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## Neonatal Exposure to Thymotropic Gross Murine Leukemia Virus Induces Virus-Specific Immunologic Nonresponsiveness

### Abstract

Neonatal exposure to Gross murine leukemia virus results in a profound inhibition of the virus-specific T and B cell responses of adult animals. Animals exposed to virus as neonates exhibit a marked depression in virus-specific T cell function as measured by the virtual absence of in vivo delayed type hypersensitivity responses and in vitro proliferative responses to virally infected stimulator cells. Further, serum obtained from neonatally treated mice failed to either immunoprecipitate viral proteins or neutralize virus in an in vitro plaque assay, suggesting the concurrent induction of a state of B cell hyporesponsiveness. The specificity of this effect at the levels of both T and B cells was demonstrated by the ability of neonatally treated mice to respond normally after adult challenge with either irrelevant reovirus or influenza virus. The replication of Gross virus within both stromal and lymphocytic compartments of the neonatal thymus suggests that thymic education plays a key role in the induction of immunologic nonresponsiveness to viruses.

### Disciplines

Congenital, Hereditary, and Neonatal Diseases and Abnormalities

# Neonatal Exposure to Thymotropic Gross Murine Leukemia Virus Induces Virus-specific Immunologic Nonresponsiveness

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## Summary

Neonatal exposure to Gross murine leukemia virus results in a profound inhibition of the virus-specific T and B cell responses of adult animals. Animals exposed to virus as neonates exhibit a marked depression in virus-specific T cell function as measured by the virtual absence of *in vivo* delayed type hypersensitivity responses and *in vitro* proliferative responses to virally infected stimulator cells. Further, serum obtained from neonatally treated mice failed to either immunoprecipitate viral proteins or neutralize virus in an *in vitro* plaque assay, suggesting the concurrent induction of a state of B cell hyporesponsiveness. The specificity of this effect at the levels of both T and B cells was demonstrated by the ability of neonatally treated mice to respond normally after adult challenge with either irrelevant reovirus or influenza virus. The replication of Gross virus within both stromal and lymphocytic compartments of the neonatal thymus suggests that thymic education plays a key role in the induction of immunologic nonresponsiveness to viruses.

Immunologic nonresponsiveness to autologous molecules and a variety of exogenous antigens can be linked to their presence during neonatal maturation (1-4). The consequences of neonatal exposure to viruses are considerably more variable (5, 6). Mice infected either neonatally or congenitally with lymphocytic choriomeningitis virus (LCMV)<sup>1</sup> or endogenous ecotropic retroviruses mount potent Ig responses and produce functional CTL precursors (7-9). However, several recent studies have shown that exposure of neonatal mice to Moloney murine leukemia virus significantly reduced the frequency of Moloney virus-specific CTL precursors and Ig levels (6, 10). These contrasting results raise important questions concerning the ability of different viruses to induce tolerance, and the contributions of T and B cell components to virus-specific tolerance. To address these questions, the immune response of mice exposed as neonates to NB-tropic Gross murine leukemia virus (GMuLV) was investigated. Exposure of neonates to GMuLV induces a state of immunologic nonresponsiveness that may be linked to intrathymic replication of this retrovirus.

Intraperitoneal injection of neonatal mice with GMuLV leads to the development of lymphatic leukemias typified by the presence of thymic lymphomas, and to a unique form of persistent infection within the central nervous system white matter (11-13). The data reported here demonstrate that neonatal exposure to these murine retroviruses also induced a state of virus-specific T cell nonresponsiveness, characterized by impaired delayed type hypersensitivity and *in vitro* proliferative responses. Concomitant with this T cell defect was the functional inactivation of virus-specific B cells, as evidenced by the absence of both neutralizing and immunoprecipitating antibody. These immunologic defects occur simultaneously with the detection of GMuLV replication within thymic stromal elements. The implication of these observations with regard to more general aspects of immunologic tolerance is discussed in the context of the specific requirements for viral replication within thymic and lymphoid cells.

## Materials and Methods

*Mice.* 6-8-wk-old BALB/cByJ mice (H-2<sup>d</sup>) were obtained from The Jackson Laboratory, Bar Harbor, ME, and breeding pairs were established. Mice were maintained on standard lab chow and water and libitum. BALB/c *nu/nu* mice were obtained from the National

<sup>1</sup> Abbreviations used in this paper: DTH, delayed-type hypersensitivity; GMuLV, Gross murine leukemia virus; HAU, hemagglutinating units; LCMV, lymphocytic choriomeningitis virus; PFU, plaque-forming units; RT, reverse transcriptase.

Cancer Institute, Bethesda, MD, and housed in a germ free facility at the University of Pennsylvania School of Medicine.

**Viruses.** Two strains of GMuLV were used in these studies: parental NB tropic GMuLV and the WB91 strain of GMuLV (12). Virus was expanded by passage in Sc-1 cells, a feral mouse embryo fibroblast line (American Type Culture Collection, Rockville, MD). Sc-1 cells were grown in MEM supplemented with 10% FCS and pre-treated with Polybrene (8  $\mu\text{g}/\text{ml}$ ) (Aldrich Chemical Co., Milwaukee, WI) for 1 h before addition of virus. To confirm infection of Sc-1 cells, supernatants from confluent monolayers were tested 7–10 d after virus addition for the presence of reverse transcriptase (RT) according to the method of Goff et al. (14). Supernatants were aliquoted and stored at  $-70^{\circ}\text{C}$ . Viral titers were determined by a modified XC cell plaque assay (15).

Virus-specific neutralizing activity was determined by incubation of virus with medium (control) or test serum at a dilution of 1:5 for 40 min at  $37^{\circ}\text{C}$ , followed by addition to Polybrene-treated Sc-1 cells plated into six-well tissue culture plates ( $10^4$  cells/well) for XC cell plaque assay. Serum neutralizing activity was expressed as a neutralization index, calculated as the difference in the  $\log_{10}$  titer of the virus samples with or without serum.

The purified PR8 strain of influenza A virus was prepared and quantitated for hemagglutination activity by Dr. Michael Cancro (University of Pennsylvania). Reovirus type 3 was prepared from infected L cell monolayers as described previously (16).

**Immunizations.** Neonates were injected intraperitoneally with  $2 \times 10^3$  plaque forming units (PFU) of live GMuLV-containing culture supernatants within the first 24 h after birth. At 6 wk of age, these animals and age-matched controls were challenged intraperitoneally with  $2 \times 10^3$  PFU of virus, and in some experiments, with 1,000 hemagglutinating units (HAU) of UV-inactivated influenza virus. Serum was collected by retro-orbital bleeding both before and 8 d after challenge. Samples were allowed to clot, spun for 10 minutes in a micro-centrifuge, serum aspirated, and stored at  $-20^{\circ}\text{C}$ .

