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Programmed Cell Death of T Lymphocytes during Acute Viral Infection: a Mechanism for Virus-Induced Immune Deficiency

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Acute viral infections induce immune deficiencies, as shown by unresponsiveness to mitogens and unrelated antigens. T lymphocytes isolated from mice acutely infected with lymphocytic choriomeningitis virus (LCMV) were found in this study to undergo activation-induced apoptosis upon signalling through the T-cell receptor (TcR)-CD3 complex. Kinetic studies demonstrated that this sensitivity to apoptosis directly correlated with the induction of immune deficiency, as measured by impaired proliferation in response to anti-CD3 antibody or to concanavalin A. Cell cycling in interleukin-2 (IL-2) alone stimulated proliferation of LCMV-induced T cells without inducing apoptosis, but preculturing of T cells from acutely infected mice in IL-2 accelerated apoptosis upon subsequent TcR-CD3 cross-linking. T lymphocytes isolated from mice after the acute infection were less responsive to IL-2, but those T cells, presumably memory T cells, responding to IL-2 were primed in each case to die a rapid apoptotic death upon TcR-CD3 cross-linking. These results indicate that virus infection-induced unresponsiveness to T-cell mitogens is due to apoptosis of the activated lymphocytes and suggest that the sensitization of memory cells by IL-2 induced during infection will cause them to die upon antigen recognition, thereby impairing specific responses to nonviral antigens.

Many acute or persistent infections caused by viruses such as measles virus, cytomegalovirus, Epstein-Barr virus (EBV), and human immunodeficiency virus (HIV) suppress the host's immune responses. This is demonstrated by the inability of lymphocytes to respond to the mitogenic lectins concanavalin A (ConA), phytohemagglutinin, and pokeweed mitogen or to specific protein antigens (9, 22, 29). Patients suffering from measles virus infection have long been known to have suppressed skin responses and immunological activity against tuberculin, *Candida albicans*, and diphtheria toxoid, and acute virus infections predispose individuals to a secondary infection by another pathogen. The focus of much current interest has been on the immunosuppressive nature of HIV. Long before the symptoms of AIDS evolve, the response of HIV-infected individuals to a variety of T-cell mitogens is reduced, and their anamnestic responses to antigens such as tetanus toxoid are severely impaired (9). A variety of mechanisms for virus-induced immune suppression have been proposed, including the generation of suppressor T cells, production of prostaglandins by macrophages, the encoding or direct induction of immunosuppressive cytokines by viruses, and the direct lysis or dysfunction of lymphoid cells caused by virus infection or virus-encoded proteins (29).

Recent studies have shown that subtle differences in intracellular signalling induced by growth factors, cytokines, or other cell surface interactions may determine productive versus abortive lymphocyte activation, resulting in a characteristic mode of programmed cell death known as apoptosis (7). During ontogeny, programmed cell death is believed to be involved in the shaping of the mature peripheral lymphocyte repertoire (14, 25, 36, 45); experiments in various T-cell receptor (TcR) or B-cell receptor transgenic mice, in which the lymphocyte antigen receptors are specific for a defined antigen, have shown the exquisite sensitivity of immature lymphocytes to die apoptotically upon antigen receptor cross-linking by the specific antigen and hence be deleted in vivo (10, 27, 40). For example, T-cell tolerance to the male minor histocompatibility antigen, H-Y, in male mice carrying an anti-H-Y TcR is achieved by deletion via apoptotic death in the thymus (38). Recent work in some systems suggests that mature T lymphocytes in the periphery may also be susceptible to apoptotic cell death upon activation through their TcR (activation-induced cell death) (6, 16, 28, 42). T-cell hybridomas undergo growth arrest and die by apoptosis upon activation through their TcR with antireceptor antibody or specific antigen (3, 35). In addition, T cells from TcR transgenic mice stimulated in vitro with antigen die apoptotically when their antigen receptors are subsequently cross-linked with antibody to the TcR-CD3 complex (30). The Vβ-specific immune response to both exogenous (bacterial) and endogenous (retroviral) superantigens results in the apoptotic death of the responding T lymphocytes subsequent to an antigen-mediated expansion in vivo (15, 46). CD5 B cells in the peritoneal cavity of mice carrying an erythrocyte-specific immunoglobulin M transgene are induced to die apoptotically upon antigen exposure (24). The mechanism(s) and immunological consequences of this activation-induced cell death of lymphocytes in the periphery are not well understood.

Recently, it has been shown that T lymphocytes from individuals with HIV or EBV infection are conditioned in vivo to die by apoptosis in vitro (9, 22, 43). It is thus possible that a major and universal mechanism of virus-induced immune deficiency may be the stimulation of T cells into apoptotic death upon antigenic challenge. To address this question, we have examined the well-defined acute lymphocytic choriomeningitis virus (LCMV) infection of the mouse. The LCMV infection results in a characteristic antiviral

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T-cell response that is concomitantly associated with a transient immune deficiency (12, 23, 29, 31, 32), characterized by a failure of lymphocytes to proliferate in response to mitogens and by impaired cellular and humoral immunity to unrelated protein antigens. We show here that the induction of immune deficiency in LCMV-infected mice correlates with the generation of the T-cell response to the acute infection and with the concomitant sensitivity of the T cells to undergo apoptosis upon stimulation through their TcR-CD3 complex. This phenomenon is greatly accelerated by preculture of the T cells in interleukin-2 (IL-2), which could sensitize memory T cells, specific for nonviral antigens, for apoptotic death.

MATERIALS AND METHODS

Infection of mice. Six- to twelve-week old C57BL/6J male mice, obtained from The Jackson Laboratory (Bar Harbor, Maine), were used in all experiments. Mice to be infected received 2×10^4 PFU of LCMV-Armstrong by intraperitoneal injection.

Cell preparations. Mice were sacrificed by cervical dislocation, and spleens were aseptically removed. Single cell splenocyte suspensions were prepared by grinding spleens between glass slides and hypotonic lysis of erythrocytes with 0.8% ammonium chloride and then resuspended in RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) containing 5% fetal calf serum (Sigma), 100 U of penicillin G per ml, 100 μg of streptomycin sulfate per ml, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol (Sigma), 0.1 μM sodium pyruvate (GIBCO), 0.1 mM nonessential amino acids (GIBCO), and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). T lymphocytes were isolated from splenocyte preparations by either of two methods. (i) For the method using nylon wool columns, 1.5×10^8 splenocytes were loaded onto a nylon wool column equilibrated for 30 min at 37°C in RPMI medium. Incubation was continued for 45 min at 37°C, and then nonadherent cells were eluted with warm RPMI medium. (ii) For antibody and complement depletion, granulocytes and B cells were removed from splenocyte suspensions by antibody J11d and complement treatment. J11d was used at 1 ml of undiluted culture supernatant per 2×10^7 cells. Cells were incubated in antibody for 30 min on ice and then washed twice with RPMI medium. Cells were then resuspended in rabbit complement (Pel-Freez Clinical Systems, Brown Deer, Wis.), diluted 1/3 in RPMI medium at 0.5 ml/2×10^7 cells, and incubated for 1 h at 37°C with constant agitation. Cells were washed twice in RPMI medium, dead cells were removed by density separation over Lympholyte-M (Cedarlane, Hornby, Ontario, Canada), and cells were then washed twice with RPMI medium.

