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Induction of lymphoma in athymic mice: A model for study of the human disease

(etiology/immunodeficiency/antigenic stimulation/lymphomagenesis/oncornaviruses)

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ABSTRACT **A murine lymphoma, designated L1, was produced in immunologically deficient *nude* mice after chronic antigenic stimulation by infection with the pinworms *Aspiculuris tetraptera* and *Syphacia obvelata*. *In vivo*, L1 involves primarily the spleen and lymph nodes, with infiltration of liver, kidney, and bone marrow also observed. It is characterized by large clusters of B cells and null cells, and by rare T cells. The lymphoma cells express murine leukemia virus antigens (gp70 and p30) on the surface. L1 can be passaged successfully both *in vivo* and *in vitro*. The lymphoblasts that proliferate *in vitro* are null, but injection back into the mouse produces a similar pattern of B cells, null cells, and occasional T cells as seen in the mouse-to-mouse transfers. Infectious viruses have been isolated from L1 cells and from tissue culture supernates and have been identified as a B-tropic murine leukemia virus and a xenotropic virus. The possibilities of this model for studying the etiology of human lymphoma are discussed.**

There is evidence (1) that some human lymphomas are abnormal responses to persistent antigenic stimulation and that they arise from situations related to immune deficiency states. Fraumeni and Hoover (2) have shown that lymphoproliferative neoplasms predominate in specific groups of diseases, most notably in renal transplant recipients treated with immunosuppressive agents and in patients with primary immunodeficiency syndromes. Gleichmann *et al.* (3) have suggested that human immunoblastic disorders may be mediated by stimulatory reactions of T lymphocytes towards other lymphocytes and macrophages whose membranes have been rendered histoincompatible by certain antigens.

We have previously shown (4) that pinworm infection of athymic mice leads to a change in the immune status of the mice. Proliferation of both T and B lymphocytes and formation of occasional germinal centers are noted, apparently without benefit of a thymus. These mice will synthesize antibody to sheep erythrocytes, a T-dependent antigen.

We have observed a high incidence of lymphomas in athymic mice that were subjected to chronic antigenic stimulation of the immune system, either by continued pinworm infection or by grafts of foreign tissue such as human tumor (nonlymphoma) transplants. In this paper we will present the history and partial characterization of one of these lymphomas, designated L1, and discuss the possibilities of it as a model for lymphoma in the athymic mouse that may lead to a further understanding of the etiology of human lymphoma.

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MATERIALS AND METHODS

Mice. BALB/c heterozygous (*nu*/+) and homozygous (*nu*/*nu*) mice were bred under pathogen-free conditions in the Athymic Mouse Research Center of this laboratory.

Histology. For light microscopy the organs to be studied were fixed in 10% (vol/vol) neutral buffered formalin. Paraffin sections were prepared and stained with hematoxylin and eosin.

Immunofluorescence Studies. Anti-T cell serum was prepared in rabbits by multiple injections of mouse thymocytes, absorbed with the cloned mouse tissue culture line RAW 307 (a non-T, non-B lymphoblastoid line), then further absorbed with normal *nude* mouse spleen cells. Anti-B cell serum was prepared by the *Bordetella pertussis* method (5). Antiserum to murine leukemia virus (MuLV) was prepared in rabbits by repeated injections of purified Abelson MuLV from RAW 307 cells (5). For staining, slides were incubated with these antisera at 1:50 dilution at 37°C for 30 min. After washing with phosphate-buffered saline, fluorescein-conjugated antiserum (goat anti-rabbit IgG, Cappel Laboratories, Cochranville, PA) was added to the slides at 1:8 dilution, and the slides were incubated and washed as before. They were examined by using a Zeiss photomicroscope with appropriate fluorescence filters.

Immune Precipitation. ^{125}I-Labeled cell surface lysates were mixed with antisera and precipitated before electrophoresis as described (5).