For delayed type hypersensitivity experiments, mice were immunized intraperitoneally at 6–8 wk of age with virus ( $2 \times 10^3$  retrovirus PFU or  $3 \times 10^6$  reovirus PFU in  $100 \mu\text{l}$ ). All animals were challenged after 6 d with  $25 \mu\text{l}$  of virus in one footpad, and  $25 \mu\text{l}$  of media in the contra-lateral footpad. 24 h later, footpad swelling was measured in a blind manner with a Fowler micrometer (Schelinger's, Brooklyn, NY). Responses were expressed as the percentage increase in the thickness of the virally challenged footpad as compared with the media challenged footpad.

**RIA for Antiinfluenza Antibody.** Doubling dilutions of serum were prepared in 20 mM sodium phosphate (pH 7.4), 0.02% sodium azide, 5% horse serum.  $20 \mu\text{l}$  of either standard PR8-specific hybridoma proteins or diluted serum was added to the wells of microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) previously coated with 20 HAU of purified virus. Antibody binding to adsorbed virus was determined with affinity-purified,  $^{125}\text{I}$ -labeled, goat antibody against mouse  $\alpha$ ,  $\gamma$ , or  $\mu$  chains, or with rabbit anti-mouse F(ab) $_2$  (kindly provided by Dr. Michael Cancro).

**Assay for Virus-specific Serum Ig.** Serum from mice treated with GMuLV and WB91-GV were assayed for virus-specific Ig by binding to the tumor cell lines Kgv and KKC, respectively. Tumor cell lines were originally derived from thymic lymphomas induced by inoculating adult BALB.K mice with these viruses (12, 17). Both lines express viral proteins, including the envelope glycoprotein, gp70, on their cell surface.  $10^6$  tumor cells were incubated for 45 min at  $4^{\circ}\text{C}$  with serum diluted 1:5 in 20 mM sodium phosphate (pH 7.4), 1% BSA, 0.02% sodium azide (PBS-BSA- $\text{NaN}_3$ ). Cells

were washed twice and pellets were resuspended in  $100 \mu\text{l}$  of a 1:100 dilution of a goat anti-mouse Ig/fluorescein conjugate (Fisher Scientific Co., Pittsburgh, PA) and incubated at  $4^{\circ}\text{C}$  for 45 min. As a positive control, tumor cells were first incubated with goat anti-Rauscher gp70 antisera obtained from the Biological Carcinogenesis Branch of the National Cancer Institute, followed by a rabbit anti-goat Ig/fluorescein conjugate. Cells incubated with buffer alone followed by anti-mouse Ig/fluorescein served as a negative control. Cells were washed three times and resuspended in  $500 \mu\text{l}$  of PBS-BSA- $\text{NaN}_3$  and then analyzed on a FACS IV flow cytometer (Becton Dickinson & Co., Mountain View, CA). The data are expressed as the change in mean channel fluorescence of cells incubated with pre-challenge vs. that for cells incubated with post-challenge samples.

**Surface Iodination of Tumor Cells.** The membrane proteins of tumor cells were surface labeled with  $\text{Na}^{125}\text{I}$  as previously described (18). Cells were then incubated with  $500 \mu\text{l}$  of lysis buffer (50 mM tris, pH 7.6, containing 0.1% BSA, 300 mM NaCl, 1% Triton X-100, 0.5% NP-40, 10  $\mu\text{g}/\text{ml}$  leupeptin, 50  $\mu\text{g}/\text{ml}$  aprotinin, 10 mM iodoacetamide, 0.4 mM sodium orthovanadate, 0.4 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, and 1 mM PMSF) and processed as described previously (18). Lysates were pre-cleared by incubating with protein A-Sepharose and then incubated with serum or purified goat anti-Rauscher gp70 at a final dilution of 1:10 for 45 min at  $4^{\circ}\text{C}$  followed by the re-addition of protein A-Sepharose. Pellets were resuspended in electrophoresis loading buffer and analyzed under reducing conditions by SDS-PAGE and autoradiography.

**Serum Immune Complex Assay.** A solid phase assay designed for the detection of human serum immune complexes (Sigma Chemical Co., St. Louis, MO) was adapted for measuring murine complexes. Briefly, doubling dilutions of normal mouse serum or serum from mice exposed to virus as neonates were prepared in  $200 \mu\text{l}$  of sample buffer.  $5 \mu\text{l}$  of a solution containing  $\sim 0.45$ - $\mu\text{g}$  equivalents of human immune complexes per milliliter was added to each sample, and  $100 \mu\text{l}$  of each was then transferred to microtiter wells pre-coated with goat Clq and incubated at room temperature for 20 min. Wells were washed three times, after which  $100 \mu\text{l}$  of a goat anti-human Ig/alkaline phosphatase conjugate was added and incubated at room temperature for 20 min. Wells were again washed three times,  $100 \mu\text{l}$  of substrate solution was added (*p*-nitrophenyl phosphate, disodium hexahydrate, 1 mg/ml, pH 9.6), incubated at room temperature for 20 min, and the reaction stopped by the addition of  $100 \mu\text{l}$  of a pH 12 alkaline solution. Absorbance at 405 nm was measured with a microplate reader (Bio-Rad Laboratories, Richmond, CA). This assay is sensitive to a serum immune complex concentration corresponding to 20- $\mu\text{g}$  equivalents/ml.

**Detection of Tissue-deposited Immune Complexes.** Kidneys from 6–8-wk-old neonatally treated or normal mice were removed, placed in Tissue-Tek II OCT compound (Lab-Tek Products, Naperville, IL), and rapidly frozen. Frozen sections 3–4  $\mu\text{m}$  in thickness were cut, mounted on glass slides, and fixed in methanol ( $-20^{\circ}\text{C}$ ). Sections were incubated with a goat anti-mouse Ig/fluorescein conjugate (Fisher Scientific Co.) diluted 1:200 in 0.5 M Tris, pH 7.6, for 45 min at room temperature and washed extensively. Slides were analyzed on a Nikon Optiphot fluorescence microscope.

**Mixed Lymphocyte Reaction.** Enriched T cell responder populations were prepared by treating spleen cell suspensions with the mAbs J11d (recognizes a determinant found on a variety of cell types including a majority of B cells) and MKD6 (anti-I-A $^d$ ), and a mixture of guinea pig/rabbit complement. Stimulator cells were prepared from the spleens of virally infected animals and irradiated (3,000 rad).  $5 \times 10^5$  responder cells were cocultured with  $0.3 \times$

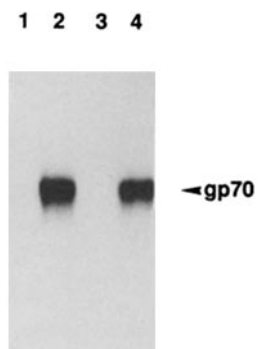
$10^5$  to  $10^6$  stimulator cells in 96-well flat-bottomed microplates (200  $\mu$ l/well of DMEM supplemented with 10% FCS,  $5 \times 10^{-5}$  M 2-ME, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 1 mM glutamine). Cultures were maintained in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C for 2 h, and were pulsed for the final 16 h with [<sup>3</sup>H]thymidine (1.0  $\mu$ Ci/well) followed by harvesting and counting.