Splenic dendritic cells were isolated as described previously (20). Briefly, 1×10^8 to 2×10^8 splenocytes were incubated in tissue culture dishes at 37°C for 2 h, and nonadherent cells were washed off. The remaining adherent cells were cultured overnight in medium alone, and nonadherent cells were subsequently aspirated off, washed, recounted, and used as a source of dendritic cells. Unless otherwise stated, all splenic T-cell cultures were supplemented with 200 to 500 dendritic cells per well.

Reagents. ConA (Sigma) was added to cultures to a final concentration of 2 μg/ml. Anti-CD3 monoclonal antibody (MAb) was either an ascites fluid or an ammonium sulfate cut of tissue culture supernatant from hybridoma 145-2C11 (17). Anti-CD3 MAb was either added directly to cultures at a final concentration of 1/20 (vol/vol) or immobilized onto plastic as follows. Plates were coated with anti-CD3 ascites fluid at 1/200 dilution (in phosphate-buffered saline [PBS]) at 4°C overnight and washed extensively with PBS prior to use. IL-2 (Cellular Products, Buffalo, N.Y., or Amgen, Thousand Oaks, Calif.), when used, was added to cultures at 10 U/ml (final concentration). When given, IL-2 concentration is presented in Amgen units per milliliter. Cyclosporin A (CsA; Sandoz, East Hanover, N.J.) was used at 200 ng/ml (final concentration). The T-cell hybridoma 2R50.2 (41) was kindly provided by David Parker, University of Massachusetts Medical School, Worcester.

Proliferation assays. T cells were cultured at 10^6 cells per ml in a 0.2-ml volume in flat-bottom 96-well plates (Costar, Cambridge, Mass.), in triplicate or quadruplicate, with various stimuli for the indicated length of time. Cultures were pulsed for the final 6 h of incubation with 1 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, Ill.) per well. The contents of each well were harvested onto glass fiber filters, and the incorporated radioactivity was determined on a 1205 BetaPlate liquid scintillation counter (Pharmacia LKB Nuclear Inc., Gaithersburg, Md.).

Flow cytometry. Cells undergoing apoptosis were identified by flow cytometry. The ability of cells to stain with the dye propidium iodide (PI) coupled with simultaneous light scatter analysis was used to identify dying cells (8, 13, 18, 34, 37). Unfixed cells, cultured variously in 48-well plates in a 1-ml volume, were stained with PI (added to a final concentration of 20 μg/ml) in RPMI medium and analyzed immediately on either a FACS 440 (Becton Dickinson, San Jose, Calif.) or FACStar Plus (Becton Dickinson). Data were analyzed by using either the Cytomation software (Cytomation Corp., Fort Collins, Colo.) or PC-Lysys software (Becton Dickinson). Light scatter data are presented on linear scales, and PI fluorescence is presented on a log scale. Apoptotic cells were also identified by their orthogonal (side) and forward light scatter, acquired and analyzed as described above.

DNA fragmentation analysis. A total of 10^6 cells were labeled with 20 μCi of ^{125}I-iododeoxyuridine (^{125}I-IUdR; ICN Radiochemicals, Irvine, Calif.) for 6 h at 37°C. Excess isotope was washed off, the cells were recultured overnight, and then cellular DNA were isolated and fractionated on 1.5% agarose gels as described previously (13). Gels were dried and exposed to Kodak X-Omat autoradiography film with intensifying screens at −70°C.

Centrifugal elutriation. Cells were separated on the basis of size at 3,200 rpm, using a Beckman JE-6B elutriation system (Beckman Instruments, Palo Alto, Calif.) as described previously (4). Large blast-size cells were eluted at flow rates above 28 ml/min, whereas small cells were eluted at flow rates between 15 and 22 ml/min.

RESULTS

Apoptotic cell death of LCMV-elicited T lymphocytes upon activation in vitro. Figure 1A shows the proliferative response of splenic T lymphocytes, isolated from mice various days after inoculation with LCMV, after activation in vitro with various T-cell mitogens in the presence of added dendritic cells from uninfected mice as a source of costimulatory signals to the T cells (19). The ability of the T cells from the acutely infected animals to mount a proliferative response to either ConA or MAb to the TcR-CD3 complex (anti-CD3) was severely impaired. This deficiency in mitogen responsiveness was transient. A proliferative response comparable to that of uninfected mice was obtained from T

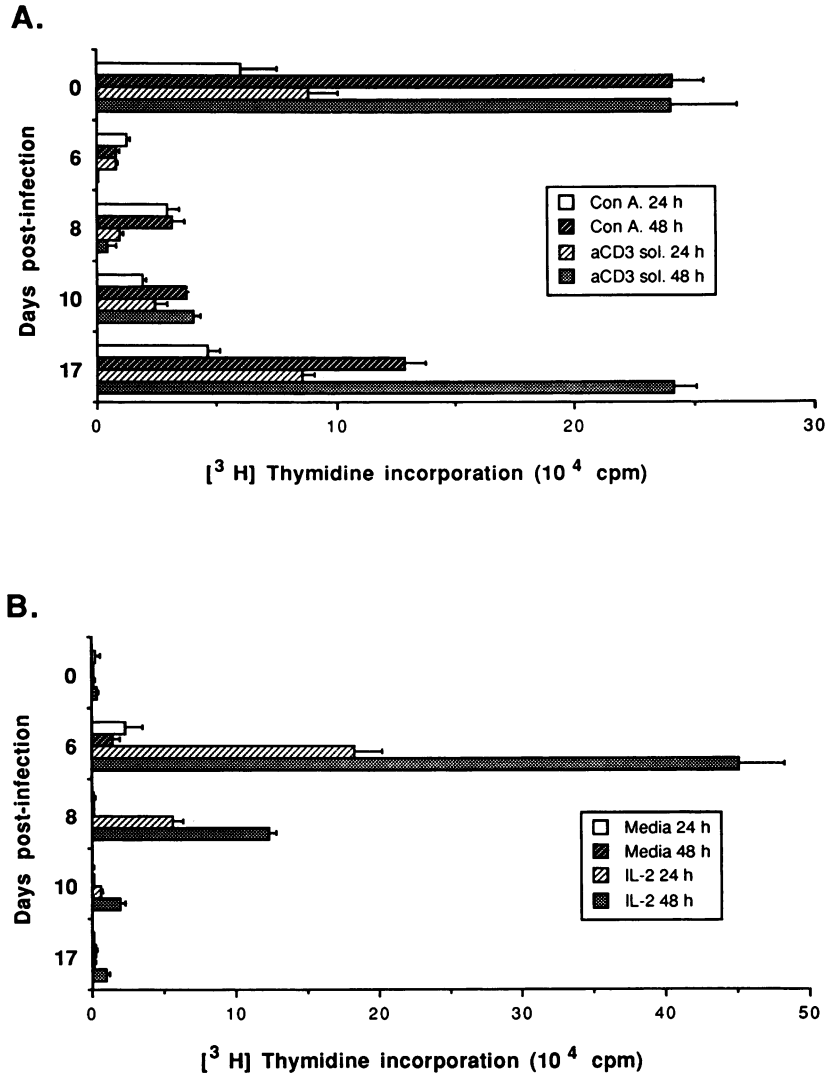
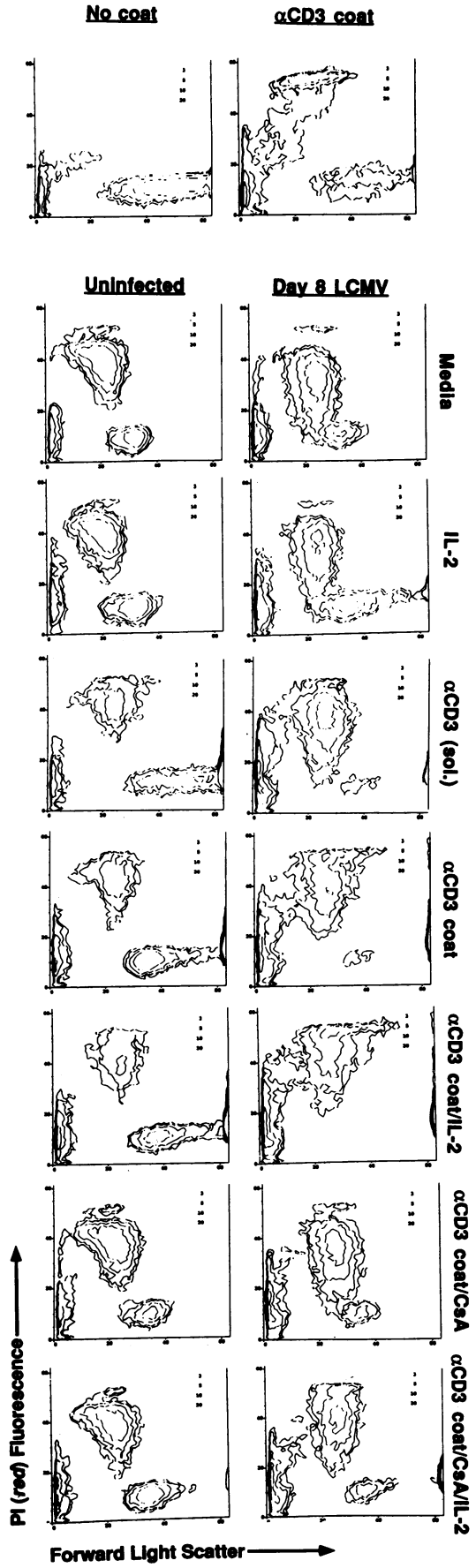


