AUTOGONOUS IMMUNITY TO ENDOGENOUS RNA TUMOR 
VIRUS 
IDENTIFICATION OF ANTIBODY REACTIVITY TO SELECT VIRAL ANTIGENS*

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It has recently been demonstrated that certain strains of mice characterized by a 
low incidence of spontaneous lymphoma produce significant levels of natural antibody 
specific for murine leukemia virus (MuLV)1 (1-3). A radioimmune precipitation 
assay utilizing 3H-labeled AKR leukemia virus (2) was used to show an age-associated 
modulation of the levels of this autogenous humoral immune response to endogenous 
MuLV. Virus-precipitating antibody levels increased as the mice developed immuno-
logic competence and then decreased as a function of senescence. The specificity of 
this natural antibody for MuLV envelope antigens was demonstrated by immuno-
electron microscopy (3), and was also associated with the chronic development of 
glomerulonephritis (1, 3). Furthermore, in a population of RFM mice we have shown 
an inverse correlation between spontaneous lymphoid neoplasia and the severity of 
glomerulonephritis, which has been demonstrated in part to be caused by deposition 
of MuLV immune complexes (1). These data provide a basis for the consideration of 
host immunologic mechanisms in the control of endogenous virus burden and of 
leukemogenesis.

Additional evidence for the significance of autogenous immunity to leukemia 
virus is provided by the fact that the Rge-1 locus, which causes resistance to 
leukemogenesis induced by Gross-passaged A virus in neonatal mice, may be 
closely associated with or identical to the Ir-i gene, which determines immune 
responsiveness to certain antigens (4), and thus may function in immune con-
rol of MuLV antigens (4, 5). The possibility must be considered that while 
the viral genome is ubiquitous in mouse cells and can be activated, and al-
though cell susceptibility to virus infection is controlled by genetic factors 
(Fv1, Fv2, etc.), an additional level of immunological control may be significant 
in preventing the intercellular spread of endogenous leukemia virus, which may 
play a major role in leukemogenesis. Therefore, the immunological factors that 
may limit endogenous virus burden should be characterized.

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1 Abbreviations used in this paper: MuLV, murine leukemia virus; SPF, specific pathogen-
free; TNE, 0.05 M Tris (pH 7.5)-0.1 M NaCl-1 mM ethylenediaminetetraacetate.
Considerable research has been directed at identifying and isolating the polypeptide components of mammalian C-type tumor viruses, as well as classifying serological reactivities against viral components. Such studies have used heterologous antisera against purified viral components (6, 7) or against disrupted virus (8), or more commonly have used antisera prepared from tumor-bearing animals in heterologous systems (9, 10). In the present studies we have attempted to identify the viral components that are specifically recognized by natural autogenous immune sera.

Materials and Methods

Animals.—Male BALB/c and B6C3F1 (C57BL/6 × C3H/Anf) of various ages were used. C57BL/6 female parents and C3H/Anf male parents of the B6C3F1 were also used; these mice were approximately 6 mo of age and were retired breeders of their respective inbred colonies. All mice were specific pathogen-free (SPF).

Test Sera.—Blood was collected by orbital bleeding or cardiac puncture and allowed to clot at room temperature, and serum was separated by centrifugation. Samples of serum were frozen at −70°C until use. (Samples of serum were never thawed more than once.)

Anti-K36 test serum was prepared by immunizing C57BL/6 males with K36 cells derived and passaged in AKR mice. The immunization regimen consisted of an initial subcutaneous injection of 2 × 10⁷ cells, followed by four intraperitoneal injections of 2 × 10⁷ cells every 2 wk. Mice were bled 10–14 days after the last immunization, and serum was prepared as described above. The various normal and test sera were tested for antibody to MuLV envelope antigen by radioimmune precipitation assay as described below and by immunoelectron microscopy as described previously (3).

Preparation of Radioactively Labeled Virus.—[3H]leucine-labeled virus was prepared as previously described (2). [3H]glucosamine-labeled virus was prepared similarly, using Eagle's minimal essential medium that was supplemented with 2 mM glutamine and 10% fetal calf serum and contained 50 μCi/ml of D-glucosamine-[6-3H]hydrochloride (12.6 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.).

