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Murine AIDS Is Initiated in the Lymph Nodes Draining the Site of Inoculation, and the Infected B Cells Influence T Cells Located at Distance, in Noninfected Organs

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The infection of cells which belong to the B-cell lineage is thought to be the primary event leading to the phenotypic and functional alterations seen in the murine AIDS (M. Huang, C. Simard, D. Kay, and P. Jolicoeur, J. Virol. 65:6562–6571, 1991). Using in situ hybridization, we studied the time course of the anatomic distribution of the murine AIDS-infected B cells in C57BL/6 mice inoculated intraperitoneally or in the foot pad with helper-free stocks of the defective murine AIDS virus. The local lymph nodes draining the injection site (the mediastinal or popliteal lymph nodes) were the primary organs in which infected B cells could be detected. From this initial site, the proliferating infected B cells were found to migrate progressively to most of the other lymph nodes and to the spleen. The bone marrow cells (containing the precursor B cells) were not found to be infected by the virus. These results suggest that the defective murine AIDS virus infects mature Ly-1$^-$ B cells present in lymph nodes. We compared the concanavalin A response of the T cells at an early time postinoculation, before all lymphoid organs are infiltrated with infected B cells. In lymphoid organs free of infected B cells, T cells were found to be hyperresponsive. In lymphoid organs in which infected B cells were present, T cells were hyporesponsive. These data suggest that infected B cells influence distant T cells, maybe by the release of a circulating factor or through another uninfected cell population activated by the infected B cells.

Murine AIDS is characterized by lymphadenopathy, splenomegaly, hypergammaglobulinemia, T- and B-cell dysfunctions, and late appearance of B-cell lymphomas and opportunistic infections (for reviews, see references 22, 31, and 33). The disease is caused by a defective murine retrovirus (3, 6) which encodes a Gag precursor protein, Pr60^{gag} (3, 6, 18). Mice depleted of B (5) or T (42) lymphoid cells have been reported to be resistant to the development of the disease. By using replication-competent stocks of the defective murine AIDS virus, in which helper murine leukemia viruses (MuLVs) (ecotropic and mink-cell focus-forming viruses) are present (2, 7, 12, 13, 29), it was shown that the development of the disease was progressive (14, 24, 34) and that the defective retroviral genome could be recovered with time from most of the lymphoid and nonlymphoid organs (7, 9, 19). In these tissues, various cell types of the immune system were reported to be infected by the defective virus, namely the B (7, 17, 24) and T (27, 40) lymphoid cells and the macrophages (4, 8). However, using the same replication-competent strain of the defective murine AIDS virus, Hitoshi et al. (17) reported that fewer than 1 in 10^4 sorted Ly-1$^+$ B220$^-$ lymph node cells (representing mostly T cells) were infected.

Using helper-free stocks of the defective murine AIDS virus, we have shown that the defective virus itself is pathogenic and that gene products encoded by the helper MuLVs are not required for the development of the syndrome (19). Using these helper-free virus preparations, we have also found that most of the cells infected by the defective virus belong to the B-cell lineage (21). Very few if any T cells are infected in this system, despite the fact that the majority of T cells are profoundly anergized (38a). In addition, the majority of the macrophages harvested from the peritoneal cavity or from the spleen of diseased mice inoculated with this virus were found not to be infected by Northern blot (RNA blot) analysis or in situ hybridization (unpublished results). The infected B cells were shown to expand clonally into the lymphoid tissues of diseased mice (19, 21), and their infection is likely the primary event leading to the phenotypic and functional alterations characteristic of murine AIDS (20, 21).

To gain more information about the initial response of these B cells to infection and about their characteristics, we studied the fate of this infected B-cell population from its primary site of infection and monitored its dissemination through the body. For this experiment, we used RNA probes which are specific and sensitive enough to detect murine AIDS virus-infected cells by in situ hybridization (21). We found that the first cells to be infected are present in the lymph nodes draining the site of inoculation, either the mediastinal lymph nodes after an intraperitoneal (i.p.) inoculation (30) or the popliteal lymph node after injection into the footpad. From these sites, they disseminate progressively to most of the lymphoid organs of the body. Taking advantage of the differential and progressive nature of this infected-cell dissemination, we could document an effect of the infected B cells on T cells located in organs not yet infected.

MATERIALS AND METHODS

Animals and viruses. Inbred C57BL/6 mice were purchased from Charles River Inc. (St-Constant, Quebec, Canada) and housed in the same environment during the experiments. Young (30- to 40-day-old) mice were inoculated once i.p. as described before (19–21) with 1.5 ml of the helper-free stocks

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of a defective murine AIDS virus, either wild-type Du5H (3) or chimeric Du5H/Mo-LTR, harboring long terminal repeat (LTR) sequences from Moloney MuLV (21). In one experiment, the mice received 0.2 ml of the chimeric Du5H/Mo-LTR virus in their right footpad or by the i.p. route. The titer of the chimeric Du5H/Mo-LTR virus stocks was determined as follows. Increased dilutions of the viral inoculum (from 10^0 to 10^{-6}) were used to infect NIH 3T3 cells. At 3 days postinfection, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline and hybridized in situ, as described below, with the ^{35}S-labeled specific Moloney MuLV U3 LTR RNA probes. Foci of infected cells were counted. The virus titer was 8.4×10^5 tissue culture infectious doses per ml.

Tissue samples. Mice were perfused at different time points (1, 2, 4, 7, 14, and 34 days postinfection) with 4% paraformaldehyde in phosphate-buffered saline. The selected organs were dissected and embedded in paraffin, as described previously (21, 23, 37). Bone marrow cells were flushed from the femurs with a 23-gauge needle. Bone marrow cells, T cells (E20/34), and NIH 3T3 cells expressing the chimeric Du5H/Mo-LTR defective virus were cytospotted on APES (aminopropyltriethoxysilane)-treated slides, as described previously (23, 37). The E20/34 cell line was established from a C57BL/6 mouse in which the defective murine AIDS and helper viruses were present. The NIH 3T3 cells contained the defective virus but no helper MuLV. Occasionally, fresh organs were excised, frozen, and stored at −80°C for later use.

