Histoproliferative Effect of Rauscher Leukemia Virus on Lymphatic Tissue. II. Antigen-Stimulated Germfree and Conventional BALB/c Mice[1] (34959)

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(Introduced by C. C. Congdon)

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Depressed immune capacity associated with murine leukemia virus infection, especially of the Friend or Rauscher type, is correlated with the selective ability of oncogenic viruses to replicate in immunologically competent progenitor cells (1–3). Our previous histologic and ultrastructural studies using a Rauscher leukemia virus (RLV) preparation in BALB/c mice strongly suggested that one of the earliest detectable tissue sites of C-type virus replication in immunocompetent progenitor cells occurs in the nonthymus-dependent areas of spleen lymphatic nodules and lymph nodes, particularly in the germinal centers (4, 5). This site of C-type virus replication was observed within the first day after injection and was associated with the capacity of antigen-trapping reticular cells to localize the virus extracellularly in close proximity to the parenchymal immunoblasts of this region of the nodule. This initial localization of the virus is attributed primarily to its antigenic and not necessarily to its biologic qualities. As a consequence of this morphologic study it was interpreted that a selective replication of the virus in proliferating cells of the nonthymus-dependent region of lymphoid follicles (specifically germinal centers) causes lymphoblastosis. Further, this involvement of nonthymus-dependent lymphoid tissue is an early and essential aspect of the splenomegaly associated with Rauscher disease.

The purpose of the present study was to test the above interpretation by injecting RLV into germfree mice; before infection, these mice had been devoid of germinal centers because of their low state of immunologic activity. Previous studies with germfree and conventional mice injected with ^{125}I-labeled human gamma globulin have demonstrated that antigen trapping and localization occur in the nonthymus-dependent regions of the follicles, with the subsequent accumulation and proliferation of immunologically competent progenitor cells in this region. This response ultimately results in the development of recognizable germinal centers (6–8). In the germfree mice, however, this development takes several days and corresponds to the growth phase of the serum antibody response. Thus the germfree mice make a good test sytem for study in the role of the nonthymus-dependent, humoral antibody mechanism in RLV infection; since we previously demonstrated that upon antigenic stimulation the primary immune response in germfree mice, in terms of antigen elimination and overall serum agglutinin production as well as proliferative response in germinal centers, is quantitatively superior to that in comparably stimulated conventional mice (6). Further, this enhanced proliferative response of immunoblasts in the *de novo* formation of germinal centers could account for the increased susceptibility to Friend virus in germfree IR Swiss mice, demonstrated by Mirand and Grace (9).

Thus, it was considered that the splenomegaly of Rauscher disease within a germfree population would be further augmented by the establishment of active germinal centers

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in mice previously immunized with sterile sheep erythrocytes. Similar comparisons were also made in conventional BALB/c mice. The results of these studies demonstrate the necessary, if not essential, role of the antigen-retaining reticular cells and immunoblasts of germinal centers in the lymphoblastosis and splenomegaly of Rauscher disease. In addition, morphologic results strongly suggest the differential effect of the Rauscher preparation on the thymus- and nonthymus-dependent regions of the lymphatic tissue.

Materials and Methods. Mice. The inbred germfree BALB/c mice used in these experiments were obtained from Life Sciences, Inc., St. Petersburg, Florida, through the courtesy of Dr. W. Farrow of Life Sciences, Inc., and Dr. Ray W. Bryan of the National Cancer Institute. The mice were received as germfree weanlings. On receipt they were transferred from their shipping containers to sterile plastic-film isolators of the Trexler type and given sterile food and water *ad libitum*. Diet was Purina laboratory chow (5010c) sterilized as a wet mash to minimize vitamin loss. Prior to use in the experiments, several mice and composite fecal samples were examined for aerobic and anaerobic bacteria, fungi, and mycoplasma. All cultures were negative. No serological tests for viruses were performed on these mice *per se*, but the colony from which the mice were received has been repeatedly found negative for the common infectious murine viruses.

Conventional BALB/c mice used in these experiments were specific-pathogen-free animals. The mice were housed 10 to a plastic pan and were maintained under filter tops in a conventional animal facility.

