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ZIDOVUDINE (AZIDO DIDEOXYTHYMIDINE) INHIBITS CHARACTERISTIC EARLY ALTERATIONS OF LYMPHOID CELL POPULATIONS IN RETROVIRUS-INDUCED MURINE AIDS[1]

DENIS PORTNOI,* ALAN M. STALL,[1]* DAVID SCHWARTZ,* THOMAS C. MERIGAN,† LEONORE A. HERZENBERG,* AND TERESA BASHAM[2†]

*From the *Department of Genetics and †Division of Infectious Disease, Stanford University School of Medicine, Stanford, CA 94305*

Using flow cytometry technology and multiparameter analyses, we report early and characteristic alterations in lymphoid cell profile in spleen and lymph nodes due to LP-BM5 retrovirus disease (murine AIDS (MAIDS)) and the effect of azido dideoxythymidine, a nucleoside inhibitor, on these changes. MAIDS has been characterized by rapid and profound lymphoproliferation accompanied by hypergammaglobulinemia and immunosuppression. As early as 2 wk postinfection, there is a selective depletion of $CD8^+$ cells whereas the total number of $CD4^+$ cells increases throughout the first 8 wk of infection although the frequency is relatively stable. These population changes were partially delayed by oral AZT therapy for 6 wk postinfection. Ly-6C (AL-21) is expressed on roughly 50% of $CD4^+$ and $CD8^+$ cells in C57BL/6 mice. In MAIDS, the residual population of $CD8^+$ cells is primarily Ly-$6C^+$. The $CD4^+$ cells have a transient increase in ratio of Ly-$6C^+$/Ly-$6C^-$ cells at 2 wk postinfection but by 6 wk are primarily Ly-$6C^-$. There was an increase in both the total number and percentage of Mac 1^+ cells and a selective depletion of certain splenic B cell subpopulations. Azido dideoxythymidine delays these early population changes.

Susceptible strains of mice infected as adults with LP-BM5 MuLV[3] develop a syndrome with many similarities to human AIDS. Murine retrovirus-induced immunodeficiency syndrome, MAIDS, is characterized by early onset of lymphadenopathy, splenomegaly, polyclonal B cell activation, hypergammaglobulinemia, and deficiencies in both B and T cell responses to polyclonal and antigenic stimuli. Death occurs at 14 to 22 wk after infection associated with enhanced susceptibility to opportunistic infections and secondary neoplasms (1–7). In the context of the ongoing pandemic outbreak of AIDS, studies of this murine retrovirus-induced disease are of interest for both testing therapeutic drugs and studying the mechanism of retrovirus induced immunosuppression.

Azido dideoxythymidine (AZT), the only drug licensed so far as a treatment of AIDS, was shown to be effective against murine leukemia C-type retroviruses several years before the outbreak of HIV (8). Ruprecht et al. have shown very effective anti-retroviral activity using oral AZT therapy (0.1 - 1.0 mg/ml) in the Rauscher murine leukemia virus complex model (9). Rauscher murine leukemia virus attacks red cell precursors, causes no immune dysfunction, and is therefore a much simpler model of retrovirus infection than human AIDS or the LP-BM5 model.

Using flow-cytometry technologies and multiparameter analyses, we report the ability of 6-wk oral AZT therapy to delay early and characteristic alterations of MAIDS lymphoid cell profile.

MATERIALS AND METHODS

Mice. Female C57BL/6 (B6) mice (8 to 10 wk of age) were obtained from The Jackson Laboratories, Bar Harbor, ME.

Production of viruses and inoculation of mice. LP-BM5 MuLV consist of a mixture of B-tropic ecotropic virus and MCF. It has been previously shown that neither cloned ecotropic virus nor MCF, although infectious, will cause the MAIDS syndrome. The virus mixture containing a defective 4.8-kb MuLV is required for development of lymphoproliferation and immunodeficiency (10, 11). Virus stocks of LP-BM5 were prepared from chronically infected SC-1 cells, generously provided by Dr. Janet Hartley, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. Titers of ecotropic MuLV were determined by XC plaque assay (12) and contained 5×10^5 plaque-forming units/ml. Titers of the MCF were determined by immunoperoxidase staining of focus formation in *Mus Dunil* cells, using mAb 514 (13). Assay (14) and mAb were kindly provided by Dr. Bruce Chesebro, Rocky Mountain Laboratories, Hamilton, MT. Titers were determined at log $10^{1.5}$ focus forming units/ml.