Cell surface labeling and FACS analysis. Freshly dispersed cells from mediastinal lymph nodes were labeled with antibodies as described previously (36). Monoclonal antibodies Ra36B2 (murine anti-B220; kindly provided by R. Coffman, DNAX Research Institute of Cellular and Molecular Biology, Palo Alto, Calif.), HB58 (187.1; murine anti-C_κ; American Type Culture Collection, Rockville, Md.), and MAC-1 (Boehringer Mannheim Inc., Montreal, Quebec, Canada) were used in an indirect assay. The second antibody was fluorescein isothiocyanate (FITC)-conjugated anti-rat immunoglobulin G (IgG) (mouse adsorbed; Kirkeengaard and Perry Inc.). FITC-conjugated murine anti-CD3 antibody 145-2C11, kindly provided by R. P. Sekaly, Institute of Clinical Research, Montreal, Quebec, Canada, was used in a direct assay. Cells were analyzed by fluorescence-activated cell sorting (FACS) with a FACScan (Becton Dickinson).

T-cell response to ConA. Concanavalin A (ConA) assays were performed on dispersed lymph node or spleen cells as previously described (20, 39) except that 10^5 cells were used in each well. The medium was RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. Triplicates were done. The mean counts in the absence of ConA (background level) were subtracted from the mean counts in the presence of ConA. Maximal counts incorporated for each sample were used.

Probes. The D30 and C_κ antisense-specific riboprobes have been described elsewhere (3, 21). Two Moloney MuLV U3 LTR antisense-specific probes were used and mixed together. One, consisting of the 58-bp *Sau*96I U3 LTR fragment, has been described before (21); the other is a 75-bp *Nla*III-*Sau*96I fragment (bases 7875 to 7949 in the Moloney MuLV LTR U3 region [38]) cloned into pGEM-4. As shown and discussed previously (21), both probes are highly specific and do not detect signals from endogenous MuLVs.

In situ hybridization. Hybridization of paraffin-embedded tissues or cytospots of dispersed cells was performed as described previously (21, 37), with ^{35}S-labeled RNA probes.

RNA extraction and hybridization. Total RNA was extracted by the method of Chomczynski and Sacchi (10), separated on 1% formaldehyde–agarose gels, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled D30 probe, as described previously (19, 20).

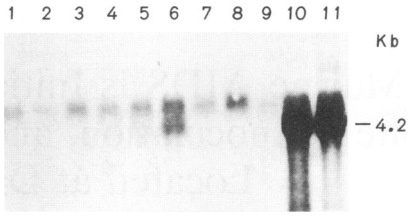

FIG. 1. Northern blot analysis of spleens of diseased mice inoculated with the helper-free defective murine AIDS virus. C57BL/6 mice, injected i.p. with helper-free stocks of the defective Du5H (wild-type) virus, were killed 2 (lanes 1 to 3), 5 (lanes 4 to 9), or 10 (lanes 10 and 11) days postinoculation. The 4.2-kb RNA species is specific to the defective virus, while the 5.2-kb RNA represents an endogenous species.

RESULTS

Fate of cells infected with the defective murine AIDS virus early in the course of the disease in C57BL/6 mice. To monitor the fate of cells infected by the defective murine AIDS virus, we used mice which had been inoculated with helper-free stocks of the defective virus. We have shown previously that, because virus reinfection does not occur in this system, the levels of defective viral RNA or DNA in the infected organs reflect the extent of proliferation of the infected cells (19–21). These infected cells have been shown before to belong to the B-cell lineage (21).

Our initial study was performed on total RNA from spleens of infected mice. Mice inoculated with the helper-free stock of the wild-type defective virus (Du5H) were killed 2, 5, and 10 days after i.p. inoculation. Total RNA was extracted from their spleen, and RNA from the defective virus was detected with the ^{32}P-labeled D30 probe. The specific 4.2-kb defective virus RNA transcript was detected as early as 5 days postinoculation in only one of the six C57BL/6 mice tested (Fig. 1, lane 6). However, at 10 days, the 4.2-kb RNA was strongly detected in both animals tested (Fig. 1, lanes 10 and 11). A dramatic increase in RNA expression occurred in the spleens over this period.

To follow the progression of the infected-cell population in more detail and especially at earlier times, we used a more sensitive assay, the in situ hybridization technique. For this experiment, the helper-free stock of the chimeric Du5H/Mo-LTR defective virus (21) was used. The genome of this virus is identical to that of the wild-type virus except that its LTR was exchanged for that of the Moloney MuLV. This chimeric virus was shown to induce a disease similar to the one induced by the wild-type virus and to target the same B-lymphoid cell population (21). The high specificity of the Moloney U3 LTR RNA probes allowed the detection of infected cells in the absence of signals from endogenous viral sequences, as shown previously (21). Table 1 summarizes the in situ hybridization data obtained from lymphoid organs of the C57BL/6 mice at different times after a single i.p. inoculation of the same virus lot. None of the control tissues from uninfected normal C57BL/6 mice were positive in situ. In addition, all the tissues labeled with the control sense probe at various time points were negative in situ (data not shown), a result confirming our previous results (21).

At 1 day post-virus inoculation, no infected cells could be detected in the lymphoid tissues of the inoculated mice.

