred to slides precoated with 0.01% poly-L-lysine solution (Sigma). Sections were deparaffinized and hydrated before being exposed to the TUNEL technique.

Detection of DNA fragmentation in tissue sections

The method was performed, with slight modifications, as described by Gavrieli et al. (17). Tissue sections were washed in 10 mM Tris-HCI, pH 8 for 5 min, followed by incubation with 5 μg/ml proteinase K for 15 min (20 units/mg protein; Boehringer Mannheim, Mannheim, Germany), to remove the nuclei proteins. The reaction was terminated by transferring the tissue sections to a bath of ice-cold distilled water. Inactivation of endogenous peroxide was performed by immersing the sections in 35% H$_2$O$_2$ for 5 min, followed by two washes in distilled water for 5 min. Sections were then dipped in TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 5 min. Thereafter, sections were incubated for 60 min with 75 nl reaction mixture containing distilled water, 7.5 nl of a 10 times diluted TdT buffer, 1 mmol biotin-dUTP (Boehringer Mannheim) and 22.5 units TdT (Pharmacia, Uppsala, Sweden). The sections were covered with cover glass to avoid evaporation and incubated in a humid chamber at 37°C in the dark. The reaction was stopped by adding 0.5 M EDTA to the sections for 15 min. Sections were then washed in PBS buffer for 5 min and incubated with a 2% human serum contain 0.03% saponin. 7-AAD (4 μg/ml in PBS-saponin) was then added and cells were incubated at room temperature shielded from light for 30 min. Thereafter, the samples were analyzed.

Samples were analyzed by the use of a FACScan (Becton Dickinson, San Jose, CA) and data on 1–5x10^4 cells were collected. Subsequent data analysis was performed using the Lysys II software program.
T cell activation in vivo is followed by apoptosis

Fig. 4. Apoptosis as a function of time after LCMV infection. BALB/c mice were infected with $10^3$ LD$_{50}$ of LCMV intravenously, and on the indicated days splenocytes were surface stained with FITC-anti-CD8 and PE-anti-CD4, then permeabilized and stained with 7-AAD. Gates were set for CD4$^+$ and CD8$^+$ cells respectively, and the fraction of cells with $<2N$ content of DNA is indicated.

Results

Cell dynamics during LCMV infection

Figure 1 summarizes the dynamics of splenic CD4$^+$ and CD8$^+$ T cell numbers as observed in BALB/c mice acutely infected with $10^3$ LD$_{50}$ of LCMV (Traub strain). For comparison the time-course of spleen virus titers is also presented. Notably, only the number of CD8$^+$ T cells changes markedly during the infection, whereas CD4$^+$ cell numbers are relatively stable.

Analysis of cell cycle status

To correlate the above changes in cell numbers with proliferation of activated T cells, analysis of cell cycle status of CD4$^+$ and CD8$^+$ T cells during infection was carried out. Initial experiments, analyzing sorted T cell subpopulations combined with propidium iodide staining, revealed that few T cells were cycling in mice infected 3 days earlier, whereas a marked increase in cycling cells was found on day 6 post-infection (data not shown). Combining staining for surface markers of T cell subsets with 7-AAD staining of DNA (16), a more extensive analysis was carried out. As can be seen from the results presented in Fig. 2, as many as one-third of the CD8$^+$ T cells are cycling at a given timepoint, whereas CD4$^+$ T cells are proliferating. In contrast, little increase in the frequency of cycling CD4$^+$ T cells was observed at any time during the infection. This is in contrast to the results of a previous study (9), but clearly consistent with our own observations on the cell dynamics during LCMV infection (Fig. 1).

Since the observed increase in CD8$^+$ cells in LCMV-infected mice has recently been found to reflect accumulation of cells expressing an increased level of VLA-4 (8), splenocytes were triple labeled using anti-CD8 and 7-AAD combined with anti-VLA-4, in order to investigate whether this marker could be used to separate cycling cells from resting cells. This analysis revealed that all CD8$^+$ cells with an increased DNA content were included in the subset characterized by increased expression of VLA-4, as shown by results representative of day 6-primed cells presented in Fig. 3 (upper panel). Similar results were obtained combining cell sorting and staining with propidium iodide (data not shown).

