Autogenous Immunity to Endogenous RNA Tumor Virus Antigens in Mice with a Low Natural Incidence of Lymphoma

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SUMMARY

The immune reactivity to endogenous (wild-type) RNA tumor virus-related antigen(s) was evaluated in RF mice, a strain with a low natural incidence of lymphoma. The purposes were to determine whether maintained humoral immunity could be detected and to evaluate the response with respect to the immune-mediated pathogenesis of glomerulosclerosis and incidence of tumors of lymphatic origin. Measurements of detectable murine leukemia virus antigen in the thymus and spleen were correlated with the development of immune competence, glomerulosclerosis, and the incidence of lymphoid neoplasia. Also, the specificity of the antibody that lodged in the kidney was determined by elution and indirect immunofluorescence with Gross virus-infected cells. A marked decrease in detectable murine leukemia virus antigen in thymus and spleen was found to correlate with development of immunological competence of the spleen as assayed by de novo germinal-center formation, antigen localization, and immune elimination from serum, which in turn correlated with occurrence of glomerulosclerosis. The antibody in the kidney was determined to be specific for Gross virus antigen(s). The role of this autogenous immunity may be considered to be beneficial in the RF mouse, as data correlating natural lymphoid neoplasia with severity of immune-complex glomerulosclerosis indicates that an inverse relationship is established in aged animals.

INTRODUCTION

Serological and ultrastructural studies have demonstrated antigenic expression of type C RNA tumor viruses in normal tissues from both fetal and adult mice of strains with a low as well as high natural incidence of lymphoma (6, 7, 13, 31). In AKR mice (a high-lymphoma strain), the virus concentration in tissue during embryonic and early postnatal life is extremely low (12, 25), yet these animals contain elevated levels of MuLV² throughout their postnatal life (12, 18). A similar pattern of age-associated increase in concentration of RNA tumor virus may occur in feral as well as in most laboratory strains of mice (31). If the leukemia virus must reach a critical concentration in the host before the leukemogenic process begins or if virus transformation of target cells depends on the multiplicity of infection, it may be assumed that some cellular or systemic modes of regulation are involved that affect quantitatively the levels of infectious virus in certain tissues. This could account for the fact that, while all laboratory mouse strains tested are positive for type C virus, the incidence of leukemia and lymphoma among these strains is highly variable.

It has been generally assumed that, with vertically transmitted tumor viruses, an initial period of tolerance exists (4, 14) to virion- and virus-induced cell-surface antigens. An alternative to this assumption is that some immunity develops to endogenous type C virus, as demonstrated by Oldstone et al. (21) during the “preleukemic” period in AKR mice. Unfortunately, it is difficult to evaluate the functional significance of the innate immunity to MuLV in AKR mice, since this is a strain with a high incidence of lymphoma. The important question is whether the balance of this host factor regulates or is directly associated with RNA tumor virus-induced pathogenesis. This question can best be evaluated by studies in mice with a low incidence of lymphoma. Two observations have led us to investigate this possibility. (a) The localization of type C virus in germinal centers of peripheral lymphatic tissue is a consistent finding in several mouse strains of low or high incidence of lymphoma (11, 27, 28). Such localization has been observed for both endogenous and inoculated RNA tumor viruses. Thus viruses localize as antigen, extracellularly, in a manner particularly associated with amplification of the immunocompetent cells of the humoral immune system (see Fig. 1b). It has been demonstrated that in truly tolerant animals such localization of antigen does not occur (15, 32). (b) Glomerulonephritis and glomerulosclerosis have been found in kidneys of mice of both high- and low-lymphoma strains (10, 21). It has also been shown in some strains that the etiology of this lesion is a specific immunological interaction of antibody and leukemia virus antigens (3, 21).

In this study we attempted to correlate detectable MuLV-related antigen in the thymus and spleen with the development of immune competence and glomerulosclerosis and with the incidence of lymphoid neoplasia. We also determined the specificity of kidney-bound antibody by elution and indirect immunofluorescence using cells infected with Gross virus. RF mice were selected for these studies because several of their characteristics led us to suspect the
presence of some resistance to, or host regulation of, type C virus expression in this strain: (a) while not subject to a high natural incidence of thymic lymphoma or myeloid leukemia, adult RF mice are susceptible to induction of these diseases by X-rays (5); (b) a high incidence of glomerulosclerosis has been described in this strain, with the incidence and severity increasing as a function of age (10); (c) a significant inverse relationship has been demonstrated between glomerulosclerosis and natural lymphoid neoplasia in a population study of aging RF mice (33); (d) type C RNA tumor viruses can be detected in these mice both serologically and ultrastructurally, and the natural myeloid leukemia of this strain has been passaged cell free (9, 29).