TABLE 1. Detection of infected cells in lymphoid organs of mice inoculated with helper-free stocks of defective murine AIDS virus[a]

Lymphoid tissue	In situ hybridization signal[b] (no. of mice positive/total) on day postinoculation:					
	1	2	4	7	14	34
Spleen	−	−	+ (1/7)	+, ++ (6/6)	++, ++++ (5/5)	ND
Thymus	−	−	−	+ (1/7)	+ (3/5)	−
Peyer's patches	−	−	−	−	−	−
GALT	−	−	−	−	−	−
Cervical LN	−	−	−	+ (4/7)	+, ++ (5/6)	ND
Mediastinal LN	−	+, ++ (4/6)	++, +++ (6/6)	++, ++++ (7/7)	+++, ++++ (6/6)	ND
Brachial LN, left	−	−	−	−	++ (2/4)	ND
Brachial LN, right	−	−	−	+ (2/6)	+ (2/4)	ND
Inguinal LN, left	−	−	+ (1/7)	+ (5/6)	+, +++ (4/4)	ND
Inguinal LN, right	−	−	−	+ (3/6)	+, +++ (6/6)	ND
Mesenteric LN	−	−	+ (1/7)	+, ++ (5/5)	+, ++++ (6/6)	ND
Lumbar LN	−	−	−	+ (5/7)	+, ++ (5/5)	ND

[a] C57BL/6 mice were inoculated once i.p. with helper-free stocks of the chimeric Du5H/Mo-LTR defective murine AIDS virus. In situ hybridization was performed on tissue sections with the ^{35}S-labeled Moloney U3 LTR antisense RNA probe. LN, lymph node.

[b] ND, not done; −, no in situ-positive cells detected; + to ++++, very few (+, <1%) to very many (++++, >80%) in situ-positive cells detected. Numbers in parentheses represent the number of positive mice/total number of mice screened.

However, 2 days after i.p. inoculation, approximately 70% of the mice showed isolated positive cells or clusters of a few positive cells in at least one of the mediastinal lymph nodes (Fig. 2A and Table 1). At this time, none of the other lymphoid organs which were screened were infected (Fig. 2B to D, Table 1). The mediastinal lymph node chain was definitively the earliest organ in which in situ-positive cells could be detected. The expansion of the infected-cell population in this lymphoid organ progressed with time. In addition, these mediastinal nodes showed progressive enlargement and contained the highest number of infected cells.

In the spleen, in situ-positive cells were first detected at 4 days postinoculation in a minority (only one of seven) of inoculated C57BL/6 mice, and only a very few isolated positive cells were observed (Table 1). A stronger signal was detected in all the spleens (six of six) at day 7 postinoculation (Fig. 2L, Table 1). At this time, the positive cells were found mainly surrounding blood vessels of the white pulp, suggesting invasion by the bloodstream. At the same time, and with an equivalent intensity, positive infected cells were detected in both the cortex and medulla of the mesenteric lymph node (Fig. 2J, Table 1). At day 7 postinoculation, when numerous clusters of positive cells were found in the spleen and the mediastinal lymph nodes, a few positive cells began to be detected in the lumbar and superficial (cervical and inguinal) lymph nodes (Fig. 2K, Table 1). Positive infected cells appeared later in the brachial lymph nodes (Table 1).

Other lymphoid organs, such as the thymus, Peyer's patches, and gut-associated lymphoid tissues (GALT), were also evaluated. None of the 26 C57BL/6 mice studied showed any signal in the intestinal lymphoid tissues as late as 34 days after virus inoculation (Fig. 2S and T, Table 1). During this period, a few dispersed in situ-positive cells were occasionally seen in the thymus (4 of 30 mice studied) (Table 1).

We have reported previously that in mice with murine AIDS, infected B cells are occasionally detected in nonlymphoid organs (21). To determine whether these organs were infected at an early time after infection, numerous nonlymphoid tissues were also hybridized, including brain, lung, heart, liver, kidney, stomach, small intestines, bladder, uterus, testis, virgin mammary glands, skin, and leg muscles. At 14 and 34 days after virus inoculation, only some livers (3 of 12) and kidneys (2 of 12) showed positive cells, which were indistinguishable from lymphoid cells (Fig. 2Q).

Bone marrow is not a target organ for the defective virus. We have reported previously that the vast majority of cells infected with the defective murine AIDS virus belong to the B-cell lineage (21), but it is not known whether B-cell precursors are infected by this virus. Since B-cell precursors are found in the bone marrow (35), we looked for infected cells in the bone marrow during the early phase of the disease. Bone marrow cells from mice perfused on days 2, 7, and 34 postinoculation were obtained from the femurs and prepared for cytospots. No in situ-positive cells were detected among the bone marrow cells from any of the 12 C57BL/6 mice analyzed on days 2 and 7 postinoculation (Fig. 2U). However, a few positive cells were seen in two of six C57BL/6 mice on day 34 postinoculation (Fig. 2V).

These results indicate that the initial development of murine AIDS does not require infection of bone marrow cells and suggest that the B cells targeted by the virus are not precursor B cells.

Lymph nodes draining the site of inoculation show signs of infection first. As shown above, the mediastinal lymph nodes are the first tissues to contain cells infected with the defective murine AIDS virus shortly after an i.p. inoculation. This suggests that these lymph nodes have the unique property of containing the specific target B cells for this virus, although this is unlikely. Alternatively, it suggests that the murine AIDS virus targets a specific cell population of the peritoneal cavity, such as Ly-1$^+$ B cells (15). Since the mediastinal lymph nodes are the natural site of lymphatic drainage of the peritoneum (30), such infected cells would then migrate to these nodes. To check this hypothesis, we chose a different route of virus inoculation. In this experiment, seven C57BL/6 mice were inoculated with 0.2 ml of the helper-free stock of the defective murine AIDS virus in the right footpad. As controls, four mice were challenged with the same amount of virus given i.p. The mice were killed 12 days postinoculation. The right and the left popliteal lymph nodes and the mediastinal lymph nodes were measured and processed for in situ hybridization.