FIG. 1. Response of splenic T lymphocytes from mice infected 0, 6, 8, 10, or 17 days previously with LCMV to mitogens or IL-2. (A) Response to ConA and soluble anti-CD3 MAb (aCD3 sol.) added to the culture without prior attachment onto plastic; (B) response to recombinant IL-2 alone. Proliferation was assayed at 24 and 48 h after initiation of culture.

cells isolated from mice 17 days postinfection, a time at which the acute infection has subsided. Similar results have been published previously by Saron et al. (31, 32) and Jacobs and Cole (12).

Coincident with the severe impairment of T cells from acutely infected mice to respond to mitogens (which cross-link the TcR-CD3 complex on the T-cell surface) was an enhanced ability to respond to exogenous IL-2 (Fig. 1B). This was probably due to activation of the T cells by the virus infection and the concomitant acquisition of IL-2 receptors (IL-2R) on these cells. Maximal response to IL-2 was at day 6 postinfection and declined thereafter. This result formally shows that the unresponsiveness of the virus-induced T cells is limited to those agents analyzed which cross-link the TcR, as there is no inherent inability of the T cells to proliferate in response to IL-2.

We examined whether this mitogen unresponsiveness was due to the induction of an activation-induced cell death upon TcR stimulation by the mitogen. Figure 2A shows the activation-induced apoptosis of a T-cell hybridoma, 2R50.2, as assessed by PI staining and light scatter analysis by flow cytometry (see Materials and Methods). As is apparent, cells not stimulated on the immobilized antibody were viable (i.e., cells are PI^{low}). When their TcR-CD3 complex was stimulated on the MAb-coated plate, a majority of the cells became PI^{high} with low forward scatter (small), characteristic of apoptotic cells. Some subcellular particles (very low forward scatter) with intermediate levels of PI staining, probably representing the DNA-containing apoptotic bodies resulting from the breakup of the dying cells, were seen also (Fig. 2A). Figure 2B shows a PI/forward light scatter analysis of splenic T cells isolated from control (uninfected) or day 8 LCMV-infected mice. The level of spontaneous cell death was relatively high in all cultures, because mouse splenic T lymphocytes die at a relatively high rate upon explant. Control T cells (from uninfected mice) underwent extensive blastogenesis in response to stimulation with anti-CD3 added to the culture for 48 h, as shown by [^{3}H]thymidine uptake



(Fig. 1A) and flow cytometry (Fig. 2). In contrast, in the T cells from the day 8 LCMV-infected mice, upon stimulation with anti-CD3 MAb, few blast cells were seen by forward light scatter (Fig. 2B) and [^{3}H]thymidine incorporation (Fig. 1A). Concomitantly, a large number of PI^{high} cells was seen, indicative of extensive cell death (Fig. 2B).

The preceding experiments were performed with the anti-CD3 MAb directly added to the T-cell cultures. Other studies have shown that the anti-CD3 MAb is presented to the T cells on the surface of accessory cells (e.g., macrophages) present in the T-cell population. Since previous work has suggested a virus-induced macrophage defect during acute LCMV infection (12), we questioned whether defects in residual accessory cells present in the T-cell population could be responsible for the aberrant response of the T cells to the mitogenic stimulus. Therefore, we presented the anti-CD3 MAb to the T cells in a highly cross-linked form by immobilization onto plastic. Splenic dendritic cells from uninfected syngeneic animals were, as in the experiments described above, included in all cultures as a source of costimulatory signal(s) to the T cells (11) (see Materials and Methods). Control T cells proliferated extensively in response to immobilized anti-CD3 MAb over several days of culture. Day 8 T cells showed some proliferation (Fig. 3), which was less than that of control cells but greater than that obtained by stimulation with nonimmobilized anti-CD3 (Fig. 1A). At 48 h, control T cells continued to undergo blastogenesis in response to immobilized anti-CD3 and the number of viable cells expanded, whereas the level of PI^{high} cells was relatively low and comparable to background levels (Fig. 2B). In marked contrast, the day 8 T cells by 48 h underwent extensive cell death, as indicated by the presence of a large number of PI^{high} cells and a small number of viable cells (PI^{low} with high forward light scatter). To further examine the effects of the presentation of the anti-CD3 MAb to the cells, we compared immobilized versus soluble (not immobilized) anti-CD3 presented to the cells in the same experiment. After 48 h of stimulation, the two treatments resulted in equivalent [^{3}H]thymidine incorporation of the day 0 cells (155,262 ± 11,492 cpm with immobilized anti-CD3 and 169,036 ± 37,655 cpm with soluble anti-CD3) and recoveries of PI^{low} cells of 48 versus 39%, respectively. However, in the day 8 cells, stimulation with immobilized or soluble anti-CD3 MAb led to reduced cell recoveries (19 versus 6% PI^{low} cells, respectively) and [^{3}H]thymidine incorporation (68,780 ± 2,501 cpm versus 1,346 ± 1,003 cpm, respectively). Of note is that immobilized anti-CD3 led to considerably more proliferation of the day 8 cells than did soluble anti-CD3, but both treatments ultimately led to cell death. Thus, although the proliferative response of LCMV-induced T cells is transiently influenced by the nature of the anti-CD3 presentation, the ultimate fate of the T cells remains unchanged.