Mice received injections with 0.2 ml of LP-BM5 MuLV containing medium at 8 to 10 wk of age. This dose of virus had previously been determined to double spleen weight in 2 wk.

Azidothymidine treatment of infected mice. Zidovudine (commercial name for AZT) was a generous gift from Dr. Philip Furman, Burroughs Wellcome, Research Triangle Park, NC. AZT was administered orally in the drinking water (0.25 mg/ml) for 6 wk, starting 24 h after inoculation of the viruses. This is equivalent to approximately 60 mg/kg/day. No detectable toxicity was found at this dose, as measured by mouse weight and hematocrit levels.

Antibodies. The mAb used were 331.12 (rat anti-IgM), AF6-122 (mouse anti-IgD of b allotype), 53-7.3 (rat anti-CD5/Ly-1), 53-6.7 (rat anti-CD8/Lyt-2), GK1.5 (rat anti-CD4/L3T4), M1/70 (rat anti-Mac 1), and AL-21 (rat anti-Ly-6C). Purification and fluorochrome conjugation of these antibodies have been described in detail (15). Biotin-conjugated antibodies were revealed with Texas red avidin.

FMF analyses. Single cell suspensions were prepared from

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[2] Address correspondence to Teresa Basham, S-156, Department of Medicine, Division of Infectious Diseases, Stanford University School of Medicine, Stanford, CA 94305.
[3] Abbreviations used in this paper: MuLV, murine leukemia virus; MAIDS, murine acquired immunodeficiency syndrome; AZT, azido dideoxythymidine. FMF, flow microfluorimetry; MCF, mink cell focus-inducing virus.

lymphoid organs, and stained with optimal amounts of mAb. FMF analyses were conducted as described on a highly modified FACS II (Becton Dickinson, Mountain View, CA), and data were collected and processed by a Digital Equipment Corp. (Marlboro, MA) Vax 11/780 computer, using the FACS-DESK software. Dead cells were stained with propidium iodide and excluded from the analyses (16, 17). For each analysis, data from 20,000 to 30,000 viable cells were collected. Data are presented as 5% probability contour maps (18).

RESULTS

Phenotypic changes in T cell populations delayed by AZT. Previous studies of T cells from spleens and lymph nodes of mice infected with LP-BM5 showed that the frequency of L3T4$^+$ (CD4$^+$) cells decreased slightly and the frequency of Lyt-2$^+$ (CD8$^+$) cells decreased more significantly at 4 to 12 wk postinfection (4, 7). Figure 1 confirms these T cell subset changes in spleen at 4 wk postinfection. CD8$^+$ cells were reduced to 4% of splenic lymphoid cells compared to 13% in uninfected control mice. AZT administered continuously at 60 mg/kg/day for 6 wk partially delays this drop in frequency (9% vs 4%).

In contrast, the frequency of CD4$^+$ cells decreased slightly, (16 vs 18%) with their total number increasing almost proportionately with the increasing total number of lymphoid cells (three- to fourfold by 8 wk postinfection). AZT therapy delays the increase in spleen size, total lymphoid cell number, and the concomitant increase in total CD4$^+$ cells (19).

It has been previously demonstrated that T cells are required to establish this disease and that athymic B6 nude mice, although infected with the carrier viruses, do not develop this immune dysfunction syndrome (2). Ly-6 is a family of differentiation Ag on lymphoid cells encoded for by genes localized to chromosome 15 (20). Recently it has been proposed that CD8$^+$ Ly-6C.2$^+$ cells may represent a distinct subpopulation of T cells that can differentiate in the absence of thymic influence (21). Therefore it was of interest to determine whether the depletion of CD8$^+$ cells involved to the same extent both Ly-6C positive and negative subpopulations, or preferentially one of them.