Virus. Rauscher leukemia virus Lot No. P-424 was originally obtained from Dr. F. J. Rauscher of the National Cancer Institute. The virus was serially passaged in BALB/c mice. The preparations used in this study were 10% extracts of leukemic spleen tissues in Tyrode's solution, with 500 units/ml of penicillin and streptomycin. The germfree mice were infected with approximately 50,000 ID_{50} as determined by the spleen weight assay (10). The conventional BALB/c animals were injected with approximately 10,000 ID_{50}. As determined in preliminary studies, lactic dehydrogenase virus (LDV) is one of the known passenger viruses in these preparations. LDV was originally obtained from Dr. Albert Notkins (National Institute of Dental Research). The preparations used in this study consisted of pooled sera from BALB/c mice injected 24 hr previously with approximately 10^8 ID_{50} of LDV; these mice were from a colony previously tested for and found free of the common murine viruses. The LDV titer of such preparations, as determined by the marked elevation of serum lactic dehydrogenase, was approximately 10^9/ml.

Radioisotope techniques. Iododeoxyuridine (IUdR) is a thymidine analog which is incorporated specifically into newly synthesized DNA; ^{125}IUdR was used as an assay of cell proliferation (11). Mice were pretreated with an intraperitoneal injection of 0.13 mg of fluorodeoxyuridine[2] in 0.5 ml of saline to deplete the thymidine pool and decrease competition for IUdR. One-half hr later the mice were injected intraperitoneally with 0.5 μCi of ^{125}IUdR in 0.5 ml of saline. Eighteen hr later the mice were killed; the organs were removed and assayed in a deep well scintillation spectrometer. This 18-hr delay permits excretion of nonincorporated ^{125}IUdR, as well as the decomposition products of ^{125}I-iodide and ^{125}I-iodouracil.

Treatment and assay. Ten-week-old germfree male and female mice were divided into two equal groups, one of which was pretreated with an intraperitoneal injection of 1 ml of 1% sheep erythrocytes (SRBC). Ten days after injection of the sheep erythrocytes, these mice, plus an equal number of nonimmunized mice, were injected intraperitoneally with 0.5 ml of the Rauscher virus suspension. Mice which received no treatment and those which received only sheep erythrocytes were studied as controls. At various times after injection of the virus suspension, mice were injected with ^{125}IUdR. Eighteen hr later, the mice were killed by an overdose of ether anesthesia and the body was weighed. Five mice were killed at each of the following intervals: 1, 2, 4, 8, 10, 14, 21,

[2] Obtained through the courtesy of the Hoffmann-LaRoche Company, Inc., Nutley, New Jersey.

28, and 30 days. The radioactivity of the samples was then counted in a Packard Autogamma counter. The tissues were processed for histological examination, and 5-μ sections stained with hematoxylin and eosin were examined.

Twelve-week-old conventional male mice were divided into three equal groups. One group was pretreated with an intraperitoneal injection of 1 ml of 1% SRBC ($\sim 2.5 \times 10^9$ cells), and the second group was pretreated with an intraperitoneal injection of 1 ml of serum containing LDV. The third group was an untreated control. All three groups were intraperitoneally injected with RLV within 1 hr of the first injection. Five animals from each group were killed at 2, 4, 8, 15, 20, 25, and 30 days after the RLV injection. The spleens were removed, weighed, and processed for histological examination.

Results. Spleen and thymus weight changes. In both the SRBC-stimulated and normal germfree RLV-infected mice, a two-phase growth of spleens occurred during the first 30 days after infection (Fig. 1). No significant difference was detected in the first phase, which occurred to day 10, during which time the spleen weight more than doubled in both experimental groups. However, this effect was much more pronounced in the SRBC-immunized mice. Between days 10 and 20 the spleens in this group had a doubling time (T_2) of approximately 4 to 5 days. At 21 days after RLV infection, there was a 65% difference in spleen weight between the SRBC-immunized and the normal germfree, RLV-injected mice. Mice began to die from ruptured spleens in the former group at 22 days, while no deaths occurred in the normal germfree RLV-injected mice until after the 30-day time point was taken. A marked difference in spleen weights still occurred in both groups between days 21 and 30.