All mice inoculated by either route showed a significant increase in the size of the draining lymph nodes, the mediastinal nodes for mice inoculated i.p. and the right popliteal node for mice inoculated in the right footpad (Table 2, Fig. 3A, B, D, and E). In addition, when the animals were inoculated in the right footpad, in situ-positive cells were first detected in the right popliteal lymph node, not in the mediastinal or left

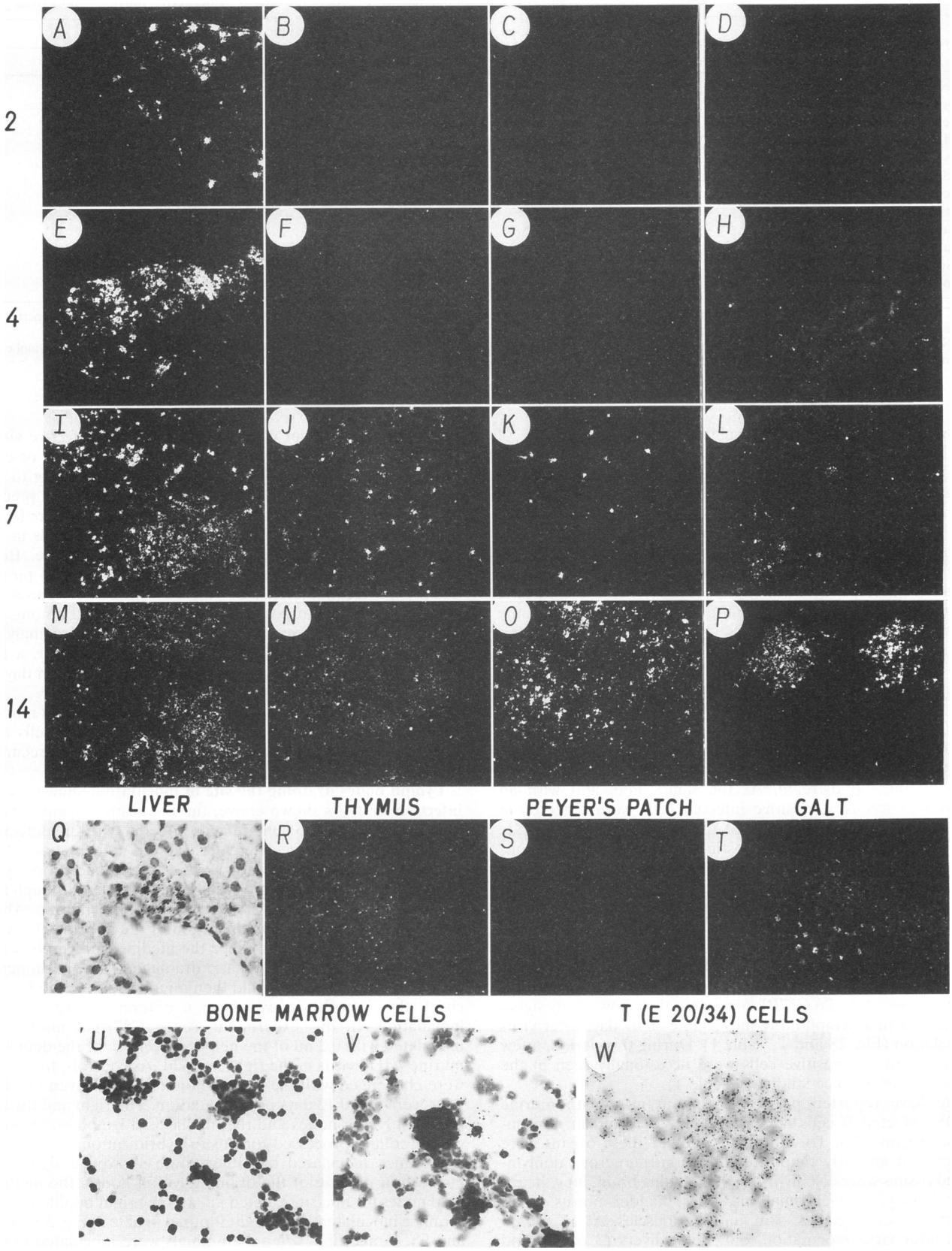
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TABLE 2. Effect of route of inoculation on expansion of draining lymph nodes[a]

Route of inoculation	Mouse no.	Lymph node size (mm^3)		
		Popliteal		Mediastinal
		Right	Left	
Footpad	1	14.1	0.5	11.9
	2	14.1	1.1	4.4
	3	11.5	0.5	16.2
	4	8.2	1.8	2.6
	5	8.2	0.5	5.6
	6	14.1	0.5	4.3
	7	9.2	0.5	7.8
	Mean ± SD	11.3 ± 2.8	0.8 ± 0.5	7.5 ± 4.9
Peritoneum	8	3.1	0.9	42.8
	9	1.1	1.1	29.2
	10	0.5	1.1	27.2
	11	0.5	0.5	32.6
	Mean ± SD	1.3 ± 1.2	0.9 ± 0.3	32.9 ± 6.9

[a] Young adult (30- to 40-day-old) mice were inoculated with helper-free stocks of the chimeric Du5H/Mo-LTR defective murine AIDS virus. A volume of 0.2 ml was inoculated into the right footpad or into the peritoneal cavity. Mice were killed 12 days postinoculation, and the lymph nodes were measured under a binocular microscope with a numeric grid to evaluate their size. Their volume was then calculated.

popliteal lymph node (Fig. 3A′ and B′). Conversely, when mice were inoculated i.p., the mediastinal lymph nodes were the first to harbor in situ-positive cells, whereas both popliteal lymph nodes were negative (Fig. 3D′ and E′).