In this set of experiments, we used rat IgG2a AL-21 that recognizes Ly-6C on a subset of macrophages, CD4$^+$ and CD8$^+$ T cells (22). Figure 1 shows that in control B6 mice, Ly-6C is expressed on roughly half of the CD4$^+$ and CD8$^+$ cells. In MAIDS mice, the remaining CD8$^+$ population is primarily Ly-6C$^+$. In contrast, the CD4$^+$ population, which increased in number and remained relatively constant as a percentage of lymphocytes, is predominantly Ly-6C$^-$. Thus, CD4 and CD8 cells undergo opposite alterations in relation to expression of Ly-6C (AL-21). This process is partially controlled by AZT treatment.

Alteration of the ratio of Ly-6C positive vs negative T cells is a sensitive and early effect of infection in MAIDS (Fig. 2) in both spleen and lymph nodes. As early as 2 wk postinfection, the majority of CD8$^+$ cells is Ly-6C$^+$ in both spleen (*A*) and lymph nodes (*C*). The ratio of Ly-6C$^+$/Ly-6C$^-$ CD8$^+$ cells remains elevated throughout the experiment. AZT therapy reduces the number of Ly-6C$^+$ cells and delays the increase by 2 to 4 wk (*B* and *D*). CD4$^+$ cells show a transient increase in Ly-6C$^+$ cells at 2 wk in both spleen and lymph nodes (*A* and *C*) that drops by 4 wk postinfection and remains lower than control. AZT therapy blocks both the transient increase and the subsequent drop in Ly-6C$^+$ CD4$^+$ cells.

Selective depletion of a subpopulation of splenic B cells in MAIDS. Klinman et al. (6) have previously reported a 10-fold increase in both the rate of B cell proliferation and the proportion of B cells activated to secrete Ig by 4 wk postinfection. These investigators found that as early as 2 wk after infection the frequency of normal B cells decreased in association with the appearance of large populations of B cell blasts that were dull Ly-5 (B220)$^+$ and dull ThB$^+$. We have analyzed splenic B cells of B6 mice 4 wk postinfection with anti-IgM (331.12) and anti-IgD (AF6-122). As shown in Figure 3, splenic B cells expressing high levels of surface IgM are depleted in spleens of infected mice (2 vs 12% in B6 controls). AZT therapy partially delays this effect (7 vs 2%).

Previously, we have demonstrated that high surface IgM-expressing B cells are found predominantly in the high scatter fraction by FMF analysis. Gating analysis