**Thymic Adherent Cell Lines.** Thymic cell lines were established from neonatal BALB/c mice injected with  $2 \times 10^3$  PFU of WB91-GV at birth. After 10 d, thymi were removed, cells dissociated, and the thymic capsule was incubated in 0.1% collagenase (Sigma Chemical Co.) in PBS for 20 min at 37°C. Cell lines were termed NTgV-Cl and NTgV-Cl/T. The cells were distributed in cell culture flasks in DMEM supplemented with 10% FCS, 2 mM L-glutamine,  $5 \times 10^{-5}$  M 2-ME, modified Eagle's medium, nonessential amino acids, sodium pyruvate, and 1 mM Hepes buffer, and maintained at high cell density for several weeks with periodic removal of nonadherent cells. Stromal monolayers of mixed morphology were evident at  $\sim 3$  wk of culture; in some cultures (NTgV-Cl/T), enrichment for epithelial cell populations was accomplished by periodic removal of contaminating fibroblasts by brief treatment with Trypsin-EDTA (Gibco Laboratories, Grand Island, NY).

Viral replication was determined by RT assay as described previously (14). Controls included culture supernatants from normal d14 BALB/c primary fetal liver cell cultures, a thymic cortical reticular epithelial cell line derived from SV40-transgenic mice designated 1308.1 (18a), or C57SV, an SV40-transformed embryonic stromal cell line (19).

## Results

**The Major Immunologic Response of GMuLV-infected Mice Is to the gp70 Envelope Glycoprotein.** The antigenic specificity of the humoral immune response of adult mice challenged with GMuLV was initially evaluated by radioimmunoprecipitation. To identify viral gene products, lysates of <sup>125</sup>I surface-labeled virus-induced tumor cells were first incubated with purified goat Ig raised against the Rauscher virus glycoprotein. This reagent reacts with gp70 molecules encoded by all murine leukemia viruses. As seen in Fig. 1, a band with an apparent molecular weight of 70,000 was detected upon incubation with immune goat serum (lane 2) compared with normal goat serum (lane 1). The Ig response of normal adult mice to challenge with GMuLV displayed a similar specificity.

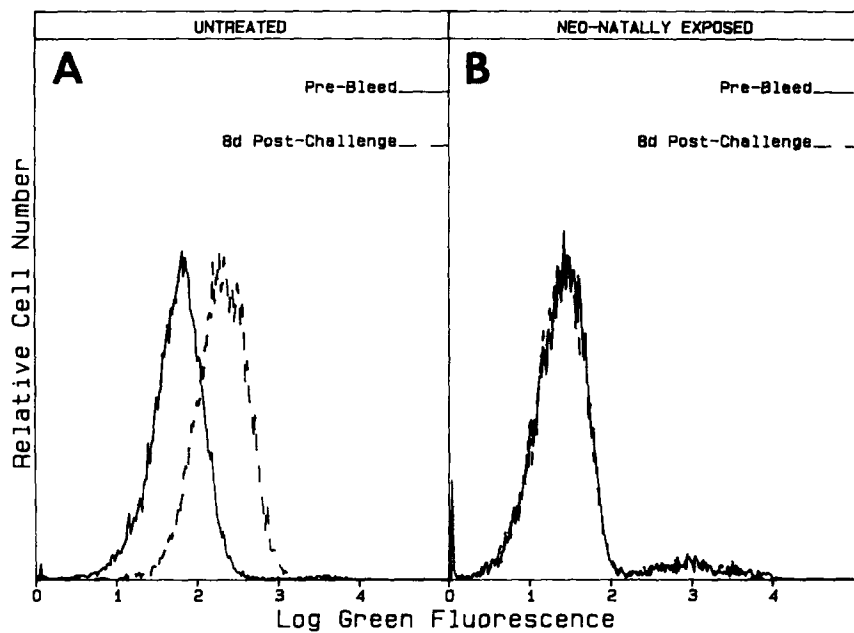


**Figure 1.** Immunoprecipitation of gp70 by GMuLV immune sera. Analysis of antisera specificity was conducted by immunoprecipitation of <sup>125</sup>I-labeled gp70-infected KKC cells. Detergent cell lysates were indirectly immunoprecipitated with normal goat Ig (lane 1), goat anti-Rauscher virus gp70 antiserum (lane 2), normal mouse serum (lane 3), or 8-d post-bleed serum from an adult BALB/c mouse challenged with  $2 \times 10^3$  PFU of WB91-GV (lane 4). Precipitates were separated by 10% SDS-PAGE run under reducing conditions and bands were detected by autoradiography.

Incubation of cell lysates with serum taken from a normal 6-wk-old BALB/c mouse 8 d after intraperitoneal injection of GMuLV resulted in the precipitation of a band of 70,000 (lane 4), compared with normal mouse serum (lane 3). This band was shown to correspond to the viral envelope glycoprotein gp70 by successful pre-clearing of lysates with goat anti-Rauscher gp70, before immunoprecipitation with mouse serum (data not shown). These results indicate that exposure of normal adult BALB/c mice to intact GMuLV elicits a humoral immune response directed predominantly against the viral gp70 molecule. However, responses to other viral proteins can be detected by this analysis. Over-exposure of the same gels illustrated weak reactivity to proteins with apparent molecular weights of 15,000, 30,000, and 43,000. These bands are believed to represent the viral proteins p15, p30, and gp43, respectively (20).