To confirm that the infected cells from mice inoculated in the footpad were of B-cell origin, as we previously found for mice inoculated i.p. (21), adjacent sections of infiltrated spleen were hybridized with the virus-specific U3 LTR probe or with the C_κ probe. The C_κ probe was chosen because we have found previously that most infected B cells were C_κ positive (21). The virus-positive cell clusters were also C_κ^+ after infection in the footpad (Fig. 3C and C′), as they were after infection i.p. (21) (Fig. 3F and F′), confirming the B-cell origin of these infected cells, as shown previously (21). These results show that murine AIDS is initiated in the first lymph node to be infected and that its initial location is dependent on the site of inoculation and is related mostly to the local drainage of the lymph. Our data also suggest that the target B cells are distributed in all the peripheral lymph nodes of the mouse.

FACS analysis of mediastinal lymph node cells early in the course of murine AIDS. Several changes in the distribution of the hematopoietic cell populations as well as in the cell surface markers on these cells have been reported previously for mice with murine AIDS inoculated with helper-competent viruses (14, 25, 32). Because the mediastinal lymph nodes are the primary site of virus infection after an i.p. inoculation, we were interested in monitoring the lymphoid and macrophage populations early after inoculation of the helper-free defective murine AIDS virus. This analysis was done at 3 and 6 days postinoculation by FACS analysis with monoclonal antibodies against cell surface antigens specific for B (C_κ and B220), T (CD3), and macrophage (Mac-1) cells on mediastinal lymph node cells from mice inoculated with helper-free stocks of the defective virus. During the first week postinfection, the percentages of $CD3^+$ T cells and $Mac\text{-}1^+$ macrophages up to 6 days postinoculation were quite stable and did not show significant variations (Fig. 4G to J, Table 3). However, the percentage of C_κ^+ and $B220^+$ B cells decreased with time (Fig. 4A to C and 4D to F, respectively; Table 3). By 6 days postinoculation, the $B220^+$ cell population represented only 60% of that in the negative uninfected mice.

These results suggest that the proliferating infected B cells downregulate their C_κ and B220 cell surface markers. In addition, it is possible that some uninfected B cells downregulate their B220 level, but we have not addressed this issue in the present experiment.

Functional assay of T cells at early stages of murine AIDS. It has been reported previously that the T-cell response to ConA, a specific T-cell mitogen, was much impaired in mice with murine AIDS (28, 34). However, these assays were performed on lymphoid tissues containing a mixture of infected and noninfected cells. To determine whether infected B cells could influence the noninfected T cells at a distance, we compared the ConA response of T cells at an early time post-inoculation (<1 week) in lymphoid tissues containing infected B cells and in those not yet infiltrated with infected B cells. To choose these organs, we took advantage of our previous observation of the sequential dissemination of infected B cells from the lymph nodes draining the site of inoculation in C57BL/6 mice inoculated with helper-free stocks of the defective murine AIDS virus. To confirm the presence or absence of the infected cells in the tested organs, Northern blot analysis (for spleens) or in situ hybridization (for lymph nodes) was performed.

In most organs in which infected cells were detected, a modest decrease (compared with the control) in the ConA response of T cells was detectable as early as 4 days postinoculation (Fig. 5), confirming previous results. Interestingly, in most of the organs free of infected cells that were tested, specifically in mesenteric lymph node and spleen cells, a significant increase (compared with the control) in the ConA response of T cells was observed (Fig. 5). These effects were independent of the route of inoculation. These results suggest that the infected B cells are capable of influencing noninfected T cells not only when both populations are located in proximity to each other, but also when they are distant from each other.

FIG. 2. Detection by in situ hybridization of infected cells in organs of diseased C57BL/6 mice inoculated with the helper-free defective murine AIDS virus. Mice inoculated i.p. with helper-free stocks of the defective chimeric Du5H/Mo-LTR virus were killed 2, 4, 7, or 14 days after inoculation. In situ hybridization was performed with ^{35}S-labeled Moloney MuLV U3 LTR-specific antisense RNA probes on tissue sections or cytospots, as described in Materials and Methods. Mediastinal, mesenteric, and inguinal lymph nodes (LN) and spleens were studied (panels A through P). An increased percentage of in situ-positive cells was seen with time (from 2 to 14 days postinoculation). (Q) Liver, 14 days postinoculation; note the focal invasion of infected lymphoid B cells. (R to T) Representative thymus (R), Peyer's patch (S), and GALT (T) from a diseased mouse killed at 14 days postinoculation; in situ-positive cells were not detected in these lymphoid organs. The few white dots observed represent background or overstained cellular structures. (U and V) Bone marrow cells harvested from diseased mice at 2 (U) or 34 (V) days after inoculation. (W) Established E20/34 T cell line expressing the chimeric Du5H/Mo-LTR defective murine AIDS virus, used as a positive control. Bright-field (Q and U to W) and dark-field (A to P and R to T) representations are shown. The counterstain was hematoxylin and eosin. Magnification: ×65 (A to P and R to T) or ×157 (Q and U to W).