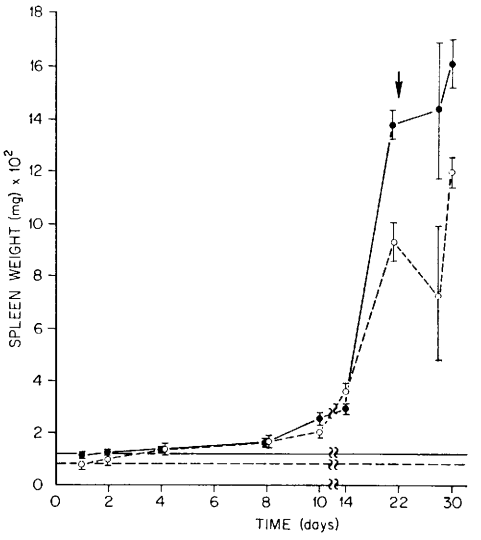

FIG. 1. Spleen weights of SRBC-stimulated (●); and normal (○) germfree RLV-infected mice. Each point represent the mean of 4 mice ± 1 standard error of the mean. Arrow indicates time of first deaths in SRBC-stimulated RLV-infected mice. Parallel solid and broken lines represent the means of non-RLV-injected, SRBC-stimulated germfree mice and normal germfree mice, respectively.

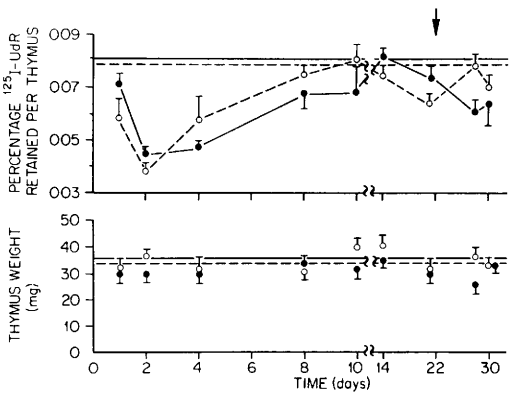

FIG. 2. Thymus weights and mean percentage of ^{125}IUdR measured per thymus of SRBC-stimulated (●); and normal (○) germfree RLV-injected mice. Each point represent the mean of 4 mice and ± 1 standard error of the mean. Arrow indicates time of first deaths in SRBC-stimulated RLV infected mice. Parallel solid and broken lines represent the mean of non-RLV-injected, SRBC-stimulated germfree mice and normal germfree mice, respectively.

No significant changes in thymus weight (Fig. 2) could be measured during the first 30 days after RLV injection, regardless of whether the germfree animals had previously been stimulated with SRBC. This lack of thymic weight changes in these two groups of experimental mice is in marked contrast to the splenomegaly observed.

The spleen weight changes in conventional mice injected with an RLV dose approximately 1/10 that received by their germfree

TABLE I. Spleen Weights (mg) of Conventional RLV-Injected BALB/c Mice.

Treatment	Days[a] 2	4	8	15	20	25	30
RLV	106.3 ± 3	142.8 ± 9	146.5 ± 4	200.3 ± 9	448.9 ± 8	517.0 ± 162	579.3 ± 182
SRBC 1 hr before RLV	134.3 ± 5	231.7 ± 1	237.2 ±11	748.9 ±143	833.7 ± 204	1563.7 ± 159	2641.2 ± 318
LDV 1 hr before RLV	127.4 ± 5	163.9 ± 8	150.2 ± 7	219.3 ± 16	511.5 ± 137	797.9 ± 40	1605.5 ± 332

[a] Each interval represents the mean of 5 mice ± 1 standard error of the mean.

counterparts is shown in Table I. A significant enhancement of the spenomegaly is detected in the mice treated with SRBC or LDV 1 hr before the RLV infecton. In the SRBC-stimulated mice, the enhanced splenomegaly is measured at all intervals between days 4 and 30; eventually a 4- to 5-fold difference in spleen size is seen. Although increasing more slowly in the LDV-pretreated mice, splenomegaly ultimately is enhanced approximately 3-fold over that in the animals injected only with RLV.

^{125}IUdR incorporation in spleen and thymus cells. An increase in ^{125}IUdR incorporation in spleen cells in both experimental germfree groups occurred between days 2 and 4 after RLV injection (Fig. 3). Thereafter, the elevated ^{125}IUdR counts in spleens remained relatively constant during the subsequent 6 days. At each of the time points the germfree RLV-infected counts remained marginally but consistently above those of the SRBC-stimulated germfree mice. A rapid increase in the rate of ^{125}IUdR incorporation occurred in both groups between days 10 and 21. The percentage of ^{125}IUdR incorporation occurred in both groups between days 10 and 21. The percentage of ^{125}IUdR retained per spleen was markedly higher in the SRBC-stimulated germfree mice, as compared to the normal germfree RLV-infected mice, at days 21, 28, and 30.