**Neonatal Exposure to GMuLV Diminishes the Ability of Adult Mice to Mount a Virus-specific Antibody Response.** The effect of neonatal exposure to GMuLV on adult immune responsiveness was determined by FACS analysis of serum Ig binding to GMuLV-induced tumor cells. The exclusive detection of gp70 in immunoprecipitates of tumor cells, as described above, ensures that FACS analysis detects specific binding of retrovirally encoded molecules. To assess the impact of neonatal exposure to GMuLV, mice were intraperitoneally injected with  $2 \times 10^3$  PFU of virus at 6–12 h after birth (these were termed neonatally treated mice) or left untreated. Untreated and neonatally treated mice were then challenged by intraperitoneal injection with  $2 \times 10^3$  PFU of GMuLV at 6–8 wk of age. Serum samples obtained at pre- and post-challenge were incubated with GMuLV-infected tumor cells over a range of serum dilutions to accurately assess antibody titer. Cells were then incubated with a fluoresceinated goat anti-mouse Ig conjugate, and subsequently analyzed by flow cytometry. Increases in the level of fluorescence between populations of cells treated with equivalent dilutions of pre- or post-challenge sera reflect the level of antibody molecules binding viral proteins on the surface of these cells. As shown in Fig. 2A, there was a 3.5-fold increase in staining of cells incubated with post-challenge serum compared with pre-immune serum. In contrast, there was no detectable difference in staining when comparing cells incubated with pre- or post-challenge serum from a neonatally treated mouse (Fig. 2B). A close comparison of the FACS profiles of pre-bleed sera for the two representative animals shown in Fig. 2 also illustrates that the level of endogenous (i.e., pre-challenge) antiviral antibody was decreased in neonatally treated mice.

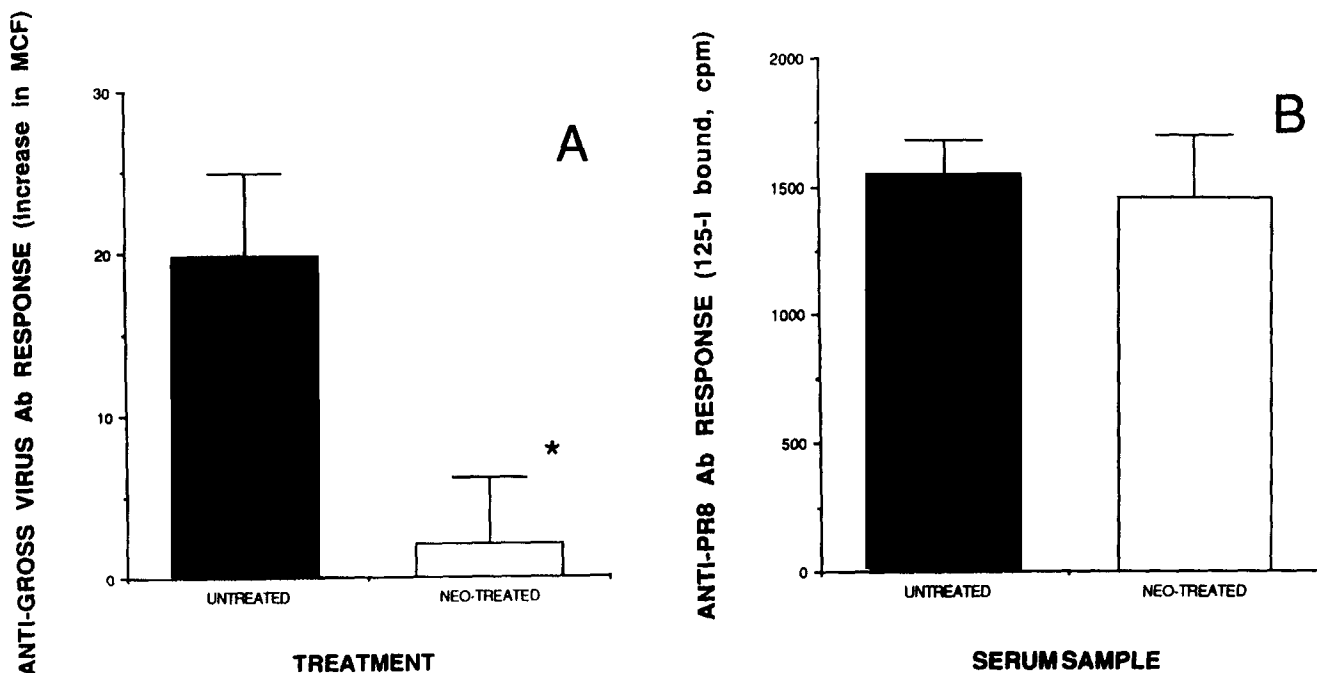
A compilation of FACS data from 10 mice treated and analyzed in an identical manner to that described in Fig. 2 is shown in Fig. 3A. Normal mice produced significantly more antibody in response to adult challenge with virus compared with neonatally treated mice. These results are in marked contrast to previous observations with LCMV (7, 21), and to studies conducted by one of us (J. Korostoff), on the effect of neonatal exposure to the PR8 strain of influenza virus (22). In this latter instance, neonatal exposure to influenza virus induced a shift in the B cell repertoire without concomitant reduction in the magnitude of the antibody response.



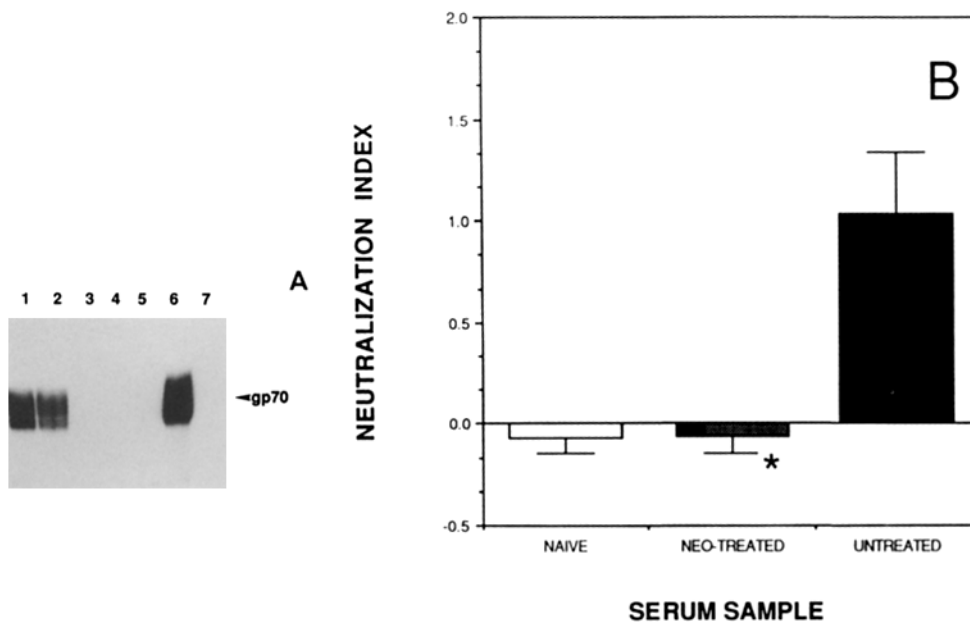
**Figure 2.** Reduced binding of serum from neonatally treated mice to gp70 on KKC cells. The level of antibody to GMuLV antigens was evaluated by FACS analysis with KKC cells. Serum was obtained from normal (A) or neonatally (B) treated BALB/c mice before and 8 d after intraperitoneal challenge with  $2 \times 10^3$  PFU of WB91-GV. KKC cells were incubated with sera at a final dilution of 1:5, then treated with fluoresceinated anti-Ig reagents and analyzed on a FACS IV flow cytometer (Becton Dickinson & Co.).