Radioimmune Precipitation Assays Against Disrupted Virus.—Intact [3H]leucine-labeled AKR virus was purified and recovered from isopycnic sucrose gradients as previously described (2). The virus was diluted fivefold with TNE buffer (0.05 M Tris, pH 7.5-0.1 M NaCl-1 mM ethylenediaminetetraacetate) and concentrated by pelleting at 4°C in a Beckman 65 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 40,000 rpm for 2 h. The viral pellet was resuspended in a minimal volume of TNE and centrifuged at 2,000 rpm for 20 min in an International PR-2 centrifuge (International Equipment Co., Needham Heights, Mass.). Aliquots of the clarified virus were frozen at −70°C until use. Immediately before radioimmune precipitation assays, aliquots of virus were mixed with an equal volume of disruption buffer (1.2 M KCl-1% Triton X-100-0.03 M β-mercaptoethanol-0.1 M Tris, pH 7.5). The virus was disrupted at 4°C for 1 h. The extract was then either diluted 10-fold with distilled water or extracted five times at 25°C with 3 vol of ether; residual ether was removed by passing nitrogen gas over the sample. In either case the extracts were centrifuged at 2,000 rpm for 20 min at 4°C in an International PR-2 centrifuge immediately before the assay. The two techniques gave essentially identical results.

For titration assays, the test serum (0.1 ml) was twofold serially diluted in TNE, and the antigen (~60,000 cpm) in 0.05 ml was added. The mixture was incubated at 37°C for 3 h and at 4°C for 14 h. The samples were then diluted with 0.9 ml of TNE and 0.2 ml of a polyspecific antilgoglobin (Cappel rabbit antimouse gamma-globulin or Cappel goat antirabbit gamma-globulin) was added. The precipitates were allowed to form at 37°C for 2 h and at 4°C for 3 h. The precipitates were collected by centrifugation at 2,000 rpm for 15 min in an International PR-2 centrifuge, and the supernate was removed for determination of radio-
activity. The precipitates were washed five times with 2-ml aliquots of TNE, resuspended in 1 ml of distilled water, and prepared for scintillation counting. All samples were counted in 10 ml of Aquasol (New England Nuclear, Boston, Mass.) in a Nuclear-Chicago scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Precipitation is expressed as the percentage of counts in the precipitate relative to the combined counts in the precipitate and the first supernate. The radioimmune precipitation titer is defined as the serum dilution at which 50% of the maximum radioactivity is precipitated.

To prepare immune precipitates for polyacrylamide gel electrophoresis, 0.05 ml of serum was added to 1.0-2.0 X 10⁶ cpm of antigen and reacted as above. The immune complexes were precipitated as above with 0.1 ml of antiglobulin, and the precipitates were collected and washed four times with TNE as above. After the last wash the precipitates were resuspended in 1 ml of TNE and overlaid on 1.0 ml of a 25% sucrose solution containing TNE and 0.5% deoxycholate. The precipitates were pelleted at 2,000 rpm for 20 min in an International PR-2 centrifuge (International Equipment Co.) and finally resuspended in 0.05 ml of a solution containing 1% SDS, 1% β-mercaptoethanol, and 0.01 M sodium phosphate buffer at pH 7.4. The mixture was incubated at 60°C for 1 h and at 37°C for 14 h to dissolve the precipitate before electrophoresis.

Radioimmune Precipitation Assays Against Intact Virus.—Titration assays against intact virus were performed as previously described (2). To determine the antigens recognized in the precipitation of intact virus, the virus was precipitated at a 1:8 dilution of test serum. The immune precipitate was resuspended in 0.5 ml of TNE, and 0.5 ml of disruption buffer was added. The mixture was incubated at 4°C for 1 h, and the immune precipitate was collected, washed, and prepared for gel electrophoresis as described above.

SDS-Polyacrylamide Gel Electrophoresis.—SDS-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (11). Bromphenol blue was used as a reference standard to determine relative mobilities and was generally allowed to migrate 8 cm. Gels of standard protein samples to calibrate the system were stained with Coomassie blue (ICI America Inc., Stamford, Conn.). Radioactivity profiles of the gels were obtained by sectioning the gels into 1-mm sections with a Mickel gel slicer, dissolving individual sections in 0.2 ml of 30% hydrogen peroxide at 75°C overnight, and determining radioactivity in a liquid scintillation counter after the addition of 10 ml of Aquasol (New England Nuclear).

gs Antiserum.—The gs-1 antigen from Rauscher leukemia virus was purified from deoxycholate-disrupted virus by G-150 gel filtration and fractional ethanol precipitation (J. N. Ihle and F. T. Kenney, unpublished results). Antiserum was prepared by immunizing New Zealand white rabbits with 0.3 mg of gs antigen in Freund's complete adjuvant injected intramuscularly, followed by intravenous injection of 0.3 mg of gs antigen 2 wk later. Serum samples were collected by ear bleeding.