MATERIALS AND METHODS

Animals. Male and female RF mice from an inbred colony were used in these studies. They were housed 8 to 10/cage and were fed Purina laboratory chow.

Serological Assay for Virus Antigen. MuLV-related antigens were assayed by the complement-fixation technique of Hartley et al. (12). Tissues were taken from mice of various ages and then homogenized, suspended in PBS (10 to 20%, w/v), quick-frozen, and stored at −70°. Before the tests, the tissue homogenates were treated in a Raytheon sonic oscillator (200 watts, 10 kC/sec) for 1 min. The tests were performed with 4 antibody units and 1.7 units of guinea pig complement in the microtiter system. The antiserum was obtained from rats carrying a transplanted Moloney sarcoma virus tumor. The serum reacts against Friend-Moloney-Rauscher and Gross virus envelope and cell-surface antigens and against the group-specific antigen (gs-I) of the murine leukemia viruses. The antiserum was obtained from Dr. Roger Wilsnack, Huntington Research Laboratories, Baltimore, Md.

Electron Microscopic Techniques. Tissue examined in the electron microscope was fixed in 2.3% glutaraldehyde buffered with s-collidine, pH 7.4. After 1 hr, the 1-cm tissue blocks were postfixed in collidine-buffered osmium tetroxide (1.3%), pH 7.4, for 90 min. The fixed tissue blocks were dehydrated through ascending grades of ethanol and propylene oxide and embedded in Epon 812. Thin sections were prepared with a SLEE HRM cryostat. Fluorescein-conjugated horse anti-mouse globulin was used in performing direct immunofluorescence on the RF kidney sections; and an eluate prepared from kidneys, followed by the fluorescein conjugate, was used in carrying out indirect immunofluorescence on the AKR thymoma sections.

RESULTS

MuLV Antigens in Thymus and Spleen. Spleen and thymus tissues from mice 7 to 280 days of age were assayed for MuLV antigens by the complement-fixation test. The spleen and thymus were particularly chosen because they represent primary sites of virus replication in many strains of mice and are the primary sites of natural and radiation-induced neoplasia in RF mice (5). The results (Chart 1) demonstrate that virus antigen can be detected in the thymus as early as at 7 days of age, with the percentage of positive thymuses increasing to 50 days of age. The percentage of thymuses positive for virus antigen drops from 97% to approximately 70% between 50 and 70 days of age and undergoes another increase between 200 and 300 days. Leukemia virus antigen is first detectable in the spleen at 30 days of age, with the highest percentage positive at 50 days. A precipitous decrease...
in the percentage of positive spleens occurs between 50 and 70 days, corresponding with a decrease in positive thymuses. In subsequent tests an increase in spleens with virus antigens was found in mice over 1 year old.

Electron microscopic examination of thymus and spleen lymphatic nodules was performed on tissue from 10- to 50-day-old mice. Virus was detected in the thymus at 10 days; and at 50 days type C viruses were detected both extracellularly and budding from cell surface membranes of parenchymal cells of spleen germinal centers (Fig. 1, a and b).

**Development of Immune Competence in RF Mice.** The pattern of leukemia virus expression in the spleen in particular suggested that the change could be related to the increase in immunocompetence, which might promote clearance of the virus. Thus, we tested the level of immunocompetence in mice at 20 to 50 days of age by 2 techniques. Chart 2 shows the clearance of $^{125}$I-labeled human $\gamma$-globulin from serum of RF mice of various ages. Tests of serum from 20- and 30-day-old mice revealed no immune elimination, while a clear immune elimination of the heterologous protein occurred in the serum of 50-day-old mice. Autoradiographic studies of the spleens of these animals showed both follicular localization of the antigen and germinal center development in the 50-day-old mice. These results are considered a morphological and serological corollary of the development of humoral immune competence in this age group and directly relate to the pattern of leukemia virus expression in the spleen (see Chart 1).

**Development of Glomerulosclerosis.** The correlation of increased immunocompetence and decreased spleen-associated virus antigen suggests that the RF mouse may be capable of mounting an immune response to endogenous leukemia virus. It has been reported recently (33) that RF mice develop a high degree of glomerulosclerosis that appears to be inversely related to natural leukemogenesis. We therefore examined the temporal development of glomerulosclerosis in RF mice.