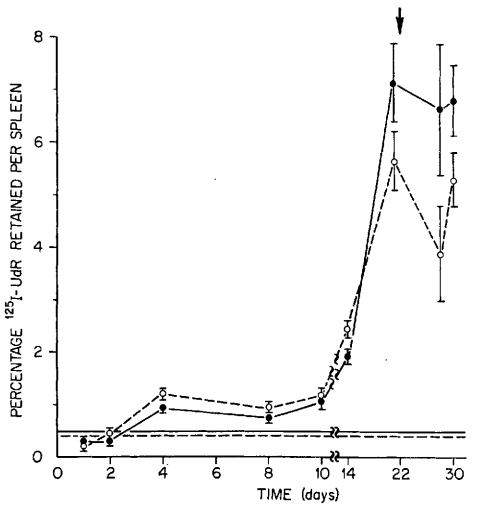

FIG. 3. Mean percentage of ^{125}IUdR measured per spleen in SRBC-stimulated (●); and normal (○) germfree RLV-infected mice. Each point represents the mean of 4 mice ± 1 standard error of the mean. Arrow indicates time of first deaths in SRBC-stimulated, RLV-infected mice. Parallel solid and broken lines represent the mean of non-RLV-injected, SRBC-stimulated germfree mice and normal germfree mice, respectively.

In both groups of animals a marked decrease in the percentage of ^{125}IUdR retained per thymus was measured at 48 hr after RLV injection (Fig. 2). The recovery appeared to be greater in those germfree mice not immunized with SRBC. A second decrease in the percentage of ^{125}IUdR retained per thymus was measured between days 14 and 30 in the SRBC-stimulated germfree mice injected with RLV.

Histology. The first paper of this series describes the histology of the spleen in conventional BALB/c mice following Rauscher virus injection (6). Therefore, the results to be reported here will be exceptions to the previous descriptions.

In normal germfree mice, except for a marked prominence of the marginal zones of the spleen lymphatic nodules, no major alterations occurred in the spleen until 2 days