Detection of apoptotic cells using flow cytometry

Staining with 7-AAD may also be used to detect apoptosis, as cells with subnormal staining reflect cells with fragmented DNA. The result of this type of analysis of T cells during the course of LCMV infection is presented in Fig. 4. It is evident that coinciding with the decrease in lymphocyte numbers around day 11-14 post-infection (Fig. 1), a marked increase in cells with subnormal levels of DNA is found. Further, CD8$^+$ T cells are much more prone to undergo apoptosis than are CD4$^+$ cells (Fig. 4) or B cells (not shown). Comparison of CD8$^+$ cells expressing high and low levels of VLA-4 revealed that the frequency of cells with subnormal levels of DNA is much higher in the former population (Fig. 3, lower panel).
This is in complete accord with previous findings demonstrating that the normalization of lymphocyte numbers primarily reflects a decline in the number of CD8+VLA-4hi cells (8). Thus, CD8+VLA-4th cells are characterized by initial proliferation, whereupon many of these cells appear destined to undergo apoptosis.

Detection of apoptotic cells in spleen sections

To exclude the possibility that the above results merely reflect an in vitro phenomenon of little relevance in vivo, sections of spleens taken from LCMV-infected mice were subjected to analysis using the TUNEL technique. This method (17) is based on the detection of DNA strand breaks—a characteristic event in apoptosis. Terminal transferase incorporates biotin-labeled dUTP to the sites of DNA fragmentation (the free 3'-OH ends). The reaction is visualized by peroxidase-conjugated streptavidin, using AEC as substrate, which gives a reddish color.

By use of the TUNEL technique on spleen sections, it was always possible to detect apoptotic cells. Apoptotic signals were seen either as red nuclei in cells with more or less intact cytoplasm, or as variable numbers of red inclusions in the cytoplasm of macrophages—apoptotic bodies of various sizes. In normal spleens (Fig. 5A), apoptotic signals were always numerous in the red pulp, generally localized in macrophages, whereas they were absent or few in the white pulp. However, in spleens from infected mice, increasing numbers of apoptotic cells were seen in the white pulp from day 7 to day 14 post-infection (Fig. 5B and C), besides numerous apoptoses in the red pulp. Apoptoses in the white pulp tended to localize in the periarteriolar areas, but could be seen in any part of the pulp, both in single cells and as apoptotic bodies (equivalent to tingiblable bodies) in macrophages. Thus, the results obtained clearly demonstrate apoptosis of white pulp lymphocytes in vivo during the recovery phase from systemic LCMV infection.

Increased cycling of persisting CD8+ VLA-4th cells

It has previously been found that a significant proportion of CD8+VLA-4th T cells persists for at least 2 months after virus inoculation (18). Furthermore, this population manifests itself both phenotypically (Pgp-1hi) and functionally (increased IFN-γ production) as a subset of primed cells (18; J. P. Christensen et al., submitted for publication). Therefore, in the light of the ongoing discussion of the cell cycle status of memory cells (22), it was of interest to study whether these cells were actively cycling. Cell sorting followed by DNA analysis using propidium iodide revealed little if any increase in the frequency of cycling cells in the CD8+VLA 4th subset late in the LCMV infection as compared with VLA-4lo cells from the same animals or CD8+ cells from naive controls (data not shown).