Between 50 and 700 days of age, animals (20/time point) were periodically killed, and their kidneys were evaluated for degree of glomerulosclerosis. We used a method similar to that of Gude and Upton (10) for grading glomerulosclerosis in these mice. Since at no time was a proliferative or infiltrative response associated with this lesion, we adhere to the term glomerulosclerosis. The end stage of the lesion was a simple sclerotic formation. A diagnosis of thickening of the capillary wall with dilation of capillaries at the hilum and periphery was assigned to Grade 1; occasionally, there was pyknosis and swelling of nuclei in intercapillary cells. Glomeruli classified as Grade 2 were enlarged and often had separations of glomerular tufts with hyalinized mesangium and dilated capillaries. In Grade 3, over 50% of the capillary tufts of the glomeruli were hyalinized. Nuclear karyorrhexis was common at this stage. The results (Chart 3) indicate an exponential increase in percentage of animals positive for severe glomerulosclerosis.

**Fluorescent Antibody- and Complement-Fixation Studies of Kidney.** Since several studies have implicated the deposition of immune complexes in the pathogenesis of glomerulosclerosis in AKR and NZB mice, we tested the kidney tissues of RF mice for the presence of MuLV-related antigens and immunoglobulin. Frozen sections of kidney from 1- and 1.5-year-old mice were examined for distribution of mouse immunoglobulin by direct immunofluorescence assay. In 1-year-old animals the incidence of glomerulosclerosis is approximately 30% (Chart 3). The majority of glomeruli showed positive reactions for immunoglobulin. The distribution was generally restricted either to the mesangial areas or to the peripheral walls of the glomerular capillaries (Fig. 1, c and d). At 1.5 years of age,
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When 70% of the surviving mice have severe glomerulosclerosis (Fig. 1e), the fluorescent reaction of the glomeruli is marked, and the granular pattern of globulin deposition occupies both the mesangial and parietal walls of the glomerular capillaries (Fig. 1f). Thus there is a gradual development of an immune-complex glomerular lesion which histologically lacks overt inflammatory reaction and is classified as an immune-complex glomerulosclerosis.

All kidneys tested from 1- and 1.5-year-old mice were positive for MuLV antigens as measured by complement fixation with the use of a broad-reactive rat anti-virus serum.

Immunofluorescent Tests for Antiviral Specificity of Globulin Eluted from RF Kidneys. Eluates from kidneys of 1- and 1.5-year-old mice were tested by the Ouchterlony gel-diffusion technique for presence of immunoglobulin, and both gave a positive reaction to rabbit anti-mouse globulin sera. The eluate was then used by indirect immunofluorescence tests against AKR embryo cells. The AKR embryo cells (C26), obtained from Dr. W. P. Rowe, NIH, Bethesda, Md., were carried at 2 passage levels: the low-passage cultures were negative for leukemia virus as determined by the X-C cell-plaque assay (16, 26) and by immunofluorescence; the high-passage cultures had spontaneously activated Gross-type leukemia virus synthesis (17).

With 1.5-year-old kidney eluate, a large number of high-passage AKR embryo cells showed a positive fluorescent reaction, suggesting specific binding of immunoglobulin of the kidney eluate with cytoplasmic leukemia virus antigens (Fig. 2, c and d). We did not detect immunofluorescent cells in the low-passage AKR preparation. Only a few positive high-passage cells were seen with eluate from 1-year-old RF kidneys. Similar results were obtained when specificity was tested against infected and noninfected Swiss embryo cells grown in culture. However, the overall fluorescent reaction was less than that obtained with AKR cells. Kidney eluate from both 1- and 1.5-year-old RF kidneys was also tested by indirect immunofluorescence against frozen sections of an AKR thymoma. A granular pattern of cytoplasmic fluorescence was detected in occasional cells with the eluate from 1-year-old kidneys (Fig. 2, a and b), and a similar pattern of fluorescence was detected with eluate from 1.5-year-old RF kidneys. More thymic cells were positive with 1.5-year eluates, suggesting either more antibody or higher-affinity antibody in the latter eluate preparation.

In general, the facts that the kidney homogenates from both 1- and 1.5-year-old RF mice reacted with leukemia virus antibody by the complement-fixation test and that antibody eluted from the kidney showed positive reactivity by indirect immunofluorescence against cells infected with Gross virus show that an antigen-antibody complex associated with the spontaneous glomerulosclerosis of these mice is a leukemia virus antigen-specific complex.