The total number of viable (trypan blue-excluding) cells recovered from the cultures was also reduced in the day 8 cells treated with immobilized anti-CD3. At an initial seeding of 10^6 viable cells, treatments with anti-CD3 for 48 h doubled

FIG. 2. Death of day 8 LCMV-elicited T cells by apoptosis upon TcR-CD3 cross-linking. Flow cytometry was performed as described in Materials and Methods. (A) Activation-induced apoptosis of T-cell hybridoma 2R50.2 upon stimulation with anti-CD3 MAb (αCD3); (B) splenic T cells from control or day 8 LCMV-infected mice analyzed as described above at 48 h postculture. sol., soluble.

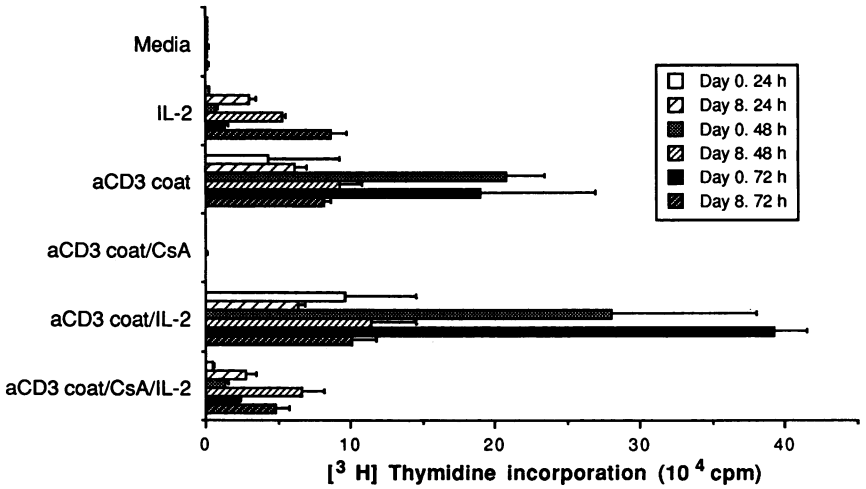


FIG. 3. Proliferative response of control (uninfected) versus day 8 post-LCMV infection-elicited splenic T lymphocytes toward immobilized anti-CD3 MAb (aCD3). Exogenous IL-2 and CsA were added as described in Materials and Methods.

the recovery of viable day 0 cells ($5.4 \times 10^5 \pm 0.4 \times 10^5$ for medium-only control versus $1 \times 10^6 \pm 0.1 \times 10^6$ for immobilized anti-CD3 treatment) but halved the recovery of day 8 cells ($1.1 \times 10^6 \pm 0.2 \times 10^6$ for medium-only control versus $4.8 \times 10^5 \pm 0.6 \times 10^5$ for immobilized anti-CD3 treatment).

One potential trivial explanation for these results could be that since the LCMV-induced T cells have cytolytic activity, they may lyse and induce apoptotic death in lymphocytes to which they are cross-linked by ConA or anti-CD3. We believe it unlikely that the plate-immobilized anti-CD3 MAb could effectively cross-link lymphocytes. Further, work by others showed that in vitro depletion of $CD8^+$ cells did not restore the response to mitogens (32), and the addition of LCMV-induced lymphocytes to control lymphocytes did not impair the proliferative response of the control lymphocytes (32). This finding strongly suggests the apoptotic death of the LCMV-induced T cells to be a cell-autonomous suicide.

Anti-CD3 MAb-induced apoptosis is not blocked by exogenous IL-2 but is blocked by CsA. T cells from LCMV-infected animals are deficient in IL-2 production upon mitogenic stimulation (32). We therefore tested whether addition of exogenous IL-2 to the cultures would alter the response of the day 8 LCMV-induced T cells. Addition of IL-2 to the day 8 T cells stimulated with anti-CD3 MAb did not block the induction of cell death in these cultures; only a few large viable (PI^{low}) cells were seen (Fig. 2B). IL-2, in the absence of TcR-CD3 stimulation, caused the day 8 T cells to undergo blastogenesis and proliferation (Fig. 1B and 3) without any increases in cell death (PI^{high} cells) over the background level (Fig. 2B). We also examined another morphologic characteristic of apoptotic cells, that of increased orthogonal (side, 90°) light scatter, a measure of the granularity of the cell surface (37). This parameter can detect the blebbing of the plasma membrane, a characteristic of apoptotic cell death. As shown in Fig. 4, in T cells from mice either day 6 or day 10 into the LCMV infection stimulated with IL-2 alone, there was a large population of cells with high forward scatter (blast cells responding to IL-2). However, when these same cells were stimulated for 48 h on an anti-CD3 MAb-coated plate in the presence of IL-2, the vast majority of the cells acquired high orthogonal light scatter (and were low in forward scatter). Hence, independent morphologic criteria showed that stimulation of the LCMV-induced T cells via the TcR delivers a signal(s) which results in apoptotic death of these cells. Note that T cells from uninfected animals (day 0 in Fig. 4; Fig. 2B) do not respond by blastogensis to IL-2 alone, whereas they undergo blastogenesis (high forward scatter) when stimulated on an anti-CD3-coated plate in the presence of IL-2.

In addition to morphologic characteristics, fragmentation of cellular DNA into oligonucleosome-size fragments is a hallmark of apoptotic cell death (7). To show that day 8 LCMV-induced T cells fragmented their DNA upon TcR-CD3 cross-linking, we labeled day 8 T cells cultured overnight in IL-2 with ^{125}I-IUdR (a DNA precursor). Hence, these cells driven in cycle with IL-2 incorporated the label into their DNA. These cells were then recultured overnight either in IL-2 alone or on an anti-CD3-coated well plus IL-2. Cellular DNA was then isolated and fractionated by electrophoresis. DNA analyses of cells from uninfected mice were not performed, as these cells respond poorly to this concentration of IL-2 (by proliferating) and hence their DNA cannot be labeled with ^{125}I-IUdR. Lane A in Fig. 5 shows DNA isolated from cells cultured in IL-2 alone. As is apparent, most of the DNA was unfragmented (and migrated only a short distance within the gel). However, oligonucleosomal fragments are still seen; this was due to the spontaneous death of murine T cells in vitro, as mentioned previously. In lane B of Fig. 5 is the DNA isolated from cells stimulated via their TcR-CD3 complex. Very little, if any, of the DNA was unfragmented, and most of the DNA was degraded into oligonucleosomal fragments. This finding supports, by a more traditional but less quantitative technique, the data presented earlier showing the apoptotic death of T cells upon TcR-CD3 signalling.