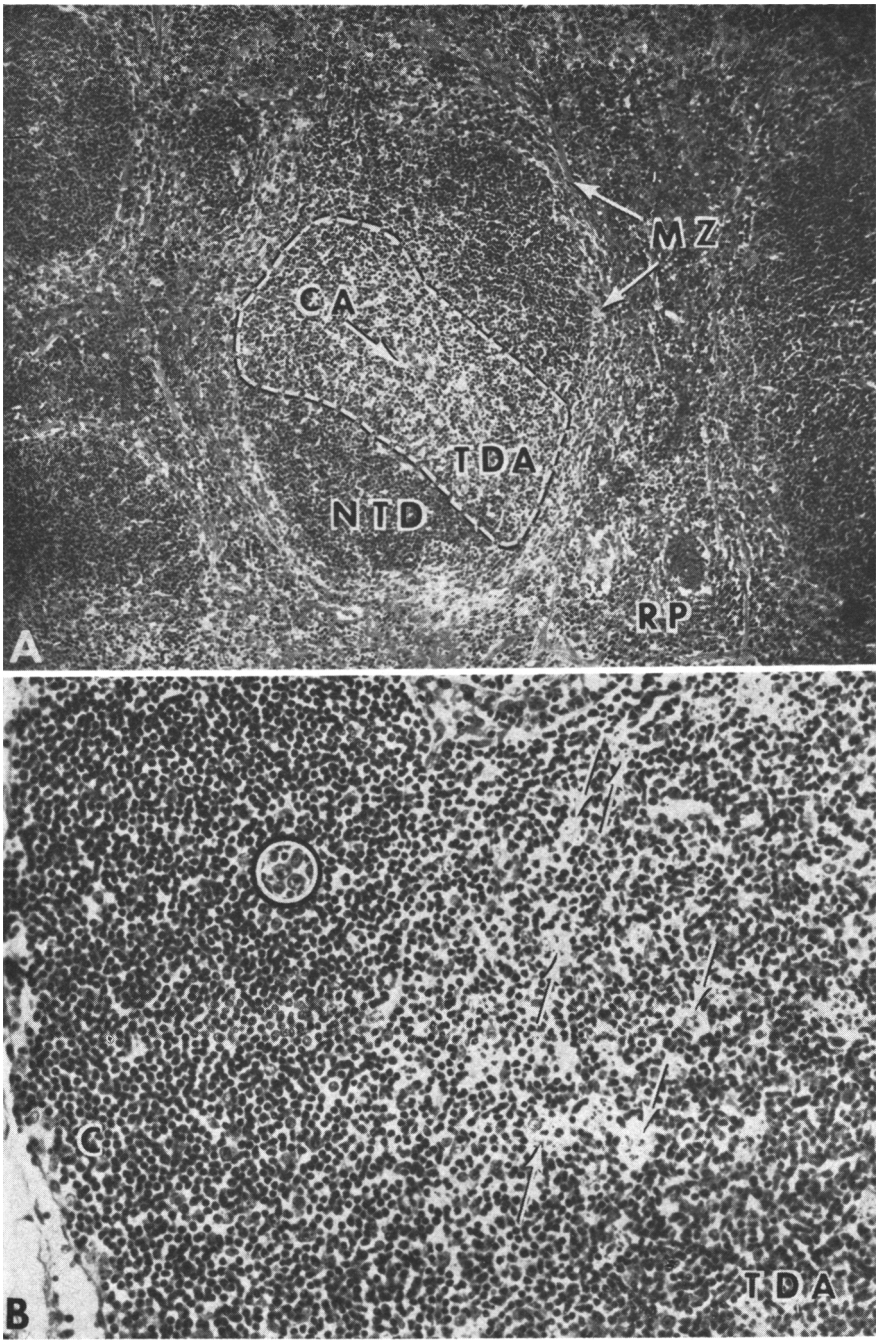

FIG. 4A. Spleen lymphatic nodule in germfree mouse 2 days after RLV injection. Broken line outlines thymus-dependent area (TDA). Note sparsity of lymphocytes and edema-like change compared to the cortical nonthymus-dependent (NTD) region. CA = central arterioles; MZ = marginal zone; RP = red pulp; × 125. (B) Mesenteric lymph node in germfree mouse 2 days after RLV injection. The arrows indicate macrophages containing nuclear debris, in thymus-dependent area (TDA). Circles in the cortical (C) nonthymus-dependent regions are two immunoblast-like cells; × 450.