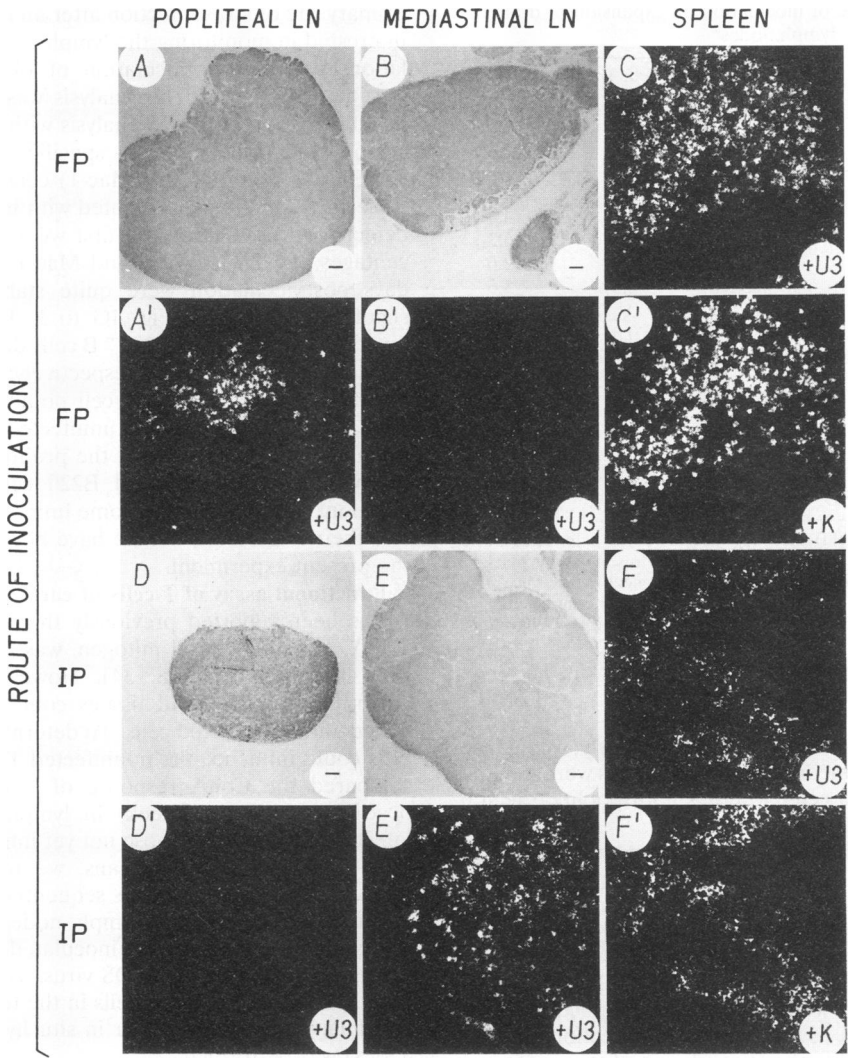

FIG. 3. Detection by in situ hybridization of infected cells in draining lymph nodes (LN) of C57BL/6 mice inoculated with the helper-free defective virus. Helper-free stocks of the defective chimeric Du5H/Mo-LTR virus were injected into the right footpad (FP) (panels A to C and A′ to C′) or intraperitoneally (IP) (panels D to F and D′ to F′). Mice were killed at 12 days postinoculation, and in situ hybridization was either not performed (−) or performed (+) with ^{35}S-labeled Moloney MuLV U3 LTR-specific antisense RNA probes as described in Materials and Methods. The right popliteal and mediastinal lymph nodes and the spleens are represented at lower (×20; A, B, D, and E) or higher (×80; A′ to F′, C, and F) magnification. (C′ and F′) Adjacent sections of spleens shown in C and F, respectively, hybridized with the ^{35}S-labeled C_κ-specific antisense RNA probe. Note the colocalization of in situ-positive cells detected with both probes: compare C and C′ or F and F′. Bright (A, B, D, and E) or dark (A′ to F′, C, and F) fields are shown. The counterstain was hematoxylin and eosin.

DISCUSSION

In murine AIDS, the expansion of the lymphoid organs results from the accumulation of various cell types, such as T and B lymphocytes and macrophages (14, 25, 28, 32). Using helper-free stocks of the defective murine AIDS virus, which prevent infected cells from releasing viruses and therefore prevent reinfection cycles, we have shown previously that the enlarged organs contain clones of infected cells that vary in size (19, 21). Most of these infected target cells were shown to belong to the B-cell lineage (21), not the T-cell lineage (38a). In the present study, we extended our knowledge about this infected B-cell population by monitoring its dissemination to the whole body from the initial site of infection with the same helper-free stocks of the defective murine AIDS virus. The very specific and sensitive in situ hybridization technique was used to detect the infected cells in their anatomic locations. We found that the first lymphoid organ to be infected by the defective virus was the local lymph node draining the injected site, namely the mediastinal lymph nodes for the peritoneum (30) and the popliteal lymph node for the footpad.

During the first few days after infection, we observed the proliferation (as small foci of clonal growth) and sequestration of the infected cells into the draining lymph nodes, which also enlarged. Between days 4 and 7 after i.p. inoculation, the infected B cells start to migrate to other lymph nodes and the spleen, and most of the lymphoid organs become infiltrated in a rather specific order. The spleen and the mesenteric lymph nodes were colonized at about the same time, followed by the superficial lymph nodes. However, other organs rich in B cells (such as the Peyer's patches and the GALT) and rich in T cells