The specificity of the inhibitory effect of neonatal exposure to GMuLV was examined by intraperitoneal challenge of mice with PR8. These results (Fig. 3 B) indicate that mice treated neonatally with GMuLV are fully capable of mounting a normal influenza-specific humoral response. Normal mice had

a 2.32-fold increase in antibody after challenge, while neonatally treated mice had a 2.34-fold increase in antibody levels. Therefore, neonatal exposure to GMuLV resulted in a specific decrease in the ability of mice to mount an antibody response to subsequent adult challenge only with the same virus.



**Figure 3.** Mice treated with GMuLV as neonates have an impairment of virus-specific Ig production. Humoral immune response to GMuLV. (A) A compilation of FACS analyses of KKC cells incubated with serum from untreated (black bar) or neonatally treated (open bar) adult mice 8 d after challenge with WB91-GV ( $n = 10$ ). Data are expressed as the change in mean channel fluorescence of cells incubated with pre-challenge serum vs. that for cells incubated with post-challenge samples. (\*)  $p < 0.05$  by unpaired student's *t*-test. Humoral response to the PR8 strain of influenza virus. (B) Data obtained from RIA of serum from untreated (black bar) and GMuLV neonatally treated (open bar) adult mice 8 d after challenge with 1,000 HAU of influenza virus ( $n = 10$ ). The data are expressed as the difference in cpm of  $^{125}$ I bound between sera obtained before and after challenge. The responses of these groups were not significantly different.



**Figure 4.** Serum from neonatally treated mice does not immunoprecipitate or neutralize GMuLV. Immunoprecipitations were conducted as described in Fig. 1. (A) The autoradiograph of  $^{125}\text{I}$ -labeled KKC cell immunoprecipitates run on 10% SDS-PAGE. Lysates were immunoprecipitated with sera from normal adult BALB/c mice challenged with WB91-GV (lanes 1 and 2), post-challenge sera of neonatally treated mice (lanes 3, 4, and 5), goat anti-Rauscher virus gp70 antiserum (lane 6), and normal mouse serum (lane 7). Analysis of the virus neutralization activity of sera was determined by direct inhibition of plaque formation. (B) Pooled data obtained from in vitro virus neutralization assays performed on naive mouse sera (open bar), and sera from neonatally treated (hatched bar) or untreated (black bar) mice challenged with WB91-GV. Neutralization indices

were calculated as the difference in the  $\log_{10}$  titer of GMuLV samples with or without serum. The neutralizing activity in the serum of normal mice after GMuLV challenge was significantly greater than that of serum from neonatally treated mice; (\*)  $p < 0.005$ . The neutralization indices determined for neonatally treated and naive mice were not significantly different.

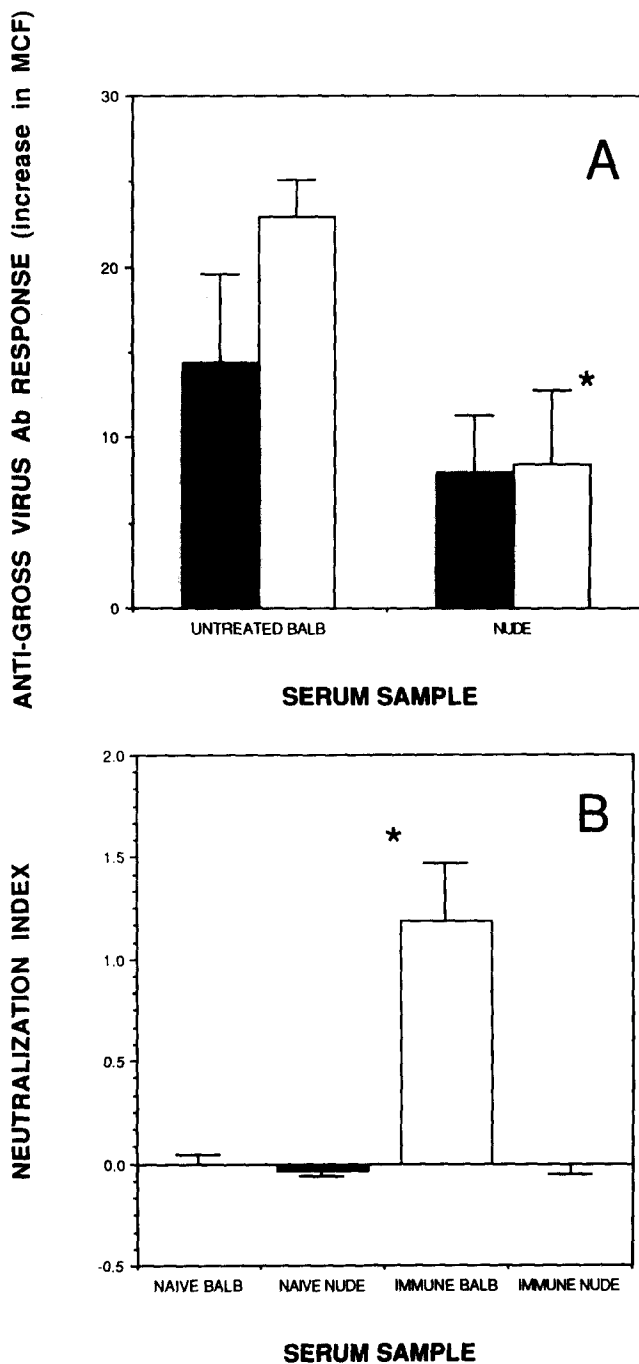
Previous studies by Oldstone and colleagues (7, 8, 21) have shown that virus-specific antibody may pass undetected due to the sequestration of antibody in the form of circulating or tissue-deposited immune complexes. To address this possibility, the serum and kidneys of mice were examined for the presence of immune complexes, in a manner similar to that described in the LCMV system. There were no detectable differences in the levels of circulating or tissue-deposited immune complexes when comparing normal adult mice with neonatally treated mice rechallenged with GMuLV as adults (data not shown).

**Neonatally Treated Animals Lack both Immunoprecipitating and Neutralizing Antibody Responses.** The observation that neonatally treated mice exhibited reduced levels of virus-specific antibody prompted analysis of the physiological significance of this hyporesponsiveness. Representative serum samples were first analyzed for their ability to precipitate viral proteins from lysates of GMuLV-infected cells. As seen in Fig. 4 A, sera from normal adult mice challenged with virus (lanes 1 and 2) contained antibody capable of immunoprecipitating gp70 from cell lysates. In contrast, sera from neonatally treated mice (lanes 3, 4, and 5) failed to immunoprecipitate gp70 or any other viral proteins under the same conditions. Similar immunoprecipitation analysis of tumor cell lysates that were metabolically labeled with  $^{35}\text{S}$ -methionine also failed to detect any response to viral proteins (data not shown). This observation shows that the humoral immune response of mice exposed to GMuLV as neonates is not redirected towards a nonenvelope viral antigen.