However, using treatment with the cell-cycle-specific drug HU, we have previously been able to demonstrate increased cell cycling in LCMV-infected mice at a time point when this is not yet detectable by use of flow cytometry (18). Therefore, mice infected with LCMV 2 months earlier were subjected to treatment with HU. As shown previously (18), short-term treatment with this drug—which markedly inhibits CD8+ cell proliferation during the acute response—does not significantly affect the relative distribution of CD8+VLA-4th and VLA-4lo cells.
cells late in the infection. However, if the animals were given HU on three subsequent days, a relative decrease in VLA-4^hi cells was observed (ratio VLA-4^hi/VLA-4^lo: 0.28, median of six mice) which was not seen in sham-treated mice (ratio: 0.71, median of five mice; p < 0.01, Mann-Whitney rank test). Thus, CD8^+ VLA-4^hi cells appear to be more susceptible to HU, suggesting that some of these cells are progressing through the cell cycle in vivo. Since a recent report (23) has indicated that cycling CD8^+ T cells in LCMV-immune mice are to be found preferentially among L-selectin^h cells, we attempted to further define cycling VLA-4^hi cells based on L-selectin expression. However, if anything, VLA-4^hi-L-selectin^h cells were less susceptible to treatment with HU than were VLA-4^hi-L-selectin^lo cells (Fig. 6)—although both were preferentially reduced in immune animals relative to naive-type (VLA-4^lo-L-selectin^h) cells.

Discussion

The present results confirm and extend previous data (9) demonstrating a preferential clonal expansion of CD8^+ cells during LCMV infection. The increase in CD8^+ cells is matched by a marked increase in cycling CD8^+ cells. In contrast, the CD4^+ population remains relatively stable and few cycling CD4^+ cells are found. More important, analysis of the CD8^+ subset revealed a marked partitioning by VLA-4 phenotype. Thus, not only are LCMV-activated effector cells to be found preferentially among L-selectin^h cells, we attempted to further define cycling VLA-4^hi cells based on L-selectin expression. However, if anything, VLA-4^hi-L-selectin^h cells were less susceptible to treatment with HU than were VLA-4^hi-L-selectin^lo cells (Fig. 6)—although both were preferentially reduced in immune animals relative to naive-type (VLA-4^lo-L-selectin^h) cells.

induced to proliferate should be drawn exclusively from a very small subset of pre-existing VLA-4^hi cells cannot be entirely excluded, but seems a much less attractive scenario given the extensive and polyclonal activation observed (8,18). In any event, a high level of VLA-4 expression is an early marker for LCMV-induced T cell activation.

Following control of the infection, lymphocyte numbers are gradually normalized and during this period many apoptotic cells can be found in the white pulp of the spleen. Although these cells could be claimed to reflect virus-infected cells killed by cytotoxic T lymphocytes, this is highly unlikely as their number only starts to go up when virus is almost eliminated, and maximal numbers are found when a very low and relatively stable level of virus can be detected. Furthermore, in a study published following completion of the present analysis, Welsh et al. (24) present evidence for apoptosis in mice infected with the Armstrong strain of LCMV, a strain that at the most infects a small proportion of CD4^+ cells and no CD8^+ cells (25).

An important question not resolved by the above mentioned findings is whether apoptosis is simply a stochastic event reflecting competition for critical growth factors or is it directly linked to prior activation. However, in view of the demonstrated partitioning of the CD8^+ subset in LCMV-infected mice, it is noteworthy that CD8^+ cells with subnormal levels of DNA are found almost exclusively among cells of the VLA-4^hi phenotype. Our results therefore indicate that previous activation is a prerequisite for apoptosis. Moreover, although this phenotype targets cells for migration to inflammatory sites (8,26), it appears that a substantial proportion of virus-activated CD8^+ cells undergo programmed cell death in the spleen once they have become superfluous due to resolution of the infection. This is in accord with previous observations demonstrating a marked preferential decrease in the number of splenic CD8^+ T cells with this phenotype (8,18).