Tissue Culture. To initiate primary cultures of lymphoma cells, spleens from mice exhibiting signs of illness were aseptically removed and cut into small pieces. They were then incubated in Falcon plastic flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in an atmosphere of 95% air/5% CO_2. Medium also contained penicillin, ampicillin, and streptomycin. After 48 hr, when adherent cells had attached to the bottom of the flask, medium was changed by removing part of the used medium and replacing it with fresh medium. Thereafter the lymphoblasts that grew out were passaged in this way or removed and placed on a new adherent layer of fibroblasts, macrophages, or both.

Mouse-to-Mouse Transfers of Proliferating Spleen Cells. Single-cell suspensions were made from the enlarged spleens of mice that had been immunologically stimulated. Sixty million cells were inoculated intravenously in the tail vein of recipient mice in 0.1 ml of phosphate-buffered saline.

Virus Isolations. L1 lymphoma cells or filtered supernates from L1 cells propagated *in vitro* were inoculated into secon-

Abbreviation: MuLV, murine leukemia virus.

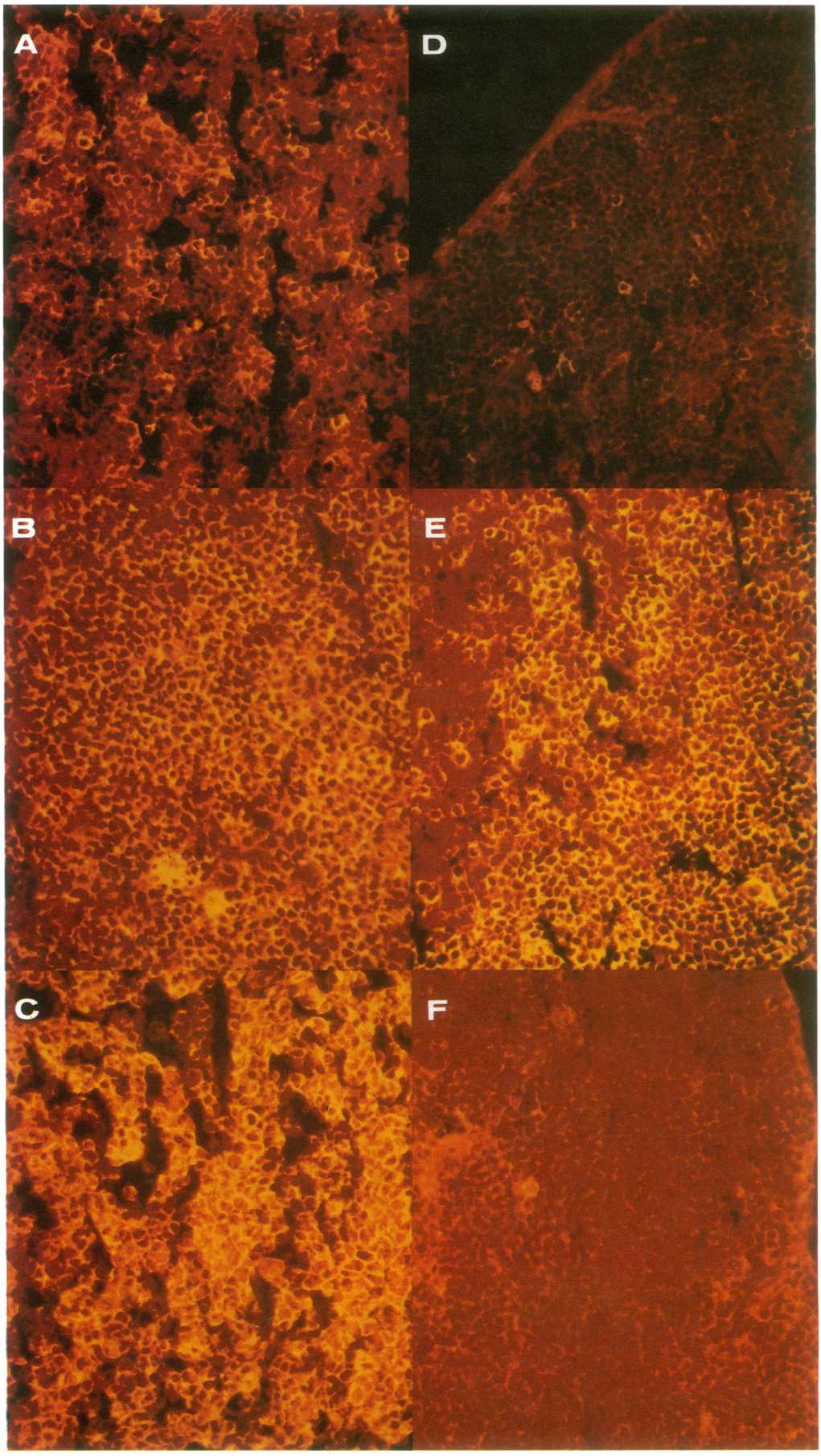

FIG. 1. (*Legend appears at the bottom of the next page.*)

Table 1. Comparison of values from control mice and those inoculated with lymphoma*

Measured value	Tissue	Normal control mice	Mice inoculated with lymphoma	Increase relative to control	Reference for method
No. leukocytes/ml	Blood	13×10^6	136×10^6	10	
IgG, mg/ml	Serum	0	0	—	
Lactate dehydrogenase, units/ml	Serum	2.1	7.19	3.4	6
Viral protein gp70, ng/ml	Serum	906	16,646	18	7
Viral protein p30, ng/ml	Serum	300	6,383	21	7
No. nucleated cells	Spleen	0.2×10^9	1.7×10^9	8.5	
Lactate dehydrogenase, units/mg protein	Spleen	1.07	7.7	7.2	6
Weight, g	Spleen	0.11	0.88	8	
Weight, g	Liver	1.53	3.47	2.3	
Weight, g	Total body	25.7	21.6	0.84	

* Eight days after inoculation of 25×10^6 spleen cells from donor mouse.

dary monolayers of NIH Swiss and BALB/c fibroblasts for the isolation and typing of the ecotropic MuLVs. Mink lung CCL 64, mink embryo fibroblasts, and rabbit endothelial cells were used for the isolation of xenotropic MuLV and for envelope gene recombinants.

RESULTS