Correlation of Glomerulosclerosis with Total Leukemia Incidence. A further evaluation was made of the pathology data from the control group of 311 RF mice in which the original demonstration of reduced leukemia in the presence of glomerulosclerosis had been made (33). Animals that died were autopsied and evaluated for incidence and degree of glomerulosclerosis, and concomitant incidence of lymphoid

![Chart 3](chart3.png)

Chart 3. Mean percentage of RF mice killed at various ages with severe (Grade 3) glomerulosclerosis. Kidneys from 20 mice were examined at each time point.

![Chart 4](chart4.png)

Chart 4. Mean glomerulosclerosis grade in animals dying with lymphoid neoplasia (●) or from other causes (○).
neoplasia such as thymic lymphoma and a mixed-type B reticulum cell sarcoma. The results (Chart 4) indicate an inverse relation between the severity of glomerulosclerosis and incidence of all lymphoid neoplasia. Between 200 and 700 days, the highest mean glomerulosclerosis grade measured was 1.8 in animals with malignant disease of lymphatic tissue, while 2.2 to 3.0 was the range of mean glomerulosclerosis grade measured in the RF mice with no detectable lymphoid neoplasia. Between 700 and 900 days, the mean glomerulosclerosis grade increased in animals bearing lymphoid neoplasia, but this increase is largely a product of the small sample sizes available at the extreme ages.

A further evaluation of these data can be made based on a comparison of lymphoid neoplasia in animals dying with severe glomerulosclerosis (Grade 3) and those dying with less than severe glomerulosclerosis: 197 animals were determined to have less than severe glomerulosclerosis and 28 of the 70 that died between 250 and 400 days had thymic lymphomas, while 127 of those that died at later intervals had reticulum cell sarcomas classified as mixed-type B. The remaining 114 mice that died during the course of this study were determined to have severe glomerulosclerosis. On the basis of the aged-matched method for calculating expected cases (33), 15 of these mice were expected to have thymic lymphomas and 75 to have type B reticular cell sarcoma. Actually, only 4 cases of thymic lymphoma were detected (26% of expected), and 46 mice had reticulum cell sarcoma (61% of expected).

DISCUSSION

Spontaneously occurring glomerulonephritis in conventional NZB mice and their hybrids has been associated with an immune complex etiology (19). These studies specifically implicated virus-specific cell-surface and soluble antigens with “G natural” antibody-complex deposition in kidney glomeruli. Oldstone et al. (21) have demonstrated that IgG eluted from kidney homogenates of preleukemic AKR mice, while not reacting with Gross virus envelope antigens, has specific reactivity with Gross virus-infected cells by immunofluorescence and with Gross virus-infected cell antigens by complement-fixation tests. Aoki et al. (1) have demonstrated the Gross leukemia virion specificity of serum antibody from a normal NZB mouse. These studies cast doubt on the general working hypothesis of a tolerant state to vertically transmitted RNA tumor virus and virus-mediated cellular antigens, which was primarily proposed because G antibody could not be detected in serum of G+ mice (2). Similar conclusions have been derived from recent findings of immune reactivity to other viruses in which tolerance was originally associated with chronic infection due to the lack of demonstrable immune reactivity (20, 22).

A critical question concerning the autogenous immunity to the wild-type RNA tumor viruses and virus-mediated cellular antigens is the functional role of such immunity in spontaneous virally induced pathogenesis, specifically, lymphoid neoplasia. In the case of the AKR strain, no specific benefits are apparent, since the natural tumor incidence is high at an early age. Furthermore, it is possible that the presence of humoral immunity might actually prompt tumorigenesis in these mice. This speculation was originally presented by Metcalf and Wahren (19), based on studies of local immune reactions in preleukemic AKR mice. We thus attempted to test the immune reactivity to endogenous RNA tumor viruses in a mouse strain with a low natural incidence of lymphoma in order to determine whether maintained humoral immunity could be detected and to evaluate the response with respect to the immune-mediated pathogenesis of glomerulosclerosis and the incidence of tumors of lymphatic origin.