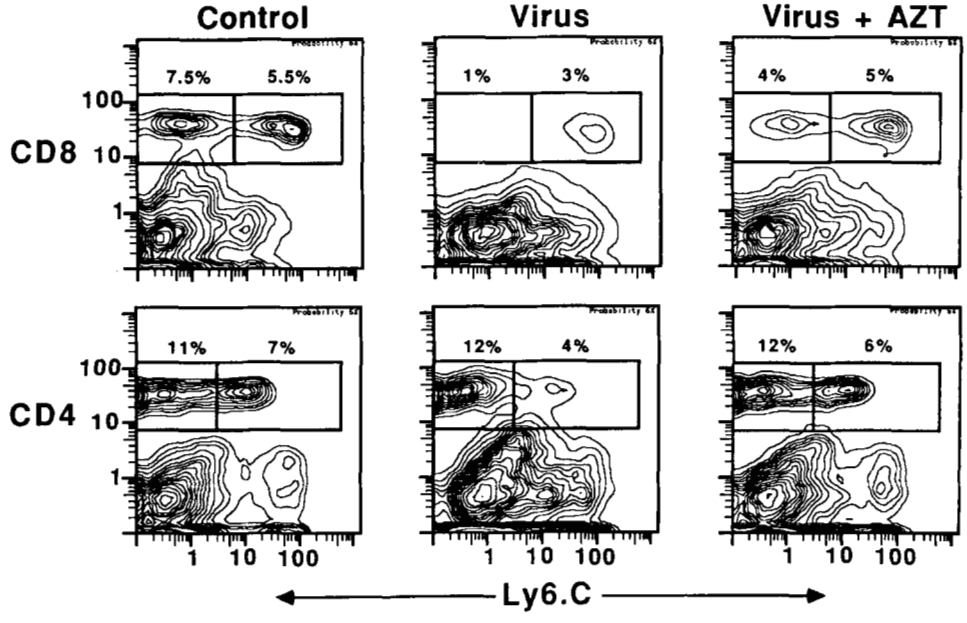

Figure 1. Alteration in expression of Ly-6C by CD4$^+$ and CD8$^+$ cells in spleens of B6 mice 4 wk after infection with LP-BM5. Single cell suspensions from control (*left*), infected (*middle*), and AZT-treated mice (0.25 mg/ml in drinking water for 6 wk; *right*) were stained with fluorescein-conjugated AL-21 (anti-Ly-6C), biotin conjugated 53-6.7 (anti-CD8) developed with Texas red-avidin, and allophycocyanin-conjugated Gk 1.5 (anti-CD4). Three-color FMF analysis were conducted on a modified FACS II and data were collected and processed by a VAX II/780 computer using FACS-DESK software. Dead cells were stained with propidium iodide and excluded from the analysis. Data are presented as 5% probability contour maps. Percent of CD8$^+$ (*top*) and CD4$^+$ (*bottom*) that are Ly-6C$^-$ (*left rectangle inset*) and Ly-6C$^+$ (*right rectangle inset*). Two mice were analyzed for each condition and the data were reproduced in a second experiment. All replicate samples were within 2%.

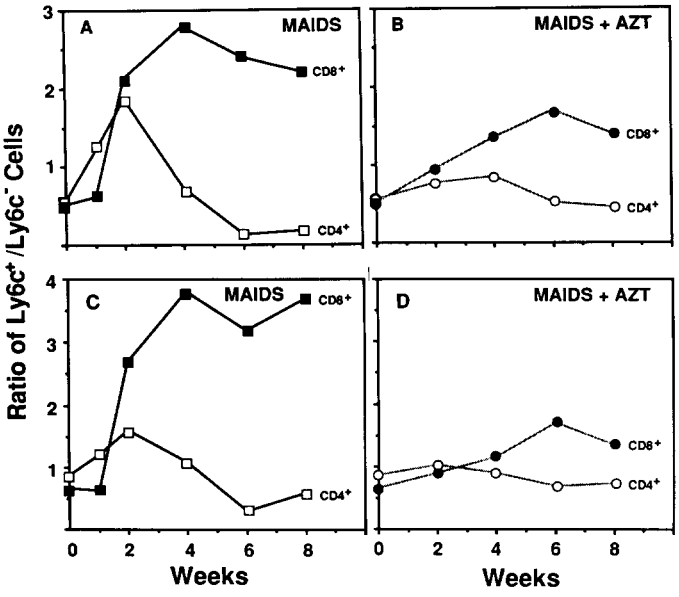

Figure 2. Time course of the ratio of Ly-6C$^+$ vs Ly-6C$^-$ T cells in spleen and lymph nodes. Single cell suspensions were stained and analyzed as in Figure 1. Ratios of CD8$^+$ (■, ●) and of CD4$^+$ (□, ○) Ly-6C$^+$ vs Ly-6C$^-$ were determined for MAIDS *A* (spleen), C (lymph node) and MAIDS + AZT *B* (spleen), D (lymph node) mice at 0, 2, 4, 6, 8 wk postinfection. No significant change was seen in a third group of uninfected littermates sacrificed at corresponding time points (data not shown). Results are the average of two to four mice per time point.

reveals an increase in the number of these high scatter cells in MAIDS mice (47 vs 16% in B6 control, *box insets at the top* of Fig. 4). Paradoxically, despite this increase in high scatter cells, the number of high surface IgM B cells is sharply decreased at 4 wk postinfection (Fig. 4 *bottom*). Thus, the difference between control and MAIDS-infected mice for this population of splenic B cells is shown most strikingly when we gate in the high scatter population. This effect was partially delayed with AZT therapy.