Serum samples were also tested for their ability to neutralize infectious virus by in vitro plaque-forming assays. As

seen in Fig. 4 B, sera from normal adult mice (untreated) challenged with GMuLV contained substantial neutralizing activity. These sera were significantly more effective at neutralizing the virus compared with sera from neonatally treated or naive normal mice, which showed no neutralizing activity. Taken together, these results indicate that exposure of mice to virus as neonates totally abrogates their ability to produce physiologically relevant virus-binding or neutralizing antibody in response to adult challenge with GMuLV.

**Nude Mice Do Not Mount Normal Humoral Responses to Challenge with GMuLV.** To determine the role of T cells in the generation of normal anti-Gross virus-specific humoral responses, 6–8-wk-old normal BALB/c or male BALB/c *nu/nu* mice were intraperitoneally injected with GMuLV on days 0 and 8, and bled on days 0, 8, and 16. The serum samples obtained from these bleeds were analyzed for the presence of GMuLV-specific antibody, and for the ability to neutralize virus in the in vitro plaque assay. Although nude mice did mount a virus-specific antibody response to GMuLV challenge, the kinetics as well as the magnitude of this response were markedly different from that seen in normal mice (Fig. 5 A). A large amount of antiviral antibody could be detected in the sera of normal mice 8 d after primary exposure, and this activity increased to a maximum level at 8 d post-secondary challenge. In contrast, sera from nude mice contained considerably less GMuLV-specific antibody after primary exposure, and failed to show any signs of a secondary response. A marked difference was also detected when the function of anti-GMuLV antibodies in nude mice was examined (Fig. 5 B). Ig from nude mice was incapable of neutralizing infectious virus, whereas the response of normal mice displayed potent neu-



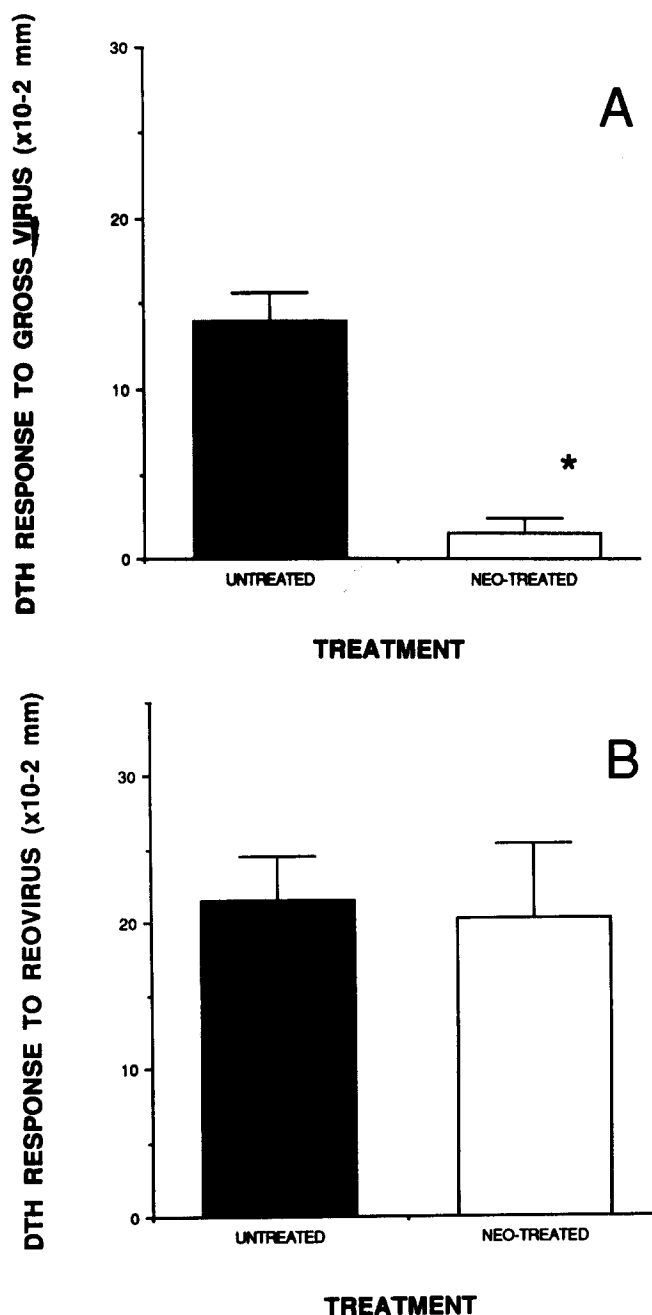
**Figure 5.** Nude mice have impaired humoral responses to GMuLV. Serum Ig responses were determined by FACS analysis as described in Fig. 2. (A) The FACS analysis of serum obtained from normal and nude mice 8 d after primary (black bar) and secondary (open bar) intraperitoneal challenge with  $2 \times 10^3$  PFU of WB91-GV. The secondary responses of normal mice were significantly greater than those of nude mice; (\*)  $p < 0.02$ . Sera were also analyzed for the presence of neutralizing antibody as described in Fig. 4. (B) The neutralizing activity of serum from naive mice, immune BALB/c mice (normal BALB/c mice 8 d after virus challenge), naive nude mice, and immune nude mice (nude mice 8 d after viral challenge). The neutralizing activity in the serum of normal mice after virus challenge was significantly greater than that of serum from virally challenged nude mice; (\*)  $p < 0.002$ .

tralizing activity. These results suggest that T cells play an important role in the generation of a physiologically normal anti-GMuLV response, and that the impaired humoral response seen in neonatally treated mice may be related to a form of T cell nonresponsiveness.

*Neonatally Treated Mice Have Impaired Virus-specific T Cell Function.* To address the effect of neonatal exposure to virus on subsequent T cell responsiveness, delayed type hypersensitivity (DTH) responses to GMuLV challenge were determined. The DTH response provides a reliable measure of in

vivo Th cell function. Normal or neonatally treated BALB/c mice were intraperitoneally injected with GMuLV, and after 6 d, animals were challenged by paired footpad injection with either virus or media. DTH responses were measured 24 h later. The data in Fig. 6 A illustrate a significant decrease in the magnitude of DTH responses elicited in neonatally treated mice compared with that of normal mice. The specificity of this inhibition was demonstrated by normal DTH responses in the same mice to antigenically irrelevant reovirus (Fig. 6 B).