Having shown that induction of apoptotic cell death in the LCMV-induced T cells required cross-linking of the TcR (with antibody), we tested whether CsA, a compound capable of blocking TcR-mediated signalling (33), would block the induction of this death. As shown in Fig. 2B, CsA blocked the induction of blastogenesis and apoptotic death in the day 8 T cells induced by CD3 cross-linking. At the dose used, CsA quenched the anti-CD3-induced blastogenesis of control T cells (Fig. 2B). This result further indicates that TcR-CD3 signaling is necessary for the induction of activa-

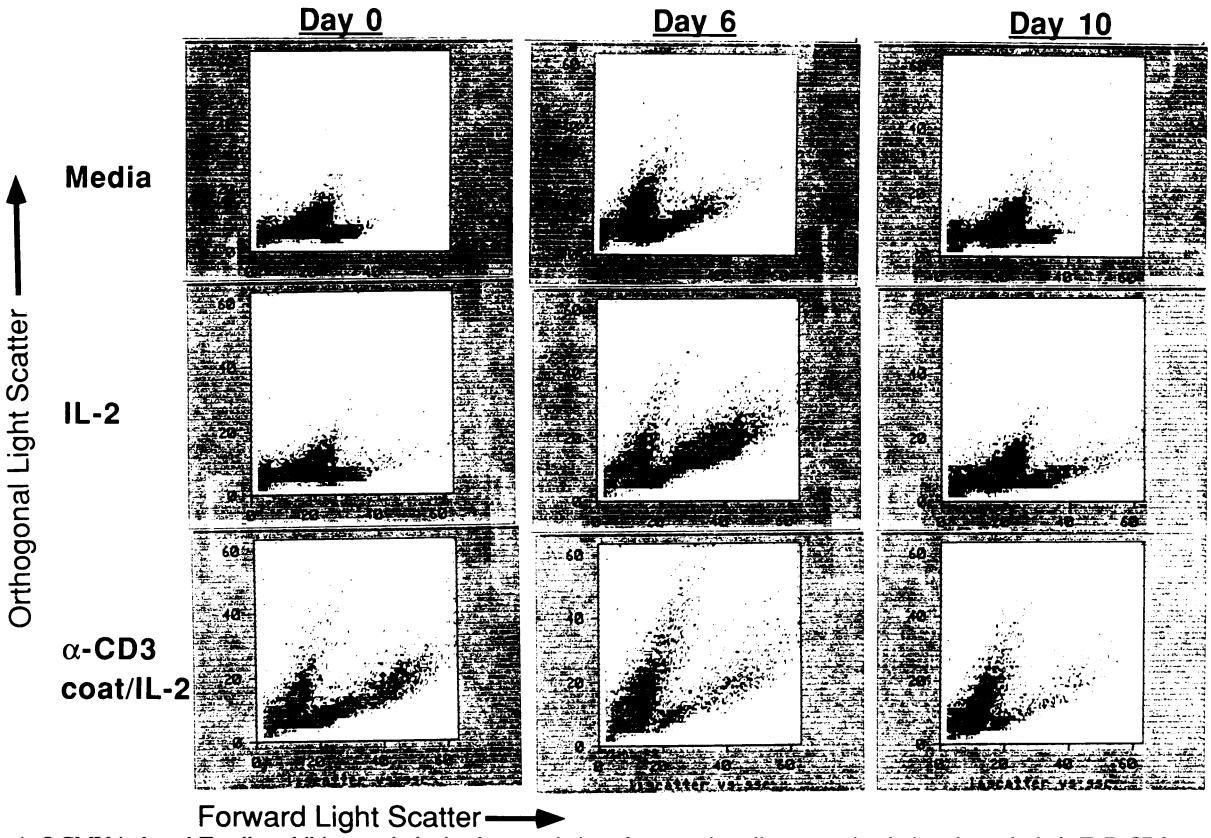


FIG. 4. LCMV-induced T cells exhibit morphologic characteristics of apoptotic cells upon stimulation through their TcR-CD3 complex. Cells were analyzed for orthogonal (*y* axis) versus forward (*x* axis) light scatter by flow cytometry 48 h after culture with the various stimuli. α-CD3, anti-CD3 MAb.

tion-induced apoptotic death in LCMV-induced T cells. Inclusion of IL-2 into the culture with anti-CD3 and CsA allowed, as did IL-2 alone, for some blastogenesis and proliferation (Fig. 3) without significant death over the background level in the day 8 T cells (Fig. 2B), again suggesting that quenching of the TcR-CD3 signal will block induction of death, even when these cells cycle in response to exogenous IL-2.

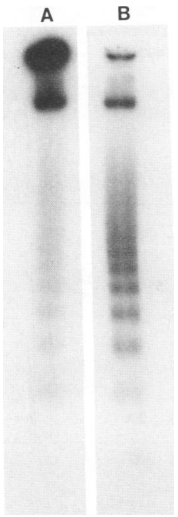


FIG. 5. DNA fragmentation analysis of LCMV-induced day 8 T cells. Lane A, cells cultured in IL-2 only overnight after labeling with ^{125}I-IUdR; lane B, cells cultured on anti-CD3 coated plates with IL-2 overnight after labeling.

TcR-induced apoptosis correlates precisely with immune deficiency during acute infection. Having shown that agents which cross-link the TcR on LCMV-induced T cells result in their death, we explored the course of acute LCMV infection in vivo in terms of the ability of the T cells to die upon stimulation. The ability of splenic T cells to proliferate without any added stimuli or in response to exogenous IL-2 alone varied during the course of the infection (Fig. 1B). In culture medium alone, the proportion of cells with high forward light scatter (indicative of blastogenesis) decreased from days 6 through 10 postinfection (Fig. 6). This decrease parallels the silencing of the immune response to infection as virus is cleared in vivo (47). The number of blast-size cells induced by exogenous IL-2 (Fig. 6) decreased from days 6 through 10 postinfection (as reflected by [^{3}H]thymidine incorporation data; Fig. 1B); this may reflect the in vivo clearance of IL-2-responsive cells elicited during the infection. Stimulation with ConA or anti-CD3 (either added to the culture or immobilized onto plastic) in the absence or presence of exogenous IL-2 (Fig. 6) allowed day 0 and 17 T cells to undergo blastogenesis with only background levels of cell death. In sharp contrast, the day 6, 8, and 10 T cells underwent extensive cell death with few viable (PI^{low}) cells present. These data, taken together with the proliferative unresponsiveness during the acute LCMV infection (Fig.