The time course for changes in the B cell population in spleen (Fig. 5A) indicates that the total number of B cells follows a biphasic curve. During the first 2 wk the number of B cells increases in parallel with the total number of lymphoid cells (data not shown). Thereafter the total number of sIgM$^+$, IgD cells drops dramatically.

Similar increases in total number of lymphoid cells in lymph nodes, resulting primarily from an increase in the number of high scatter cells, was observed as reported previously (6). B cells found in the lymph nodes of normal B6 mice are almost entirely low sIgM, high sIgD expressing cells. In MAIDS lymph nodes, in contrast to spleen (Fig. 5*B*), the number of these B cells increases throughout the infection. These population changes in spleen and lymph nodes were partially controlled by AZT therapy.

AZT therapy decreases frequency of Mac-1$^+$ cells. It has been previously reported that the frequency of Mac-1$^+$ cells in nodes of mice infected with the LP-BM5 MuLV is significantly and persistently increased 4 to 12 wk postinfection (4). Figure 6 *A* and *B* confirms that the number Mac-1$^+$ cells increases constantly throughout the infection in both spleen and lymph nodes. This alteration was also partially controlled by AZT treatment in spleen (*A*) and delayed in lymph nodes (*B*).

DISCUSSION

We report early and consistent alterations of lymphoid cell populations in the MAIDS, and the effect of a nucleoside inhibitor, AZT on these changes. We confirm previous studies that there is a progressive increase in the number but not percentage of CD4$^+$ cells and a dramatic drop in the frequency of CD8$^+$ cells after 4 wk of infection. This pattern is quite different from human AIDS where the CD4$^+$ cells are lost. Although the population of CD4$^+$ cells is increased, Th cell-dependent responses are strongly suppressed in these mice (7) similar to AIDS where many CD4-dependent responses are suppressed in HIV-infected patients before these cells are lost (23). Continuous AZT therapy administered orally for the first 6 wk partially delays both the drop in frequency of CD8$^+$ cells and the increase in total CD4$^+$ cells.

Changes in expression of the differentiation Ag Ly-6C among CD4 and CD8 T cells is also an early characteristic marker of this disease. Antibodies to Ly-6 Ag on T cells, including Ly-6C, have been shown to effect cell activation (21). IFN-γ that is expressed at high levels in spleen cells from MAIDS-infected mice by 1 wk postinfection and throughout the course of this disease (24), has been shown to induce Ly-6C on resting macrophages but not on fully differentiated macrophages. Therefore, expression of Ly-6C may be regulated in a complex manner during differentiation and activation (22). Previous studies indicate that expression of Ly-6A/E determinant on spleen and lymph node cells from MAIDS mice was also greatly enhanced at 8 wk postinfection that may correlate with increased IFN-γ production (24).

Normal B6 mice express Ly-6C (AL-21) on approximately 50% of both CD4 and CD8 cells. We observed a simultaneous increase in the percentage of CD4$^+$ and