That apoptosis should be a late consequence of activation is physiologically reasonable, as it would not only prevent accumulation of useless cells, but also limit the pathogenic potential of accidentally activated autoreactive cells (14; J. P. Christensen et al., submitted for publication), and since recent
findings in patients infected with Epstein-Barr virus point to the same conclusion (12,27), indications are that this may be a general way of re-establishing homeostasis following viral infection. Similarly, high-dose immune exhaustion in LCMV-infected mice (2,28), and perhaps in HIV-infected patients (29), may be explained by activation-induced apoptosis. In this context it should be added that although we have not documented that the rules which apply to the bulk of activated CD8+ cells also apply to the minority that are LCMV specific, previous results have revealed a substantial decline in CTLp frequency following resolution of the infection (3), thereby suggesting that this is indeed the case.

Nevertheless, a significant number of CD8+VLA-4hi cells persists in LCMV-immune mice and, at least for the time period studied here, this subset appears to contain all LCMV-primed cells as determined both phenotypically and functionally (18, J. P. Christensen et al., submitted for publication). Cell cycle analysis did not differentiate this subset from CD8+VLA-4lo cells or from CD8+ cells in unprimed mice, which is consistent with previous phenotypic characterization of these cells as expressing little or no transferrin receptor (18), a marker for cell proliferation found on VLA-4hi cells early in the infection. Similarly, few of the cells express a functional IL-2 receptor (18). However, treatment with HU on three subsequent days did induce a reproducible decrease in their number relative to VLA-4lo cells. Consequently, it would seem that at least part of these cells are progressing through the cell cycle in vivo. It is not apparent whether only a subset of these cells are cycling or there is an interconversion between a resting subset and a cycling subset. In a recent report (23), evidence was presented suggesting that cycling LCMV-primed cells in mice previously infected with LCMV-Armstrong are to be found in a subset of CD8+L-selectinhi cells. However, our analysis of the CD8+VLA-4hi subset subdivided based on expression of L-selectin did not support the idea that L-selectin would differentiate the cycling subset. Since a return of L-selectin expression on primed cells seems to occur gradually with time from antigenic stimulation (30,31), it is tempting to explain the apparent difference between our results obtained using LCMV-Traub and those obtained in LCMV-Armstrong-infected mice, by comparing the biology of these two strains. Thus, LCMV-Traub causes a much more prolonged infection and tends to persist at low levels in different organs (32 and unpublished results). Low-grade antigenic stimulation may therefore prevent the return of L-selectin expression, apparently observed in LCMV-Armstrong-infected mice.

The above findings are clearly pertinent to our understanding of immunological memory. Whereas it was classically assumed that memory cells were long-lived, resting cells (33), it has in recent years become popular to view memory cells as rapidly dividing cells, depending on some kind of stimulatory signal for their maintenance (22,34–36). Our results indicate that primed cells at least occasionally undergo cycling, although we cannot exclude that this may reflect a continued antigenic stimulation in our model.
In Fig. 7 we have summarized our present view on the T cell dynamics in LCMV-infected mice. It would appear that LCMV infection is associated with activation of CD8+ cells resulting in up-regulation of VLA-4 expression. These cells—of which only a part is virus specific—then proliferate extensively and give rise to the increased number of activated CD8+ T cells. Some activated cells migrate into infectious foci by virtue of their pattern of adhesion molecule expression (18, 26, 37), while others apparently remain in the spleen where a substantial part eventually die from apoptosis. However, a significant proportion somehow escapes programmed cell death. Whether continued antigenic stimulation is required for their maintenance is not known, but at least part of these cells are progressing through the cell cycle, suggesting some degree of ongoing activation. Eventually, both phenotypic and functional evidence for priming may be lost, and the cells may revert to a naive state (not depicted) (30, 31).

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Abbreviations

AEC 3-amino-9-ethylcarbazole
HU hydroxyurea
HSA human serum albumin
LCMV lymphocytic choriomeningitis virus
PE phycoerythrin
SEB staphylococcal enterotoxin B
TdT terminal deoxynucleotidyl transferase
TUNEL terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling
VLA-4 very late antigen 4
7-AAD 7-aminoactinomycin-D

References

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