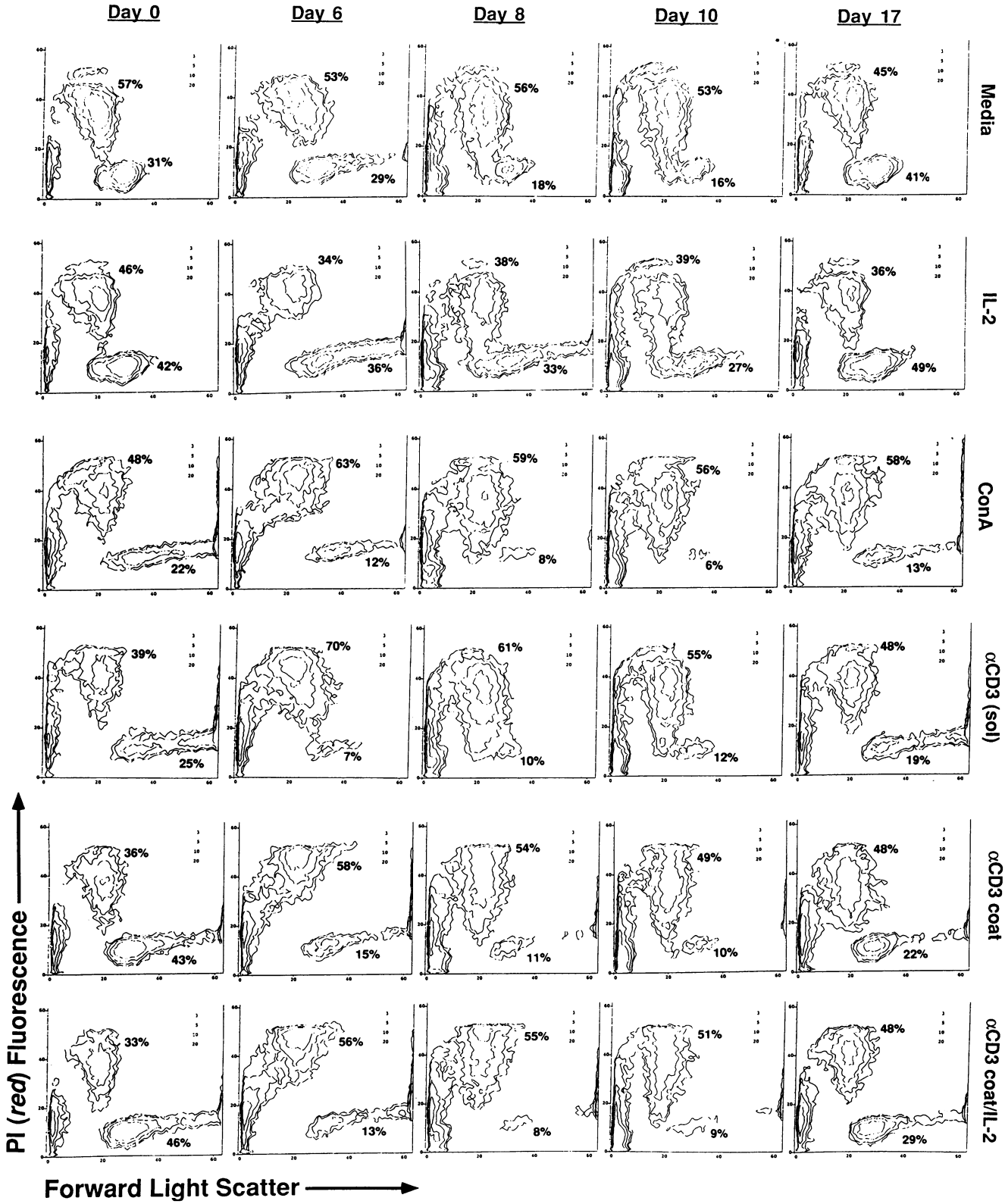


FIG. 6. Time course of LCMV infection in terms of ability of splenic T lymphocytes to proliferate or die by apoptosis. Shown is flow cytometric analysis of T cells from mice days 0, 6, 8, 10, and 17 post-LCMV infection, assayed at 48 h after culture with the indicated stimuli. Percentages refer to PI^{low} and PI^{high} cell populations obtained by electronic gating. αCD3, anti-CD3 MAb; sol, soluble.

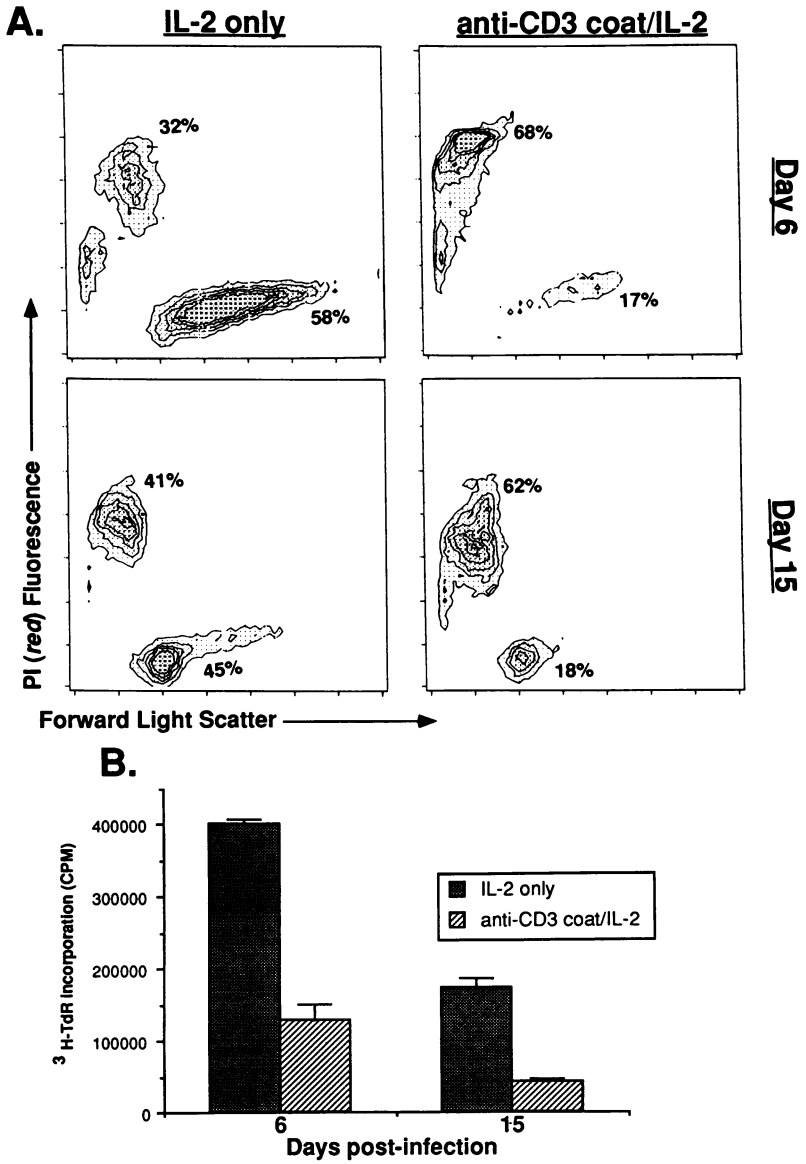


FIG. 7. Effects of IL-2 preculture on priming T cells from mice during (day 6) and after (day 15) LCMV infection to die by apoptosis. (A) PI/forward light scatter analysis of splenic T lymphocytes cultured for 96 h in 10 U of IL-2 per ml alone or on an anti-CD3 MAb-coated well for the last 12 to 16 h of culture; (B) [^{3}H]thymidine incorporation by the day 6 or 15 T cells in a parallel culture during a 6-h pulse. No exogenous dendritic cells were added to any of the cultures. Percentages refer to PI^{low} and PI^{high} cell populations obtained by electronic gating.

1A), show a precise correlation between the TcR-induced apoptosis of the LCMV-induced T cells and the proliferative unresponsiveness to mitogen seen during the acute viral infection.

IL-2 preculture primes LCMV-induced T lymphocytes isolated during and after acute infection to a rapid TcR-induced apoptosis. Lenardo (16) has recently demonstrated that exposure of murine Th1 clones or lymph node T cells in vitro to high concentrations of IL-2 primes cells for apoptosis upon subsequent TcR-CD3 cross-linking. This finding suggests that T-cell cycling driven by IL-2 predisposes to abortive lymphocyte activation upon antigen receptor cross-linking. In the experiments described below, we were interested in specifically examining the antigen receptor-induced death of activated T cells cycling in IL-2. Hence, in all subsequents experiments, in order to prevent the activation and proliferation of the small resting T cells (11) and the resulting ambiguity of the analysis, no exogenous dendritic cells were added.

We tested whether the sensitivity to TcR-induced death of the LCMV-induced T lymphocytes, which express IL-2R, could be enhanced by preculture in IL-2 prior to TcR stimulation. Figure 7A shows that T cells isolated from day 6 post-LCMV infection mice became blast sized when cultured in IL-2 alone (without any exogenous dendritic cells added) at 10 U/ml for 96 h. However, these IL-2-cultured cells died (PI^{high} cells with low forward scatter accumulate) when, during a short overnight incubation, their TcR-CD3 complex was cross-linked with plate-immobilized anti-CD3 MAb in the continued presence of IL-2. The kinetics of the

induction of death under these conditions were very rapid in that death occurred within 12 h. These results show that cell cycling in IL-2 primes in vivo-elicited T cells to die rapidly upon TcR stimulation in vitro.