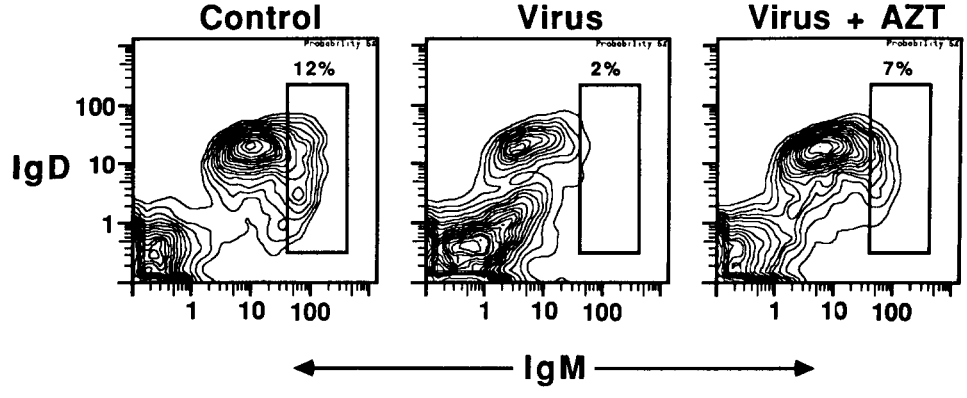

Figure 3. Depletion of high surface IgM cells in spleen of B6 mice 4-wk postinfection. Single spleen cell suspension from control (*left*), virus (*middle*), and AZT-treated mice (*right*) were stained with fluorescein-conjugated 331.12 (anti-IgM), and biotin-conjugated AF6-122 (anti-IgD) and developed with Texas red-avidin. FMF analysis were conducted as described Figure 1. Two mice were analyzed for each condition and the data were reproduced in a second experiment. The percentage of high expressing IgM spleen cells are indicated with the *rectangular insets*.

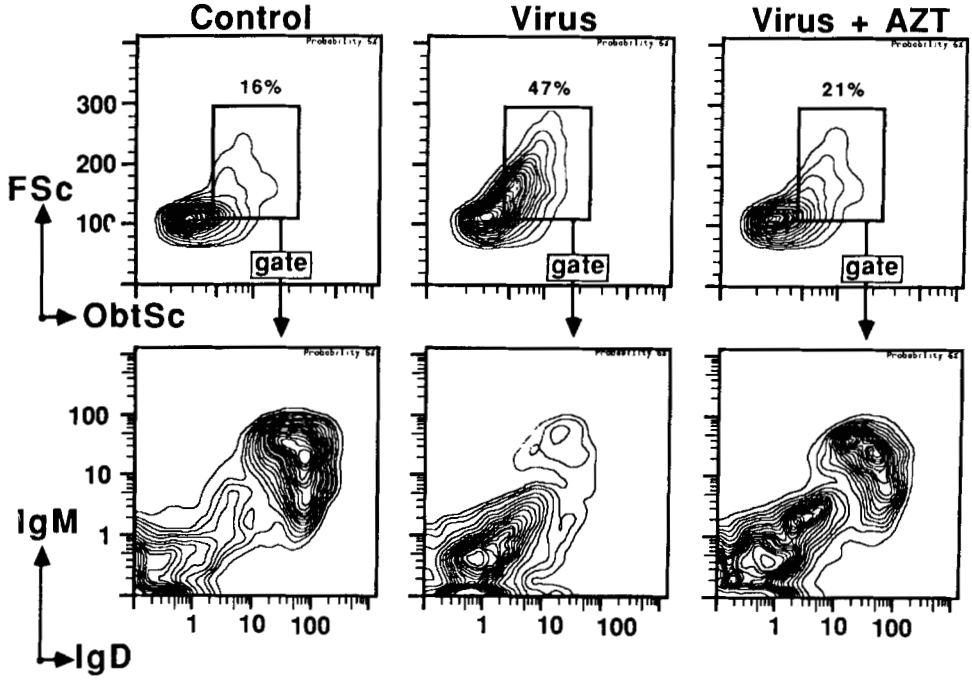

Figure 4. Polyclonal activation of spleen cells and depletion of high surface IgM B cells in high scatter cells of MAIDS mice 4 wk postinfection. Cell suspensions were stained and analyzed as described in Figure 3. The *top panels* show the forward and obtuse light scatter of the cells. The *boxes* delineate cells that are both high forward and obtuse scatter (activated cells). The *bottom panels* show the surface Ig profile of the gated high forward/obtuse scatter cells.