RESULTS

Standard 1.5 yr B6C3F₁ serum titrated against [³H]leucine-labeled, intact MuLV precipitated 90% of the label at high serum concentration and had a radioimmune precipitation titer of 320 (Fig. 1). However, when the same serum was reacted with solubilized proteins from virus disrupted with Triton and high salt, considerably less of the radioactivity was precipitated and the concentration dependence for precipitation yielded a more complex curve. At high serum concentration approximately 8% of the radioactivity in the solubilized preparations was precipitated. This percentage dropped to 1.5% at a 1:80 dilution, remained above 1% to a dilution of 1:640, and then dropped again at higher dilutions. In contrast to these results, a titration of antiserum prepared in rabbits against gs antigen gave a typical immune precipitation curve with the
same solubilized viral extract. Normal rabbit serum titrated against the same extract failed to precipitate any radioactivity. These data suggest that only a small fraction of the solubilized viral proteins is recognized by the B6C3F1 serum. Also, the complex nature of the titration curve suggests that two or more components are probably involved.

To determine which viral proteins were being recognized and precipitated by B6C3F1 serum, immune precipitates were examined by SDS-polyacrylamide gel electrophoresis. The gel profile of an immune precipitate obtained when B6C3F1 serum was reacted with disrupted virus is shown in Fig. 2 A. Three viral protein peaks are discernible, which migrate during electrophoresis with mobilities (relative to bromphenol blue) corresponding to mol wt of approximately 68,000, 43,000, and 17,000, based on the standard curve shown in Fig. 3. In Fig. 2 B the gel profile of the total extract of viral proteins is shown for comparison. It is apparent that normal B6C3F1 serum reacts only with minor components of the virus proteins.

To ensure that the components observed were being specifically precipitated by the B6C3F1 serum, reactions of disrupted virus with two control sera were examined. In Fig. 4 is shown the gel profile of an immune precipitate of rabbit anti-gs serum reacted with disrupted virus. As expected, most of the radioactivity (85%) migrates with an apparent mol wt of 31,000. Also shown in Fig. 4 is the gel profile of normal rabbit serum reacted with disrupted virus, which yields no discernible peaks of radioactivity. These data indicate that the proteins observed with B6C3F1 immune precipitates are specific and reflect a natural immunological reaction with these viral components.

The above results indicate that normal B6C3F1 serum reacts immunologically
Fig. 2. (A) SDS-polyacrylamide gel electrophoresis profile of an immune precipitate of B6C3F1 serum reacted with a Triton-ether extract of AKR virus. (B) Electrophoretic profile of the total Triton-ether extract. Mobilities are plotted relative to the migration of bromophenol blue.
with specific viral components, but they do not show which of the antigens observed are responsible for precipitation of the intact virus in the radioimmune precipitation assay. To resolve this question we reacted intact virus with B6C3F1 serum as in a standard radioimmune precipitation assay. The immune precipitates, which contained 90% of the initial radioactivity, were washed as described in the Materials and Methods. The immune precipitates were subsequently treated with Triton and high salt to disrupt the virus and solubilize the viral proteins. After disruption, the immune precipitates were again collected and washed as described above. In control experiments, when labeled virus was added in the presence of Triton and high salt to immune precipitates formed in the absence of labeled virus, no radioactivity was precipitated. Furthermore, antigen-antibody complexes that formed initially were not dissociated. Therefore, under these conditions only immune precipitates formed upon reaction with the intact virus are preserved. These manipulations resulted in solubilization of approximately 95% of the initially precipitated radioactivity. The final immune precipitate was subsequently analyzed by gel electrophoresis (Fig. 5). The three viral proteins seen initially (Fig. 2 A) are present, although the ratios of the 17,000 to the 68,000 and to the 43,000 mol wt components are somewhat decreased. This difference might reflect altered affinities to the respective antibodies for solubilized antigens, compared to antigens in their natural state. Secondly, two additional components are observed with apparent mol wt of 19,000 and 15,000. These two peaks are occasionally present when B6C3F1 serum is reacted with disrupted virus but are more generally

Fig. 3. Standard curve for determination of molecular weights by SDS-polyacrylamide gel electrophoresis. Standards include lysozyme (14,300), chymotrypsinogen (25,700), glyceraldehyde phosphate dehydrogenase (36,000), ovalbumin (43,000), and bovine serum albumin (68,000). Mobilities were determined relative to bromphenol blue.
detected as shoulders on the peak representing the 17,000 mol wt component, apparently due to a low natural titer against these two antigens, as discussed below. These data demonstrate that the antigens recognized by the B6C3F1 serum in preparations of disrupted virus are also recognized in the intact virus, suggesting that they are viral envelope antigens.