Since IL-2-dependent cell cycling poises T cells from acutely infected mice to die upon TcR engagement, we questioned whether T cells from the spleens of mice after acute infection would be similarly susceptible to TcR-induced death upon IL-2 preculture. We therefore cultured in IL-2 splenic T lymphocytes from mice 15 days after inoculation with LCMV. At this time (in contrast to day 6 postinfection), only a small percentage of cells should be IL-2 responsive. After 96 h of culture, a subpopulation of about 12% of the cells were large in size, representing cells which were in cycle under the influence of the added IL-2 (as reflected by [^{3}H]thymidine incorporation; Fig. 7B). When these cells were stimulated for the last 12 h of culture on an anti-CD3-coated well in the continued presence of IL-2, the large viable cells (PI^{low}, high forward scatter) disappeared. This disappearance in blast-size cells was also reflected by the decrease in [^{3}H]thymidine uptake (Fig. 7B). Concomitantly, the percentage of apoptotic cells (PI^{high}) increased. Most of the small cells remained small upon TcR-CD3 occupancy (no blast-size cells seen by forward scatter), since, as mentioned above, no additional dendritic cells were added to these cultures, and hence costimulation was not delivered to these resting T cells for their activation. These observations suggest that IL-2-responsive cells from mice during the acute LCMV infection are indistinguishable from the much smaller percentage of cells after the acute infection in terms of their ability to be primed by IL-2 to die a TcR-induced death.

To formally show that the IL-2-responsive blast-size cell population, and not the resting cells, died apoptotically upon TcR-CD3 stimulation, these populations were separated by centrifugal elutriation and assessed for apoptosis upon receptor triggering. Splenic T cells from mice either 17 or 60 days after LCMV infection were precultured in 10 U of IL-2 per ml for 4 or 5 days, respectively. The IL-2-responsive blast-size cells were then separated from the small cells by

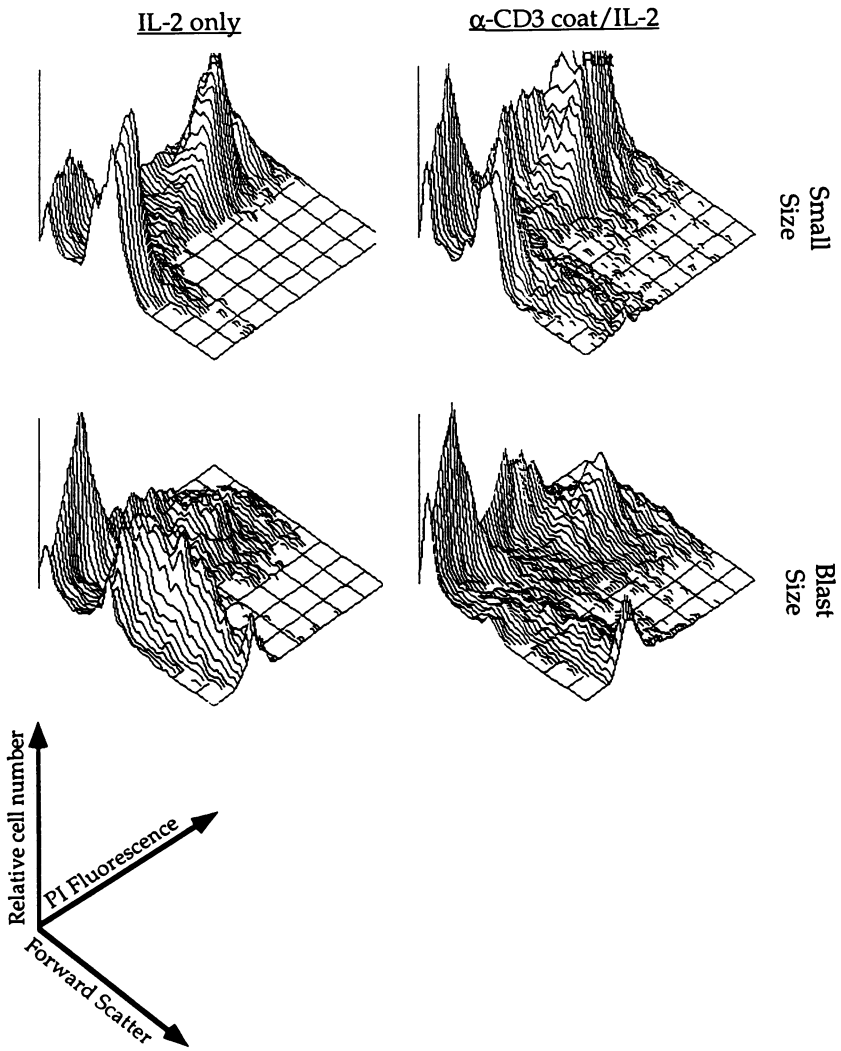


FIG. 8. Flow cytometric analysis of cell death of T cells from mice 17 days after infection with LCMV. Splenic T cells were precultured in IL-2 and separated on the basis of size by centrifugal elutriation. The small and blast-size fractions were then recultured for 48 h in IL-2 alone or on an anti-CD3 MAb (α-CD3)-coated plate plus IL-2 and subjected to PI/forward scatter analysis. No exogenous dendritic cells were added to any of the cultures.