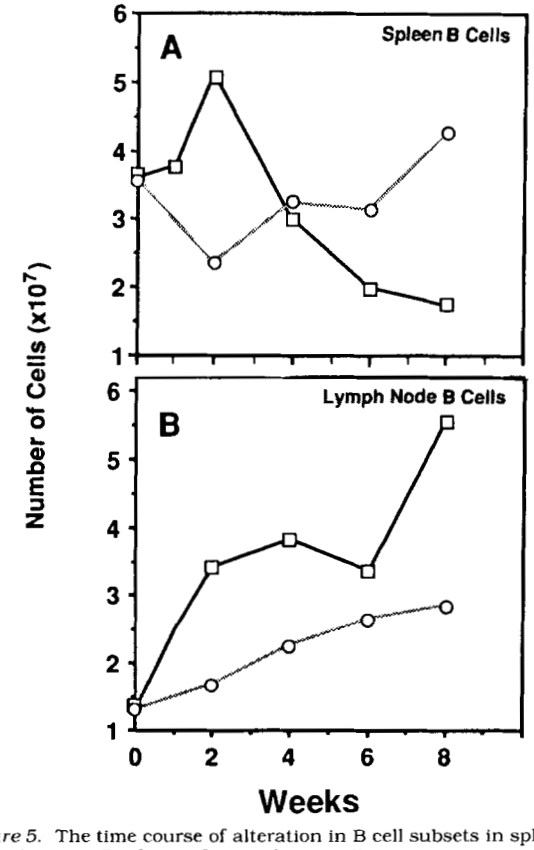

Figure 5. The time course of alteration in B cell subsets in spleen (*A*) and lymph nodes (*B*) of MAIDS mice. Single cell suspensions were stained and analyzed as described in Figure 3. The number of B cells in MAIDS (□), and MAIDS + AZT (○) at 0, 2, 4, 6, 8 wk postinfection. No significant change was seen in a third group of uninfected littermates killed at corresponding time points (data not shown). Data are an average of two to four mice at each time point.

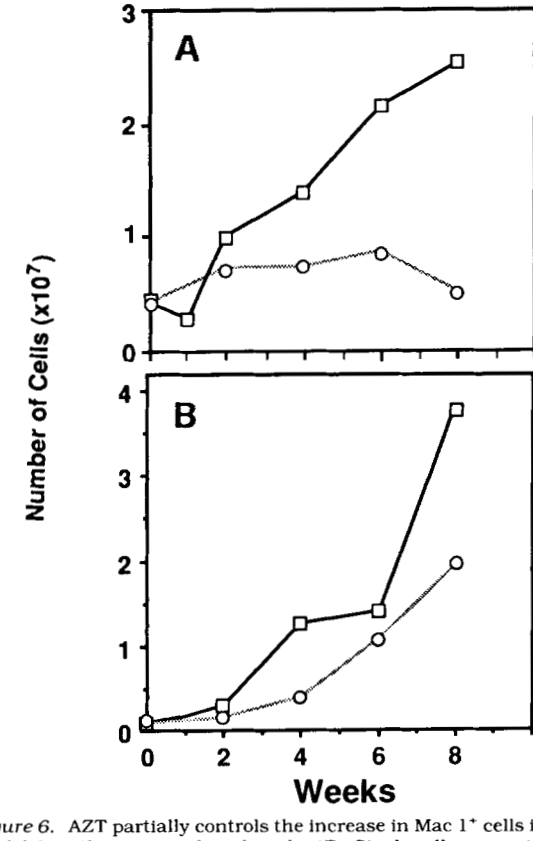

Figure 6. AZT partially controls the increase in Mac 1⁺ cells in spleen (*A*) and delays the increase lymph nodes (*B*). Single cell suspensions were stained with fluorescein-conjugated M1/70 (rat anti-Mac 1) and analyzed as previously described. Number of Mac 1⁺ cells is shown at time 0, 2, 4, 6, 8 wk postinfection for MAIDS (□), and MAIDS + AZT (○). No significant change was seen in a third group of uninfected littermates killed at corresponding time points (data not shown). Data are an average of two to four mice at each time point.