Lymphoma L1 resulted from inoculation of a cell suspension prepared from an enlarged spleen from a mouse that had been infected with pinworms (*Aspiculuris tetraptera* and *Syphacia obvelata*) for 6 months. Eight days after inoculation, the recipient mouse exhibited a dramatic wasting, hind leg paralysis, and measles-like rash. On autopsy the spleen and lymph nodes appeared greatly enlarged. Histology of the tissues revealed extensive infiltration of lymphoblasts in the spleen, lymph nodes, liver, kidney, and bone marrow. Subsequent transfers *in vivo* showed similar patterns of involvement. Immunofluorescence of serial frozen sections of the lymphoma in the lymph node showed rare single T cells, large clusters of B cells, and clusters of null cells (Fig. 1 *A* and *B*). Almost all cells expressed MuLV antigens (Fig. 1*C*). Fig. 1 *D–F* depicts control immunofluorescence of normal lymphoid tissue in the athymic mouse with anti-T, anti-B, and anti-MuLV antisera.

Table 1 shows a comparison of organs and sera from normal healthy control athymic mice and their littermates, 6 days after *in vivo* passage of lymphoma by inoculation of 25×10^6 cells from spleen of donor mouse. The leukocyte count increased 10-fold; the serum lactate dehydrogenase was elevated 3.4-fold over control; and the viral proteins gp70 and p30 were elevated 18- and 21-fold, respectively. As in controls, there appears to be no IgG secreted in the serum. The spleen weight and nucleated cell number increased 8-fold; the cellular levels of lactate dehydrogenase in the spleen were elevated 7-fold.

Attempts to grow the lymphoma in tissue culture resulted in the eventual proliferation of lymphoblasts that expressed no T or B antigens on the cell surface or intracytoplasmically. However, MuLV antigens were expressed. These null lymphoblasts would not grow autonomously, but required a feeder layer of adherent cells for growth. Both fibroblasts and macrophages promoted growth.

When these null lymphoma cells were injected back into the mice, similar profiles of organ involvement were seen as in the mouse-to-mouse transfers. Immunofluorescent staining of frozen sections of the lymphoma again revealed a few single T cells, but mostly clusters of null cells and B cells. Again, most cells expressed MuLV antigens.