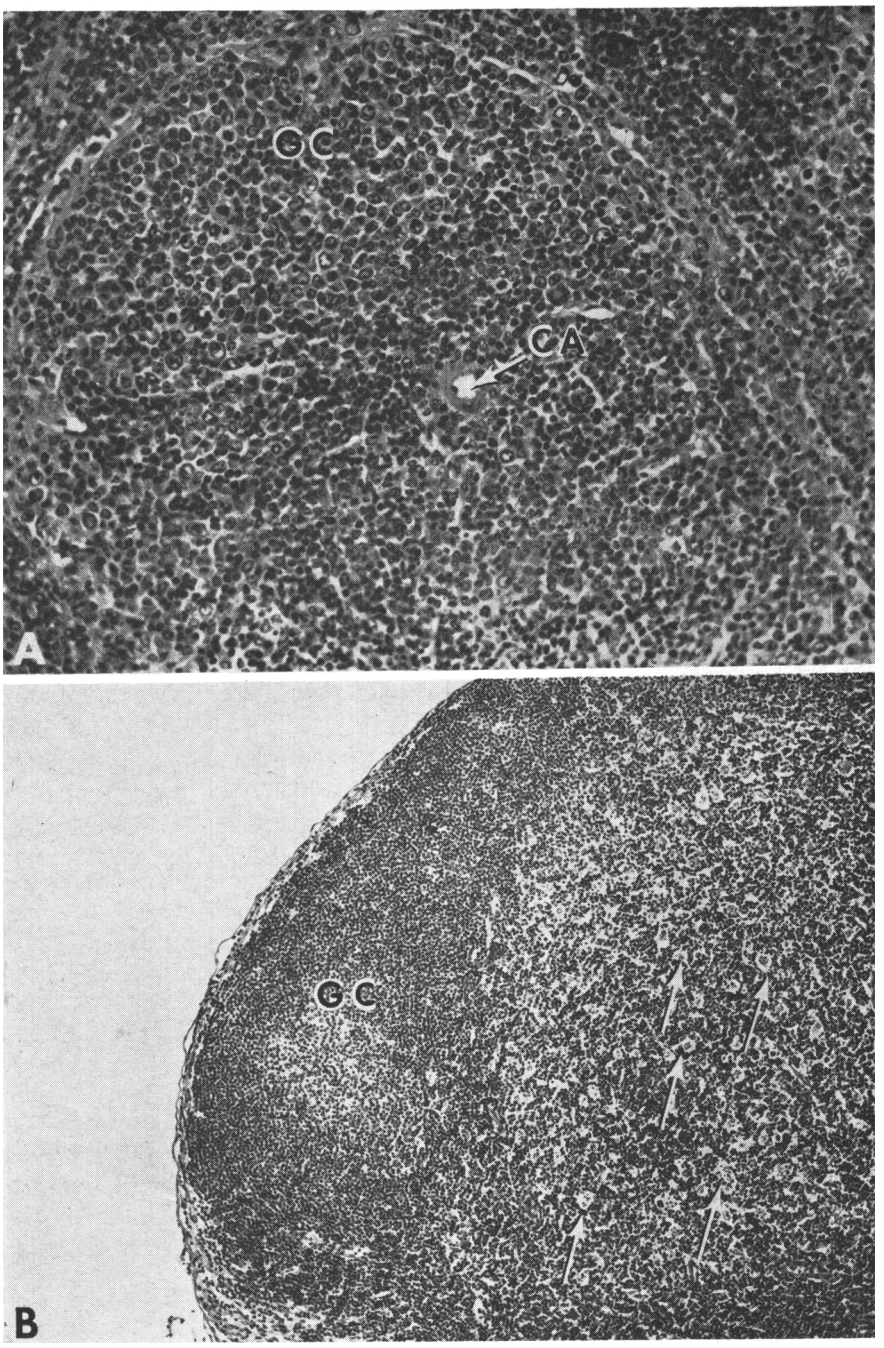

FIG. 5A. Spleen lymphatic nodule in a germfree mouse 4 days after RLV injection. Note the lymphocyte-depleted thymus-dependent area around the central artery (CA) and the active germinal center (GC) with numerous mitotic figures; × 450. (B) Mesenteric lymph node in SRBC-stimulated, germfree mouse 2 days after RLV injection. Note the germinal center (GC) in the cortex. Arrows indicate macrophages laden with nuclear debris; × 125.

after RLV injection. At this time there was a massive necrosis of the cells in the thymus-dependent region (*i.e.*, the associated lymphocyte mass surrounding the central arteriole) of the spleen lymphatic nodules (Fig. 4a). This effect was further characterized by prominent reticular cells and macrophages containing nuclear debris, presumably in part from destruction of mature lymphocytes. Completely spared from this effect was the nonthymus-dependent cortical region of the nodule, which in these animals was devoid of germinal centers. The marked necrosis of cells in this thymus-dependent region, with subsequent phagocytosis, was also observed in the mesenteric lymph nodes of the same animals (Fig. 4b).

At 4 days the nuclear debris of the thymus-dependent regions in both the mesenteric lymph nodes and spleen lymphatic nodules was cleared. Although at this time there was no evidence of the previous cell death, there was also very little reconstitution observed in these regions at the 4-day time point. Proliferation of large blast cells, primarily in the cortical regions of lymphatic tissue, occurred with subsequent development of germinal centers (Fig. 5a). In previous studies these cells have been designated as immunoblast, and morphologically they are characteristic of the parenchymal cells which normally constitute the lymphatic tissue germinal centers. Large numbers of these immunoblast-like cells were observed in the perivascular regions of the lymphatic nodules, as well as in the marginal zones. Also at this 4-day interval, accumulations of immunoblast-like cells were situated along trabecula in the spleen red pulp.

At 7 days after injection, hyperplasia of the germinal centers was very prominent in the spleen lymphatic nodules, as well as in the mesenteric lymph nodes. In many respects the disorganization of the cells in these centers corresponds to the "dissociative growth" of germinal centers characteristic of the normal primary immune responses. After this time point the progressive splenomegaly associated with Rauscher disease, characterized by lymphoid and hematopoietic hyperplasia, was observed throughout the course of the study.