$CD8^+$ T cells expressing this marker during the first 2 wk postinfection. By wk 6, an opposite effect was observed on $CD4^+$ vs $CD8^+$ T cells: whereas the vast majority of remaining $CD8^+$ cells express this marker, $CD4^+$ T cells, which increase progressively in the following weeks, tend to be Ly-$6C^-$. Lack of expression of this marker on $CD4^+$ cells may relate to previous findings that most of $CD4^+$ cells found in MAIDS mice are not functionally competent (7).

However, most of remaining $CD8^+$ cells express Ly-6C. It may be that these cells are actually activated, and eventually cytotoxic (7). Alternatively, $CD8^+$, Ly-$6C^+$ cells may represent a distinct subpopulation that can differentiate in the absence of thymic influence (21), and this subpopulation may be more resistant to the process of deletion. Actually these two alternatives are not mutually exclusive. Therefore, it is tempting to think that Ly-6C molecules are somehow linked to an alternative process of activation (25), and inversely that their expression on T cells is an indicator of activation.

AZT therapy stabilized the ratio of Ly-$6C^+$/Ly-$6C^-$ cells in the $CD4^+$ population in spleen and lymph nodes over the 8 wk these mice were analyzed. AZT reduced the magnitude of the change in Ly-6C expression in the $CD8^+$ population in both spleen (2.5:1 vs 1.5:1) and lymph nodes (3.5:1 vs 1.5:1).

Macrophages have been previously shown to be a primary site of infection in MAIDS (5). We confirm a progressive increase in the number of Mac 1^+ cells, both in spleen and lymph nodes (4). By scatter analysis and the fact there was no overlap between $CD4^+$ and Mac 1^+ cells (data not shown) these cells are presumed macrophages. AZT therapy for 6 wk postinfection strongly reduced the increase in Mac 1^+ cells in spleens of MAIDS mice over the 8-wk period these mice were followed. AZT only delayed by 2 wk the increase in Mac 1^+ cells in the lymph nodes of MAIDS mice.

In MAIDS, we confirm within the first 2 wk after the infection that there is an increase in the number of B cells, resulting mostly from polyclonal proliferation (4, 6). However, after 2 wk of infection the total number of splenic B cells was found to decrease. Fine analyses of the subpopulations of B cells in spleen show a preferential depletion of B cells expressing high level of surface IgM (331.12). Remaining B cells are mostly of lower level of surface IgM and high level of surface IgD (AF6-122). The kinetics of this biphasic change of splenic B cells differ somewhat from a previous report (6). It may be that differences in doses of virus inoculum and/or in the viruses themselves could explain these differences. Alternatively, in the previous report different mAb, B220 and ThB, were used to detect B cells in the spleen that may not detect this subpopulation change.

In lymph nodes, which contain almost exclusively high surface IgD-expressing B cells, there is a constant increase in the number of B cells throughout the infection. AZT therapy prevented the loss of B cells expressing high levels of surface IgM in spleens of MAIDS mice; however, AZT only delayed the increase in high surface IgD expression B cells in lymph nodes of infected mice. In human AIDS, characteristic phenotypic changes in peripheral B lymphocytes have also been reported (26): increased cell size and increased percent of cells bearing the transferrin receptor and development of a circulating immature CD10 (CALLA) positive B cells.

This model of retrovirus-induced lymphoproliferative syndrome with immunodeficiency is sensitive to the antiretroviral agent, AZT. Doses used here are not toxic; AZT lowers virus titers by two to three orders of magnitude in spleen and lymph nodes at 4 wk of infections and induces a significantly longer survival time of 6 wk (19). During the first 8 wk most of the alterations found by FMF analyses are partially controlled by AZT. There was a trend for AZT to be more effective in delaying population changes in the spleen than in the lymph nodes. This may relate to the rate of phosphorylation of AZT to an active form in these two organs. The ability of drugs to prevent these early FMF alterations in the LP-BM5 model could prove useful in the initial screening of candidate antiretrovirus treatments. Further studies are underway to compare other nucleoside inhibitor drugs.

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