Infectious virus has been isolated from L1 cells and from supernates. The ecotropic MuLV has been typed as B-tropic, and a xenotropic MuLV was also isolated.

Heterozygote siblings of the athymic mice were much more resistant to tumor development after inoculation of the lymphoma cells and did not always succumb, in contrast to the athymic mice, whose mortality rate was 100% with as few as 100 cells per inoculum. Of heterozygotes inoculated with 10^3–10^5 cells, 34% did not succumb (Table 2). Histological samples from the heterozygotes that appeared to be unaffected by the inoculum of lymphoma cells exhibited no signs of lymphoma. Their lymphoid tissues were hyperplastic but showed no evidence of abnormal growth. There appeared to be little relationship between the size of inoculum and mortality.

DISCUSSION

Antigenic stimulation of the immune system of athymic mice leads to a proliferation of functional T and B lymphocytes (4). A high incidence of spontaneous lymphoma in athymic mice has been documented. Outzen *et al.* (8) have reported a higher incidence of spontaneous lymphomas in athymic mice housed in germfree conditions than in those housed conventionally. Their mice were germfree, but not necessarily antigen free. Parker *et al.* (9) have reported that the method of housing appeared not to influence the incidence. Both groups agree that there is a virtual absence of similar tumors in the heterozygote littermates. Induction of murine B-cell lymphomas by injection of Crohn disease lymph node homogenates in athymic mice and by hyperimmunization in a new "double congenic" strain of mice, B10 H-2^a H-4^b p/wts, has been reported (10, 11).

Germfree conditions should not be equated with antigen-free conditions. We believe that the high incidence of lymphomas in athymic mice on the BALB/c background is related to antigenic stimulation of an abnormal immune system. If this is the case, we believe that we have a model for studying the etiology of lymphoma. In addition to L1, the history of which is presented in this paper, we have several more lymphomas that have arisen in animals undergoing antigenic stimulation by human tumor xenografts as well as pinworm infection. Preliminary observations indicate that there is a variation in the

FIG. 1. (*on preceding page*). (*A–C*) Serial frozen sections of diffuse cortex of a lymph node from an athymic mouse with lymphoma involvement. Sections were stained with anti-T antiserum (*A*), anti-B antiserum (*B*), or anti-MuLV antiserum (*C*). Most cells stained (yellow) with anti-B and anti-MuLV antisera. There are a few scattered cells stained with anti-T antiserum. (*D–F*) Frozen sections of normal athymic lymphoid tissue stained with anti-T antiserum (*D*), anti-B antiserum (*E*), or anti-MuLV antiserum (*F*). There was normal staining in the B-dependent follicles, a few single T cells in the cortex, normally seen in *nude* mice, and no staining with anti-MuLV antiserum. (×190.)

Table 2. Mortality in heterozygote and homozygote athymic mice inoculated with 10^3–10^5 L1 lymphoma cells

Genotype	No. of cells injected	No. of mice	% mortality*
nu/+	10^5	5	66
nu/nu	10^5	5	100
nu/+	10^4	16	66
nu/nu	10^4	16	100
nu/+	10^3	12	67
nu/nu	10^3	12	100

* At termination of experiment 43 days after inoculation.

cell type involved. Study of the prelymphoma stages of lymphoproliferation and the activation of endogenous leukemogenic viruses should further clarify the sequence of events that leads to lymphomagenesis.

At this time we have no evidence that the lymphomas we have observed were induced by the viruses found in the proliferating cells. Whether or not these viruses reproduce a similar disease in normal and antigenically stimulated athymic mice as well as their heterozygote litter mates remains to be investigated. If the virus seems to be the final pathway to lymphomagenesis, then the role of antigenic stimulation in an abnormal immune system leading to activation of endogenous oncornavirus will be interesting to pursue. If the virus appears to merely be a passenger, then we must try to explain lymphomagenesis as a product of antigenic stimulation of an abnormal immune system in the absence of any other "transforming" signal. We hope that this model will give us a further understanding of the events that lead to lymphomagenesis in humans.

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