**Figure 6.** Neonatal exposure to GMuLV abolishes virus-specific DTH responses. DTH reactions of untreated and neonatally treated BALB/c mice were measured by a footpad swelling assay. (A) Untreated (black bar) or neonatally treated (open bar) mice were immunized intraperitoneally with  $2 \times 10^3$  PFU of WB91-GV and were rechallenged 6 d later by paired footpad injection with either virus or media. Footpad swelling was measured after 24 h. The response of these same mice to subsequent challenge with  $3 \times 10^6$  PFU of reovirus is shown in B. The response of untreated mice to GMuLV was significantly greater than that of neonatally treated mice; (\*)  $p < 0.001$ . The response of neonatally treated mice was not statistically significant compared with background. The responses of reovirus immunized mice were not significantly different.

As a more direct measure of isolated T cell activity, in vitro proliferative responses of enriched T cell populations prepared from the spleens of normal and neonatally treated mice were measured. Antigen-primed T cells were cocultured with syngeneic, GMuLV-infected, or uninfected spleen cells for 48 h. [ $^3$ H]Thymidine was added for the last 16 h of culture, after which cells were harvested and the counts incorporated determined. Data shown in Table 1 demonstrate a significant diminution in the GMuLV proliferative response of enriched T cells from neonatally exposed mice compared with that of cells from normal mice. Thus, neonatal exposure to GMuLV induces a state of severe T cell nonresponsiveness in adult mice.

*Thymic Stromal Cells Support the Replication of GMuLV In Vivo* Previous work has shown that GMuLV infects cells of lymphoid origin within the thymus (23, 24). Replication of virus within nonlymphoid thymic cells was investigated by measuring RT activity in supernatants from adherent cell lines established from the thymi of mice infected with GMuLV at birth. As shown in Table 2, significant levels of RT activity were detected in the supernatants of two such cell lines: NTgV-CI and NTgV-CI/T. Both lines were enriched for thymic epithelial cells. RT activity was not detected in supernatants from uninfected cell lines, including 1308.1, a thymic cortical reticular epithelial cell line. Immunohistochemical analysis of the adherent stromal cell cultures showed that 25–30% of these cells expressed viral gp70 on their cell surface, and that these cells were of a CD4<sup>-</sup>/CD8<sup>-</sup>/CD3<sup>-</sup> phenotype

**Table 1.** GMuLV-primed T Cells Fail to Proliferate in Response to Coculture With Virally Infected Syngeneic Splenocytes

	[ $^3$ H]TdR incorporation	
	Normal mice	Neonatally treated mice
	<i>cpm</i>	
Normal splenocytes*	3,493	3,741
GMuLV-infected splenocytes*	5,502	3,689
Virus-specific response <sup>†</sup>	1,829	0 <sup>§</sup>
Con A	28,924	41,978

Pooled antigen-primed T cells were prepared from the spleens of either normal or neonatally treated BALB/c mice 2 wk after intraperitoneal challenge with  $2 \times 10^3$  PFU of GMuLV. Normal responders alone incorporated 3,165 cpm and neonatally treated responders incorporated, 3,673 cpm (means of triplicate samples).

\* The data represent mean values of [ $^3$ H]TdR incorporation of 12 samples.

<sup>†</sup> Virus-specific response was calculated as: response to GMuLV infected splenocytes minus response to normal splenocytes.

<sup>§</sup> The virus-specific proliferative response of cells from normal donors was significantly greater than that of cells from neonatally treated animals ( $p < 0.05$ ) as determined by *F* test statistics.

**Table 2.** Replication of GMuLV in Thymic Stromal Cells

Cell type	Description	RT activity
Media		<i>cpm</i> 135 ± 33*
Kgv	GMuLV-induced T lymphoma	4,677 ± 270
NTgV-C1	Mixed thymic stromal cells	1,449 ± 6†
NTgV-C1/T	Enriched thymic epithelial cells	1,068 ± 15†
Fetal Liver	Primary culture from normal day 14 embryos	43 ± 11
1308.1	SV40-transformed thymic cortical reticular epithelial cell line	80 ± 7
C57.SV	SV40-transformed embryonic stromal cell line	62 ± 11

\* Data represent mean and SEM of <sup>32</sup>P-TTP incorporation of duplicate samples.

† These values are significantly different from the media control ( $p < 0.001$ ), as determined by the student's *t* test.

(data not shown). Greater than 95% of cells from both lines reacted with a mAb (ER-TR5) that exclusively detects thymic medullary epithelial cells (data not shown). These data indicate that GMuLV infects and subsequently replicates in non-lymphoid thymic stromal cells.

### Discussion

Analyses of multiple parameters of B and T cell function showed that neonatal exposure of mice to GMuLV dramatically altered immune responses to adult challenge with this virus. Exposure of naive BALB/c mice to GMuLV at 6–8 wk of age stimulated a vigorous anti-Gross virus humoral response. The predominant response was towards the viral glycoprotein, gp70, as detected by immunoprecipitation from lysates of radiolabeled GMuLV-induced tumor cells. The antibody molecules generated are potent at neutralizing infectious virus. It is interesting to note that the antiretrovirus immune response in a naive adult mouse does not necessarily protect an animal from virus infection. Upon immunization with parental GMuLV, BALB/c-H-2<sup>k</sup> (BALB.K) mice develop a persistent infection despite the generation of virus-neutralizing antibody (25). The basis of this appears to lie with the development of variant viruses that differ within the neutralization-sensitive epitopes expressed on the gp70 molecule. The result of these antigenic variations is that BALB.K mice do not generate an effective antiviral immune response. Preliminary studies suggest that similar types of variants arise in BALB/c and BALB/c-H-2<sup>b</sup> (BALB.B) mice.

In contrast to the response of naive adult mice, the sera of identically immunized but neonatally treated mice, contain barely detectable levels of antiviral antibody by direct binding analysis, and were not capable of either precipitating viral proteins or neutralizing infectious virus. This effect is antigen specific in that neonatally treated animals exhibited normal humoral responses to influenza virus. The inability to detect normal levels of antiviral antibody in neonatally treated mice was not due to the formation of circulating serum or tissue-deposited immune complexes.

These observations demonstrate that the low level of antibody in the serum of hyporesponsive, neonatally treated animals are of extremely low affinity and are therefore of little physiologic relevance. This hypothesis is supported by prior descriptions of the presence of low affinity tolerogen-binding antibody in naturally occurring and experimentally induced models of immunologic nonresponsiveness (26, 27). These observations also bear on the presence of endogenous anti-MuLV antibody (20, 28). GMuLV crossreactive antibody is present in the serum of naive, normal mice but was not detected in the neonatally treated mice, indicating that neonatal exposure to GMuLV also abrogates the ability to respond to related endogenous MuLVs. The presence of anti-GMuLV-specific antibody in AKR mice (8), which endogenously express this virus, seems to argue against this hypothesis. However, the presence of maternally transmitted virus-specific antibody and low level of endogenous virus production in newborn AKR mice (29) probably preclude the development of neonatal tolerance.