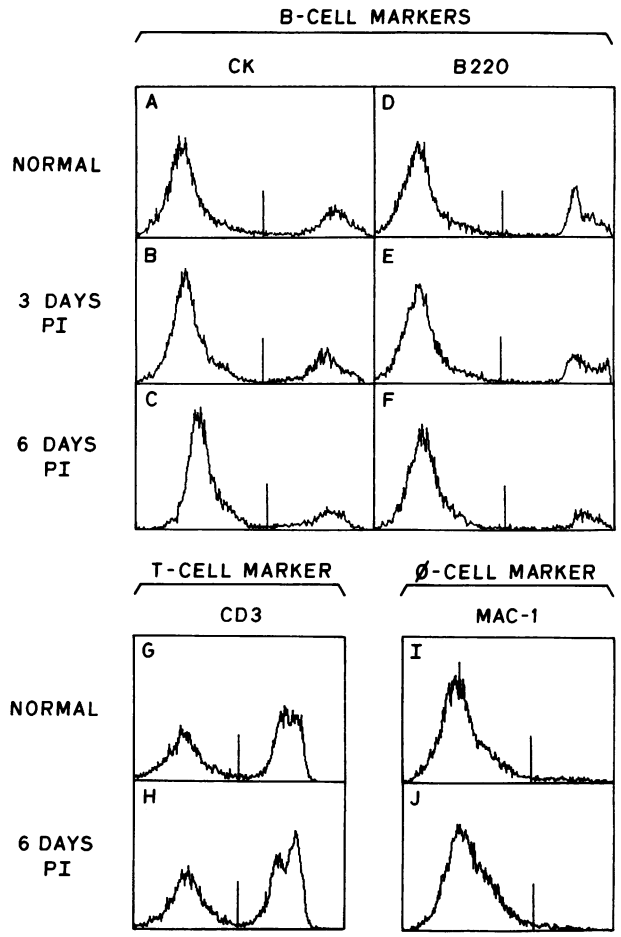

FIG. 4. FACS analysis of mediastinal lymph node cells from C57BL/6 mice inoculated i.p. with the defective murine AIDS virus. Uninoculated mice or mice injected with helper-free stocks of the chimeric Du5H/Mo-LTR defective virus were killed 3 or 6 days postinoculation (PI). The mediastinal lymph nodes were collected, and FACS analysis was performed with monoclonal antibodies specific for B-cell (C_κ and B220), T-cell (CD3), and macrophage markers as described in Materials and Methods. The vertical bar indicates the lower limit of positivity, as determined by labeling of the same cells with the second antibody (FITC-conjugated goat anti-rat IgG).

(such as the thymus) remained free of infected cells during the first month after an i.p. inoculation. Therefore, it seems quite unlikely that the Peyer's patches play a role in murine AIDS, as suggested previously (27). In addition, infection of thymocytes does not appear to be required for the proliferation of the infected B cells. In previous studies with replication-competent stocks of the defective murine AIDS virus, the T cells were found to be infected by the virus, and T-cell lymphomas were found to arise in a relatively high percentage of diseased mice (27, 40). However, using replication-deficient helper-free stocks of the defective virus, we recently found that less than 0.001% of $CD3^+$ or Thy-1^+ T cells are infected by the defective virus (38a), confirming the data obtained in the present study.

TABLE 3. FACS profile of mediastinal lymph node cell population early after i.p. inoculation of helper-free chimeric Du5H/Mo-LTR defective murine AIDS virus

Day post-inoculation	No. of mice	% of cells			
		C_κ^+	$B220^+$	$CD3^+$	Mac-1^+
0	3	26.4 ± 4.4	29.5 ± 4.2	52.2 ± 0.9	5.4 ± 0.1
3	2	27.0 ± 2.3	23.7 ± 2.8	ND[a]	4.0 ± 0.8
6	2	21.7 ± 4.8	17.4 ± 1.4	49.8 ± 8.7	3.5 ± 0.1

[a] ND, not determined.

Although very few if any T cells are infected by the defective murine AIDS virus inoculated as helper-free stocks, they seem to be very affected by the infected B cells, even when those are located far away. Taking advantage of the progressive nature of infected-B-cell dissemination and the absence of infected B cells in some lymphoid organs (mesenteric lymph nodes or spleen) early after an i.p. or footpad injection, we studied the ConA stimulation response of T cells obtained from organs free of infected B cells. T cells from the mesenteric lymph node and from the spleen were found to be hyperactivated, whereas T cells from the mediastinal lymph nodes (containing infected B cells) were less reactive, suggesting that a circulating factor or another uninfected cell population was affecting the activity of these distant T cells. This putative factor may be released by the infected B cells or by another uninfected cell population activated by the infected B cells. Later, at 10 days postinoculation, when infected B cells were present in the mesenteric lymph nodes and in the spleen, hyporesponsiveness of T cells was also observed in these lymphoid tissues, suggesting that the proximity of the infected B cells is required to decrease T-cell functions. Alternatively, T-cell hyporesponse may be the consequence of the initial hyperresponsiveness, as reported for T cells in another context (16).

Although the present experiments were not designed to study the nature of the cells infected by the defective murine AIDS virus, they confirm and extend our previous data identifying these cells as belonging to the B-cell lineage. Our present results indicate that infection of a particular cell population of the peritoneal cavity is not required for the development of murine AIDS, since a similar expansion of the infected B cells occurs after footpad inoculation. The peritoneal cavity is known to harbor numerous cell types of the immune system, including macrophages and T and B lymphocytes (41). A specific subset of B cells, Ly-1^+ B cells, is present in this site in large amounts in adult mice (10 to 40% of total cells), in contrast to adult spleen (1 to 2%) and lymph nodes (undetectable) (15). With replication-competent stocks of the defective murine AIDS virus used for inoculation, it was reported previously that the Ly-1^+ B-cell population was infected by the defective virus (17) and that B-cell lines established from mice with murine AIDS inoculated with a similar virus mixture were Ly-1^+ (24). In contrast, with replication-incompetent helper-free stocks of the defective virus used for inoculation, we found no evidence for infection of Ly-1^+ B cells.

We observed that the peripheral (popliteal) lymph nodes were highly susceptible to infection by the defective murine AIDS virus, despite the fact that in normal mice, Ly-1^+ B cells are not usually detected in the lymph nodes (15). In addition, we have recently established, from a mouse infected with helper-free stocks of the defective virus, a nonproducer B-cell line expressing the defective murine AIDS virus $Pr60^{gag}$ protein, which is Ly-1^- (39a). Together, these data suggest that the infection of Ly-1^- B cells by the defective virus is sufficient to lead to the initiation of murine AIDS. When a replication-competent virus is present, it appears that numerous other cell types, including T cells (27, 40), Ly-1^+ B cells (17, 24), and macrophages (4, 8), can also be infected, as expected. How-