To identify further the viral components recognized in the autogenous immune reaction, we utilized [3H]glucosamine-labeled virus. In contrast to [3H]-
leucine-labeled virus, when B6C3F1 serum is titered against disrupted [3H]glucosamine-labeled virus (Fig. 1), approximately 40% of the label is precipitated at high serum concentrations. The percentage of radioactivity subsequently drops and a plateau value is not evident. The lower titer of this serum against the [3H]glucosamine-labeled viral proteins is a result of the lower radioactive specific activity of these preparations. When the immune precipitations were analyzed by SDS-polyacrylamide gels, the results shown in Fig. 6 were obtained. Two antigenic reactivities are evident, with apparent mol wt of 68,000 and 43,000, and presumably are the same antigens that were seen when [3H]leucine was used. The results suggest that these two antigens are glycoproteins while the 17,000 mol wt antigen is not. When normal B6C3F1 serum was reacted with intact [3H]glucosamine-labeled virus, the virus was precipitated and disrupted as above, and the immune complexes were analyzed by SDS-polyacrylamide gel electrophoresis, profiles identical to that in Fig. 6 were obtained. This again suggests that these proteins are viral envelope antigens.

We have previously reported that marked differences in age-associated levels of the autogenous immune response and mouse strain differences in the immune response can be detected by the radioimmune precipitation assay (2). These differences could be due either to quantitative differences in the same antibodies or to antibodies which recognize different viral antigens. The age-associated differences in autogenous immunity of B6C3F1 serum were examined by gel electrophoresis of immune precipitates formed with 14-wk, 1-yr, and 2-yr sera. All of the profiles were identical to that shown in Fig. 2 A, indicating that any age-associated differences in the autogenous immune reaction against intact virus probably involve quantitative differences against identical antigens.

Sera from various strains of mice were examined by radioimmune precipitation (Fig. 7) and by electrophoresis (Fig. 8) to determine the consistency of the recognition of viral antigens by natural antibodies. The profile obtained with C57BL/6 serum (Fig. 8 A) is qualitatively similar to that obtained with
B6C3F1 serum but shows additional components with mol wt of 19,000 and 15,000. A profile obtained with 1 yr BALB/c serum (not shown) was identical to that found with C57BL/6 serum. The reactivity of C3H serum (Fig. 8 B) is identical to that of C57BL/6 with respect to the antigens precipitated but differs quantitatively with respect to the ratio of the 68,000 and the 17,000 mol wt components. When B6C3F1 and C3H sera were compared against identical virus preparations, the amount of radioactivity in the 17,000 mol wt component was similar for both, indicating that the difference in reactivity is in the 68,000 mol wt fraction. It may be that the C3H serum reacts with an additional antigen which fortuitously migrates in the same manner.

We also examined C57BL/6 anti-K36 serum to determine whether immunization against a MuLV-producing tumor alters the autogenous immune reaction. The radioimmune precipitation titer of C57BL/6 anti-K36 serum is significantly higher than that of control C57BL/6 serum (Fig. 7), but gel electrophoresis of an immune precipitate results in an almost identical profile compared to the control serum (Fig. 8 C).

**DISCUSSION**

There is now considerable evidence against the concept that mice are immunologically tolerant to endogenous leukemia virus. Initially, glomerular lesions observed in aged mice of certain strains, such as RFM and B6C3F1 (1, 3), NZB (12), and AKR (13, 14), were shown to be in part a result of chronic deposition of immune complexes between specific antibody and virus antigens. In some strains as much as 70% of the antibody that is eluted from kidneys of aged mice may be specific for MuLV antigen (15), and intact C-type virus has been detected in these glomerular lesions (16). The specificity of this antibody in serum and kidney eluates and the presence of free antibody specific for MuLV envelope antigens have more recently
Fig. 8. SDS-polyacrylamide gel electrophoresis profiles of immune precipitates of AKR virus disrupted with Triton and ether and reacted against C57BL/6 serum (A), C3H/Anf serum (B), and C57BL/6 anti-K36 serum (C). Mobilities are plotted relative to the migration of bromphenol blue.

been demonstrated by immunoelectron microscopy (3). The subsequent development of a radioimmuno precipitation assay has allowed further demonstration of the existence of free antibodies against MuLV envelope antigens and has allowed a quantitative evaluation of the extent of autogenous immunity as a function of age and
strain (2). The studies presented here further explored the autogenous immune reaction by attempting to identify viral proteins that are naturally antigenic. We have concentrated our studies on the B6C3F1 hybrid mouse strain, which has a mean survival time of ~884 days and a low natural incidence of thymic lymphoma (17); a similar hybrid from a reciprocal mating (C3H × C57BL)F1 has a much lower Gross virus susceptibility than either parental strain, and this resistance has been genetically associated with the Rgs-1 locus.