In the germfree mice injected with heterologous erythrocytes 10 days before RLV, there was also a marked alteration of the thymus-dependent region of the spleen lymphatic nodules and mesenteric lymph nodes. This response occurred independently of marked hyperplasia in the germinal centers of the nonthymus-dependent regions of this lymphatic tissue (Fig. 5b). As in the normal germfree mice, this alteration of the nonthymus-dependent region was cleared by day 4, and this perivascular region of the lymphatic nodules was infiltrated with immunoblast-like cells characteristic of those observed in the germinal centers. In these animals the dissociative growth of the nonthymus-dependent regions to the thymus-dependent regions in red pulp was much more easily detected. Between days 8 and 30 the characteristic splenomegaly observed after RLV was documented in these animals. Although the difference could not be detected histologically, it is apparent from the spleen weight changes and the ^{125}IUdR incorporation that the red pulp hyperplasia as well as cell number was greater in the antigen-stimulated germfree mice compared to the normal germfree mice injected with RLV.

Similarly, in the conventional RLV-infected animals, at 2 days there was a massive necrosis of the cells in the thymus-dependent region of the spleen lymphatic nodules. This effect was also characterized by macrophages containing nuclear debris. In all of the animals there were distinct, hyperplastic germinal centers and this region of the follicle was completely spared from the cytotoxic effect. No significant difference could be detected histologically in the intensity of alteration in the thymus-dependent regions, regardless of whether the animals were pretreated with SRBC or LDV.

Thymus. In neither germfree RLV-infected experimental group could any necrosis be observed in the thymus. The morphologic alteration that could be attributed to this organ was thymocyte depletion of the medulla at the 4- and 8-day intervals. In most respects the cortex to medulla ratio, from a subjective view, was constant throughout the course of the study.

Discussion. These studies clearly demon-

strate that, in both germfree and conventional BALB/c mice, prior injection with heterologous erythrocytes results in enhanced splenomegaly by subsequent RLV infection. Spleen weights in early RLV infection have been shown to be an accurate index of the progress of the disease (12), and in otherwise untreated mice it is directly proportional to the size of the virus inoculum (10). Thus the results of the present study suggest that a prior antigenic stimulation contributes to accelerated splenomegaly of RLV. A similar observation has been made by Siegel and Morton (13), using one injection of BSA and Freund's adjuvant prior to RLV.

There are several possible explanations for this accelerated RLV-induced splenic hyperplasia, one being an inhibition of reticuloendothelial functions due to a competition between the first antigen and the oncogenic virus in the RLV preparation. Although this explanation cannot be entirely ruled out, its importance is diminished by a 10-day interval occurring between the heterologous erythrocyte injection and the RLV in the germfree mice. Previous studies have demonstrated clearance of SRBC during this period, with complete restoration of phagocytic capacity in the peritoneal macrophages (14). Furthermore, studies of Perkins *et al.* (15) have demonstrated a deficiency in peritoneal macrophage reaction to SRBC in normal germfree as compared with stimulated germfree mice. Thus, if a state of competition with respect to RLV existed at this level, the advantage would have been in favor of unstimulated germfree mice infected with RLV.

A second explanation for the augmented leukemogenesis by antigen stimulation is antigenic competition for cells capable of synthesizing virus-neutralizing antibody. Other workers, however, have reported that prior stimulation with Zymosan or BCG brings about a lack of host protection to subsequently administered murine leukemia virus (16, 17).

A favored explanation, based on our previous studies with RLV, is that the prior antigenic exposure stimulates a proliferative expansion of immunocompetent precursor cells in lymphatic tissue and especially in the germinal centers of the nonthymus-dependent areas of the tissue. These cells, as a result of their proximity to the virus, have become the primary target cells for viral replication. Thus the degree of the leukemogenic response in the animal becomes a function of the immune progenitor cell compartment.