The B cell hyporesponsiveness seen in neonatally treated mice can be explained by a number of mechanisms, all of which have been demonstrated experimentally by others. Possibilities include the physical elimination (30) or functional silencing of antigen-reactive B cell clones (31, 32). Determination of the frequency of anti-GMuLV-specific B cell precursors by limiting dilution analysis in normal and neonatally treated mice would resolve this issue. However, the presence of low but detectable levels of antiviral antibody in the serum of some neonatally treated animals argues against the absolute deletion or silencing of reactive clones in our system. A more likely hypothesis is that B cell hyporesponsiveness is linked to the abrogation of GMuLV-specific T cell function. For example, it has recently been reported that B cell nonresponsiveness in transgenic mice expressing a vesicular stomatitis virus cell membrane-associated glycoprotein is dependent on the loss of T cell help (33).

To address this possibility, the role of T cells in the generation of a physiologically normal anti-GMuLV antibody response was first determined. These studies showed that nude

mice failed to express typical levels of neutralizing antibody and secondary responses after GMuLV challenge, analogous to the response of neonatally treated mice. The inability of serum from nude mice to either neutralize infectious virus, or immunoprecipitate viral antigens (J. Korostoff, unpublished data), suggests that these antibodies are of low affinity, and most likely of the  $\mu$  isotype. The weak antibody responses of nude and neonatally treated mice may therefore be due to direct activation of B cells recognizing T cell-independent epitopes within the structure of the gp70 molecule. A similar model was previously described for the nucleocapsid antigen of Hepatitis B virus (34).

The requirement for virus-specific T cell help in the generation of a normal anti-GMuLV antibody response lead to direct analysis of the effect of neonatal exposure to GMuLV on CD4<sup>+</sup> T cell function. It has been previously shown that neonatal exposure to Moloney murine leukemia virus resulted in severe reduction in the frequency of precursors to virus-specific cytotoxic (CD8<sup>+</sup>) T cells (10). In this regard, GMuLV-specific DTH and *in vitro* proliferative responses were determined. Neonatally treated mice did not show a significant DTH response after challenge with GMuLV, despite normal responses of the same mice to antigenically irrelevant reovirus. Further, enriched T cells prepared from the spleens of neonatally treated mice failed to show any proliferative response to GMuLV-infected syngeneic splenocytes despite normal responses to Con A. The observation of antigen-specific CD4<sup>+</sup> T cell nonresponsiveness supports the hypothesis that B cell hyporesponsiveness is a reflection of the absence of T cell help (32, 33, 35).

Recent work from a number of laboratories has shown that T cells can be rendered nonresponsive by intrathymic clonal abortion (36, 37), functional inactivation (38), or the induction of peripheral immunoregulatory phenomena such as suppressor T cells (39). The data presented here do not allow us to define the mechanism operative in this system. However, by determining the frequency of precursors to GMuLV-specific CD4<sup>+</sup> T cells, as well as studying the effects of adoptive transfer of cells from neonatally treated mice on the immune competence of normal recipient mice, it will be possible to elucidate the events leading to the T cell nonresponsiveness seen after neonatal exposure to GMuLV.

Although our data agree with the results of others regarding the induction of neonatally induced nonresponsiveness to retroviruses (10, 40, 41), other systems have been described in which neonatal or congenital exposure to virus does not alter virus-specific immune responsiveness. The most notable example is LCMV. Oldstone and colleagues (reviewed in reference 21) have shown that mice persistently infected with LCMV from birth produce similar amounts of antibody with the same specificities to that found in normal LCMV immune mice. However, most of the antiviral antibody in persistently infected mice was sequestered in the kidneys in the

form of immune complexes. Jamieson and Ahmed (9) have also shown that persistently infected animals are capable of mounting antiviral cytotoxic T cell responses after adoptive immunotherapy to reduce the viral load, suggesting that the precursors to these cells are neither clonally deleted nor irreversibly inactivated. Why then does one type of virus, e.g., GMuLV, induce immunologic nonresponsiveness, while another, e.g., LCMV, does not?

It is commonly accepted that the thymus is intimately involved in the induction of T cell nonresponsiveness to self-antigens. Therefore, it follows that the more efficient a molecule is at reaching (and subsequently being processed and presented in) the thymus, the more potent its tolerogenic effect. The analysis of stromal cell lines derived from the thymi of mice neonatally infected with GMuLV showed infection and replication of virus within nonlymphoid cells of the thymus (Table 2). GMuLV also appears to infect cells at many stages along the T cell differentiation pathway, including the CD4<sup>-</sup>/CD8<sup>-</sup> precursors that give rise to functionally mature T cells (24). Preliminary results from our laboratory indicate that at 8 d of age, 5% of thymocytes isolated from neonatally treated mice are infected with GMuLV. This increases to 17% by 3 mo of age. The dual tropism for lymphoid and nonlymphoid thymic cellular compartments allows rapid accumulation of GMuLV within thymic cells, some of which are intimately involved in shaping the T cell repertoire. The continuous production of GMuLV throughout the life of a neonatally infected mouse would serve to maintain the nonresponsive state by peripheral inactivation of any high affinity antiviral-specific lymphocytes that escaped tolerance induction (42, 43).

The presence of LCMV-specific antibody in congenitally and neonatally infected mice can be explained in a reciprocal fashion. LCMV infects mature peripheral CD4<sup>+</sup> T cells (44), and would therefore be predicted to be far less efficient at accumulating in the thymus compared with viruses that infect cells early in the T cell differentiation pathway. It has been shown that <1% of thymocytes from 6-mo-old LCMV carrier mice are actively infected with virus (45). There is no evidence of LCMV infection of thymic stromal elements. Recently, it has been demonstrated that neonatally infected transgenic mice delete CD4<sup>+</sup>/CD8<sup>+</sup> thymocytes that bear a high affinity anti-LCMV TCR- $\alpha/\beta$  (46). The work of others indicates, however, that the low level of intrathymic LCMV infection is insufficient to physically eliminate the majority of LCMV-specific T cells (7, 9, 21). To determine the validity of this model, we are currently analyzing thymocytes and stromal cell lines derived from neonatally infected mice to more accurately quantitate the rate of accumulation of viral material within the thymus. Cloning of GMuLV-infected thymic stromal cell lines is also in progress in order to determine the phenotypic characteristics and functional properties of these cells.

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