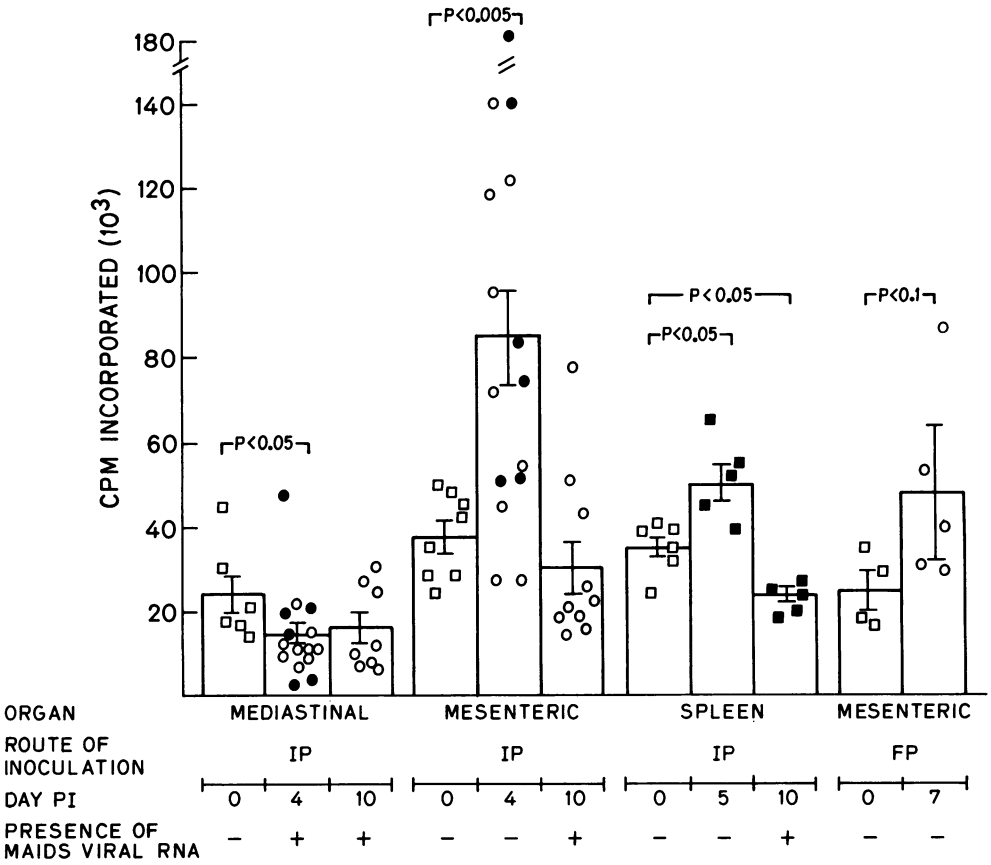

FIG. 5. ConA response of T lymphocytes from lymphoid organs of mice injected with the defective murine AIDS virus. Cells from spleens and from mesenteric and mediastinal lymph nodes (LN) of C57BL/6 mice inoculated i.p. or in the right footpad (FP) with tissue culture medium (□) or with helper-free stocks of the wild-type Du5H (■) or the chimeric Du5H/Mo-LTR (○, experiment 1; ●, experiment 2) defective virus were dispersed into RPMI 1640–10% fetal calf serum with antibiotics. ConA assays were performed as described in Materials and Methods. Values are represented for each mouse analyzed. The bars represent the mean and the standard error of the mean for each group. Wilcoxon-Mann-Whitney (two-tailed) tests were performed for each group. Significant differences between groups are shown above the respective bars. In situ hybridization with the ^{35}S-labeled Moloney U3 LTR-specific antisense RNA probe or Northern analysis was used to detect the presence of defective viral RNA in the dispersed lymph nodes or spleen cells, respectively.

ever, it is not clear which of these infected cell populations, if any, contribute to the pathogenesis of murine AIDS.

Our results also suggest that the target B cells of the defective murine AIDS virus are not immature B-cell precursors. The immature B cells are located in the bone marrow and are not normally seen in the peripheral lymphoid tissues (35). The bone marrow cells of the mice inoculated with helper-free stocks of the defective virus were found not to be infected early after inoculation of the virus. The few in situ-positive cells detected later (34 days postinoculation) had most likely migrated from the periphery. In addition, we found that lymph nodes draining two different sites of inoculation were highly infected soon after virus inoculation. These lymph nodes are known to harbor a high percentage of mature B cells. In a previous study, Cerny et al. (5) showed that mice treated with antibodies directed against surface IgM (a treatment which eliminates mature B cells) were resistant to murine AIDS. Moreover, we have previously shown that cells infected with the defective murine AIDS virus exhibited C_κ and J_H gene rearrangements (21), a phenotype of mature B cells. Therefore, it seems likely that the target cells of the defective virus are mature B cells.

However, these infected mature B cells in mice with murine AIDS appear to have a different phenotype than the majority of uninfected mature B cells. We found that, despite the fact that they express high levels of C_κ RNA (21), they are expressing low levels of the C_κ and B220 antigens in the cytoplasm (21), confirming earlier observations on the expansion on dull C_κ^+ and dull $B220^+$ B cells in lymphoid organs of mice with murine AIDS (14, 25). In addition, we have occasionally found that some of these infected B cells have a C_κ rearrangement in the absence of a J_H rearrangement (21), a phenotype initially observed in a small fraction of Abelson virus-transformed B-cell lines (1) and in human pre-B cells transformed by the Epstein-Barr virus (26), which appears to be the phenotype of a subset of normal mature B cells (11).

Together, the present and previous results indicate that the defective murine AIDS virus directly infects Ly-1^- mature (rearranged C_κ and/or J_H) B cells present in the draining lymph nodes. This infection leads to the oligoclonal proliferation of the infected B cells, to a downregulation of their C_κ and B220 cell surface proteins, and to the production of a factor(s) which directly or indirectly anergizes uninfected T cells.

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