We have previously demonstrated that the close association of the C-type virus, either of endogenous origin or presumably from injected RLV preparation, is a function of the extracellular antigen-localizing capacity of the dendritic reticular cells which form the stroma of the germinal centers. In germfree mice the antigen-trapping potential of these cells exists in the absence of active germinal centers. However, active germinal centers develop in these germfree animals after a latent period which seems to be a function of the quality and dose of the antigen (3 days for 2×10^9 SRBC, 7 days for 0.5 mg of HGG). After the RLV infection in normal germfree mice, germinal centers were detected in spleen and mesenteric lymph nodes at 4 days. We suggest that the latent period in the development of immunoblasts in the nonthymus-dependent cortex of the mesenteric lymph node and spleen lymphatic nodules, in the presence of localized virus, contributes to the decreased infectivity of the RLV and depressed leukemogenesis in the unstimulated as compared with the SRBC-stimulated germfree mice.

In the conventional BALB/c mice injected with SRBC or LDV 1 hr before RLV, a suggested splenomegaly was detected. This could be correlated with the fact that stimulation by antigen of parenchymal cell proliferation in germinal centers can be detected as early as 1 hr after an intravenous injection of SRBC (18). By autoradiographic analysis it was determined that mice injected with SRBC showed a significant increase over untreated controls in the percentage of labeled immunoblasts in germinal centers.

The most important aspect of this study was the observed differential effect of the RLV preparation on the thymus- and nonthymus-dependent areas of lymphatic tissue. Whereas early and continuous cell proliferation was an essential aspect of the RLV-

induced change in the thymus-independent region, selective destruction of small lymphocytes and development of a prominent reticular network were early alterations in the thymus-dependent areas of the mesenteric lymph nodes and spleen lymphatic nodules. Ultrastructural studies of these thymus-dependent areas demonstrate the presence of LDV and suggest that this virus has a role in destruction of the thymus-dependent tissue (19). Moreover, histologic studies of Proffit and Congdon (20) have shown that high doses of LDV alone will produce a similar pattern or cell death in the thymus-dependent areas of lymphatic tissue.

Histologically, the pattern of destruction in these lymphoid tissues parallels that after antilymphocyte serum injection in mice (21). Pretreatment with heterologous antithymocyte serum has also been shown to have a marked potentiating effect on Rauscher virus infection, as shown by increased spleen size. These results suggest that cell-mediated immunity must be very important in the host defense to Rauscher virus, as is the case with other tumor of leukemia viruses (22). Thus, in light of the selective effect of LDV as a passenger virus in Rauscher virus preparation, it becomes an important consideration when evaluating various RLV preparations. Along with the state of antigenic stimulation and the level of cell proliferation in the thymus-independent regions (those areas primarily responsible for humoral antibody production), the degree of cell destruction from passenger virus in the thymus-dependent regions (those regions primarily responsible for cell-mediated immunity) is also a critical aspect of Rauscher virus infectivity. In the animal, the synergistic interaction of the oncogenic virus and the LDV in RLV infectivity needs clarification in light of these results.

An interesting correlation to these observations of cytotoxicity in the thymus-dependent regions of lymphatic tissue is the lack of gross histologic alterations in the thymus during these early intervals after RLV injection. The only effect of RLV detectable in the thymus was a twofold decrease in the percentage of ^{125}IUdR retention at 2 days, the interval of peak cell destruction in the spleen and mesenteric lymph node thymus-dependent regions. Studies of Proffitt and Congdon (20), using high doses of LDV in BALB/c mice, demonstrate a depletion of cells and/or cell death in the thymus in association with cell death in the thymus-dependent areas of lymphatic tissue. These studies, taken in relation to the present data, suggest a dose–response effect of LDV in these various tissues.

Summary. Normal and antigenically stimulated germfree BALB/c young adult mice were used as a test system for studying the role of the nonthymus-dependent and thymus-dependent lymphoid tissue in Rauscher leukemia virus infection. It was considered that the splenomegaly of Rauscher disease within a germfree population would be enhanced by the establishment of active germinal centers in the nonthymus-dependent region of the lymphatic nodules of mice previously immunized with sterile sheep erythrocytes (SRBC). Similar comparisons were made in conventional BALB/c mice in which SRBC and lactic dehydrogenase virus were used as the test antigens. Results of these studies demonstrate the necessary, if not essential, role of the antigen-retaining reticular cells and immunoblasts of lymphatic tissue germinal centers in the early lymphoblastosis and subsequent splenomegaly of Rauscher disease. In addition, morphologic results strongly suggest the differential effect of the Rauscher preparation on the thymus- and nonthymus-dependent regions of the lymphatic tissue.

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