In the present study MuLV antigens could be detected in approximately 20% of the thymuses tested from 7-day-old RF mice. We assume that the broad-reactive antisera, as used in the complement-fixation test, has a certain degree of resolution with a relatively high quantity of leukemia virus antigens. Thus the increase in the percentage of thymuses positive for viral antigen can be reasonably interpreted as an increase in the quantity of antigen with age. No detectable viral antigen was measured in the spleen until the mice were 30 days of age, and the peak antigen concentration was reached at 50 days. The decrease in antigen in both the thymus and spleen definitely correlates with development of the immunological competence of the spleen, as assayed by de novo germinal center formation, antigen localization in germinal centers and, more specifically, immune elimination of antigen from the serum. We interpret that the developed immunological competence could also be functional for MuLV. Further, this suggested immune elimination correlates well with the development of histologically detectable glomerulosclerosis.

Although complement fixation tests of the kidney homogenates revealed that 100% of the kidneys from 1- and 1.5-year-old mice were positive for leukemia virus antigens, it was important to test the specificity of the kidney-bound globulin for RNA tumor virus antigens. We feel that the immunofluorescent reaction of AKR spontaneous Gross virus-replicating cells, AKR thymoma, and Gross virus-infected Swiss embryo cells, when kidney eluate globulin is used, strongly suggests that some fraction of the immunoglobulin is specific for Gross-type antigens. A comparison of the degree of positive reaction between high-passage AKR cells, spontaneously producing Gross-type virus cells, and Swiss embryo cells infected with Gross virus revealed that the AKR cells exhibited a stronger reaction by immunofluorescence. The same situation was demonstrated with direct rat anti-MuLV serum, suggesting that the wild-type virus infection in the AKR tissue-culture cells is a better system for testing kidney eluate immunoglobulin produced against wild-type endogenous leukemia virus. We conclude from these data that a contributory factor in the glomerulosclerosis that develops in RF mice is a fixation of antigen-antibody complexes resulting from a chronic humoral immune response to endogenous type C RNA tumor virus. Experimentally, this condition can be induced in BALB/c mice by immunization at an early age with extraneous RNA tumor viruses (3).

The role of this innate immunity appears to be beneficial in the RF mouse, as judged from data showing an inverse relationship between severe glomerulosclerosis and lymphoid neoplasia (see Ref. 33 and Charts 3 and 4). We do not consider this inverse correlation as comprising unequivocal evidence for immunity as a host regulation of the etiological agent of
lymphoid neoplasia. While empirically evident, the biological basis of the inverse relationship is yet to be established. In addition to the possibility that an adequate endogenous immune response, as estimated by glomerulosclerosis development, reduces the viral burden and therefore the probability of developing lymphoid neoplasia, it is possible that the reverse situation obtains. Lymphoid neoplasia prevents development and/or expression of glomerulosclerosis. Although unlikely, this possibility cannot at present be excluded.

The question of whether autogenous immunity can regulate pathogenesis in a positive or negative manner may be a function of the quality of the humoral response. Only in one specific instance has it been shown that the immune specificity is directed toward Gross virion antigens (1), and little data exist demonstrating development of a virus-neutralizing antibody during the course of the response to endogenous (wild-type) RNA virus infection. However, the possibility that this autogenous immune response to endogenous RNA tumor virus may be a component of host functions (23, 24) that control or regulate pathogenesis serves as a possible explanation of the diverse lymphoma patterns in various mouse strains in the presence of apparently similar viral burdens.

REFERENCES


Fig. 1. a, budding type C virus particle (arrow) from thymocyte of 10-day-old RF mouse. X 25,000. b, extracellular type C virus particle and a budding type C virus in a spleen germinal center from a 50-day-old RF mouse. X 60,000. c and d, kidney glomeruli from 1-year-old RF mice. Glomeruli are stained with fluorescein-conjugated horse anti-mouse globulin. X 300. e, section of kidney from a 1.5-year-old RF mouse illustrating severe glomerulosclerosis. H & E, X 400. f, kidney glomeruli from 1.5-year-old RF mouse stained with fluorescein-conjugated horse anti-mouse globulin. X 425.

Fig. 2. Indirect immunofluorescence assay for detecting antibody specific to Gross virus antigens. Kidney eluates from 1- and 1.5-year-old RF mice and fluorescein-conjugated horse anti-mouse globulin were incubated with frozen sections of AKR thymoma (a, b) and AKR high-passage tissue culture cells spontaneously producing Gross virus (c, d). Note granular cytoplasmic fluorescence of several thymoma cells in a and b. X 425. Arrows, fluorescent-positive AKR tissue culture cells in c and d. X 425.
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