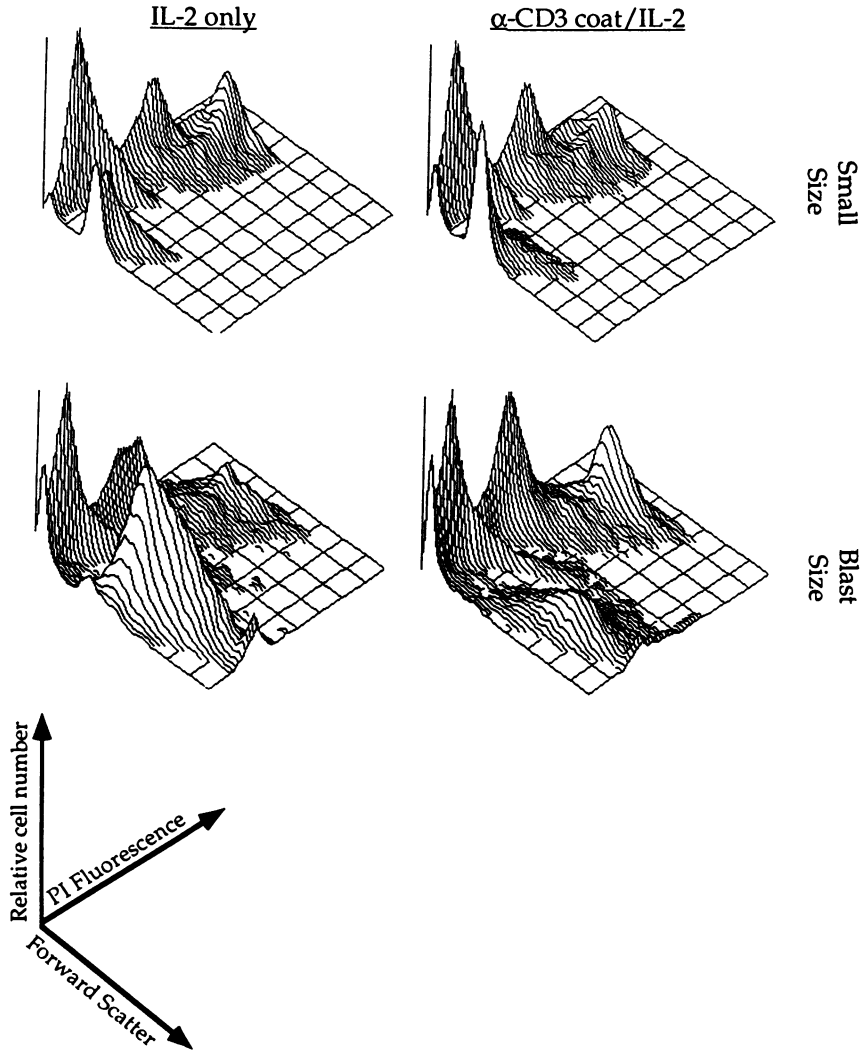


FIG. 9. Flow cytometric analysis of cell death of T cells from LCMV-immune mice (60 days postinfection). Splenic T cells were precultured in IL-2 and separated on the basis of size by centrifugal elutriation. The small and blast-size fractions were then recultured for 24 h in IL-2 alone or on an anti-CD3 MAb (α-CD3) coated plate plus IL-2 and subjected to PI/forward scatter analysis. No exogenous dendritic cells were added to any of the cultures.

elutriation, and the cells were recultured for 24 to 48 h either in wells containing IL-2 alone or onto immobilized anti-CD3 mAb-coated wells in the continued presence of IL-2. These results are graphically displayed in three-dimensional plots in Fig. 8 and 9. The blast cell fractions (all cells eluting above 28 ml/min) contained cells capable of responding to IL-2 alone (with background levels of cell death). When these same cells were stimulated by anti-CD3 and IL-2, extensive levels of cell death were seen. Also apparent from Fig. 8 and 9 is that the small cell fraction (fraction 2) did not respond to IL-2 alone by blastogenesis, and after anti-CD3–IL-2 stimulation, because of the absence of dendritic cells, very few of the small cells had undergone blastogenesis. The majority remained small and viable (PI^{low}). The PI^{high} cells were further shown to be undergoing apoptosis by their increased orthogonal and reduced forward light scatter (data not shown).

Hence, IL-2-mediated expansion of IL-2-responsive T cells from mice shortly after the acute infection or from immune mice 60 days postinfection stimulated them into the cell cycle and primed only the cycling cells to die apoptotically upon receptor triggering. As IL-2R molecules are expressed on activated T cells and on T cells expressing the memory cell phenotype (1, 2, 5; unpublished data), these results suggest that T cells elicited during the acute infection are indistinguishable from memory cells from immune mice in their susceptibility to a TcR-induced death subsequent to cell cycle progression induced by IL-2.

DISCUSSION

We describe here the induction of apoptotic death in LCMV infection-elicited T lymphocytes upon stimulation through their TcR-CD3 complex and show that this death correlated precisely with the induction of immune deficiency in the T-cell compartment during the acute infection. As many viral infections are potent stimulators of T-cell activation, it is likely that a similar pathway of activation-induced cell death is associated with immune deficiency in those infections (29). This universal mechanism for mitogen unre-

sponsiveness would require only that a significant proportion of the isolated T cells be activated in vivo. Such a mechanism for immune deficiency excludes the need for direct infection of lymphoid cells or other virus-induced immunopathologies.

In contrast to the lethal effects of TcR triggering, LCMV-induced T cells proliferated in response to IL-2. IL-2 did not, however, prevent their apoptotic death but instead sensitized T cells to die upon TcR triggering. T cells isolated from spleens both during and after the acute infection were indistinguishable in terms of their susceptibility to TcR-induced apoptosis subsequent to IL-2-driven expansion. That cell cycling in IL-2 alone did not induce cell death but instead primed cells to die upon subsequent TcR cross-linking is in agreement with the work of Lenardo, who first demonstrated that vigorous T-cell cycling driven by IL-2 results in sensitivity to death upon antigen receptor cross-linking (16). These results point to a pivotal role of the TcR in determining the fate of the T cell, depending on its cell cycling status.

Resting G_0 T cells are normally productively stimulated to enter the cell cycle, proliferate, and differentiate upon TcR signalling in the presence of the appropriate costimulatory signal(s), but at least in some cases, activated T cells progressing through the cell cycle are stimulated to die upon antigen receptor triggering (30). It is paradoxical that TcR signalling can drive the cell into such diametrically opposed fates: either entrance into a proliferative/differentiative cycle or exit from cycle and subsequent death. That immature T cells die in the thymus upon antigen recognition shows that antigen receptor signalling can, under the correct circumstances, drive a cell into death in vivo. However, only recently has the concept of death of mature peripheral lymphocytes upon antigen receptor signalling emerged. Antigen recognition by mature T cells can clearly result in expansion and differentiation of the lymphocyte precursors and the development of an immune response. However, the fact that antigen (in the appropriate context) can productively drive a resting cell does not necessarily imply a similar outcome on cells which are already activated and progressing through the cell cycle, and it seems from our work and that of others (16, 26, 30) that strong cross-linking through the TcR of actively cycling cells can deliver a signal for death. An example is our demonstration of the effect of IL-2 in kinetically accelerating TcR-induced death. Perhaps cells not actively cycling do not receive a signal leading to their death and instead give rise to the memory pool.

T lymphocytes from EBV- or HIV-infected individuals have recently been shown to die by apoptosis in vitro (9, 22, 43). In the EBV study (43), spontaneous apoptotic cell death upon explant was exclusively manifest in the activated T-cell pool (expressing CD45RO). In the study with asymptomatic, HIV-infected individuals (9), the proliferative unresponsiveness to mitogen and resulting apoptosis was partial and not as profound as that seen in the acute LCMV infection. This moderate unresponsiveness may have occurred because only a relatively small percentage of the T cells are actually activated in the persistent asymptomatic HIV infection. Since during the acute LCMV infection as many as 90% of the splenic T cells are activated and responding to the infection (21), a vast population of T cells is susceptible to TcR-induced apoptosis, as we have observed.

Antigen-specific immune deficiency has been associated with acute viral infections in many systems (29). As mentioned earlier, individuals immunized with particular antigens, such as tuberculin, may not mount a recall (memory) response to these antigens during acute virus infections; this phenomenon has been referred to as anergy. Recall responses to tetanus toxoid are severely impaired in HIV-infected individuals undergoing chronic T-cell responses to HIV (9). Cells bearing the memory phenotype have been shown to express high-affinity IL-2R and are believed to have less stringent activation requirements compared with naive T cells (44). Memory cells, in the presence of stimulating antigen, enter the cell cycle in the presence of low levels of IL-2 (2, 39). However, cells expressing high-affinity IL-2R can, at a higher concentration of IL-2, actively cycle in the absence of added antigen. The production of high levels of IL-2 during acute or chronic infections may thus impair the responses of IL-2R-bearing memory T cells by sensitizing them to undergo TcR-induced apoptotic death. These hypotheses are supported by our demonstration that IL-2-responsive T cells isolated from immune mice long after the acute infection has subsided are still susceptible to TcR-induced apoptosis.

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