The immunological reactions we have observed with B6C3F1 serum are primarily with viral antigens having approximate mol wt in SDS-polyacrylamide gel electrophoresis of 68,000, 43,000, and 17,000. Furthermore, the ability of the serum to react with these antigens in intact virus suggests that they are viral envelope antigens, and the observation that only the 68,000 and 43,000 mol wt antigens are detected when [3H]glucosamine-labeled virus is used suggests that those two antigens are glycoproteins. The 68,000 mol wt antigen we have observed here may correspond to the viral envelope glycoprotein M2, which has an approximate mol wt of 70,000 by gel filtration in guanidine hydrochloride (6, 7, 18) and has been reported in Rauscher MuLV to have a mol wt of approximately 80,000 by SDS-polyacrylamide gel electrophoresis (19).

The identity of the 43,000 mol wt glycoprotein is equivocal. A minor glycoprotein fraction with an apparent mol wt of approximately 42,000 has been reported for Rauscher MuLV by SDS-polyacrylamide gel electrophoresis (19). A comparable fraction is not observed by gel filtration in guanidine hydrochloride (18), but a 100,000 mol wt glycoprotein fraction is observed and has been termed M3. The M3 fraction appears to be an aggregate under these conditions and will partially dissociate upon subsequent SDS-polyacrylamide gel electrophoresis. The products of this dissociation are thought to be a 43,000 mol wt glycoprotein and a glycolipid fraction (D. P. Bolognesi, personal communication). In agreement with these observations, we have observed that SDS-polyacrylamide gel electrophoresis of [3H]glucosamine-labeled virus results in a major peak with an apparent mol wt of 68,000 and a minor peak at 43,000, with considerable radioactivity remaining at the origin of the gel. However, treatment of the virus with serum apparently facilitates solubilization of this complex, and with our immune precipitates all of the radioactivity migrates into the gel and a substantial increase in the 43,000 mol wt component is evident.

A viral envelope antigen with an apparent mol wt of 17,000 has not to our knowledge been previously reported. The ability of antibodies to bind to the antigen in intact virus suggests that it is an envelope antigen. However, since this antigen is not detected with [3H]glucosamine-labeled virus, it presumably is not a glycoprotein. This antigen is routinely precipitated by serum from all normal strains of mice we have examined; thus it would be interesting to determine whether it exhibits type-, group-, and interspecies-specific determinants similar to those of other viral antigens (7, 20, 21).

Although these experiments indicate which viral proteins are naturally antigenic, they do not indicate the quantitative differences in a particular serum for each antigen.
Such information will be obtainable when techniques are developed by which each antigen can be isolated and still maintain its natural antigenicity, and that knowledge should help us to understand the role of the autogenous immune response in viral replication in vivo. Although suitable purified antigens are not now available, we have attempted to compare the titer of B6C3F1 serum against the three components we have identified by quantitating on SDS-polyacrylamide gels the amount of each antigen precipitated at various serum dilutions. Preliminary results suggest that the M2 reaction is predominant, and it appears to be responsible for the radioimmune precipitation titer against intact virus. The titer against the 17,000 mol wt antigen is somewhat less, followed by a weak reaction against the M1 component.

The most important point to determine with respect to these results, is the efficacy of each reaction. The natural sera we have used in these experiments have only weak and variable, complement-independent neutralizing activity against AKR or Moloney MuLV in tissue culture, as assayed by the XC plaque assay (3). The lack of a strong neutralizing activity of these sera could be due either to the limited sensitivity of the in vitro assay or to the ratio of noninfectious or infectious particles in the virus preparations. Lastly, the observation of an inverse relationship between the severity of glomerulonephritis, caused in part by immune complexes with MuLV, and the incidence of lymphoma in some mouse strains (1) suggests that a lack of in vitro neutralization may not preclude a functional immunological response in vivo.

The experiments reported here were done with unfractionated serum. We are currently attempting to classify each reaction in terms of specific type(s) of antibody. This point will be of particular interest in assessing the strain variations in the autogenous immune response and may indicate whether qualitative as well as quantitative differences in reactivity to viral antigens determine the in vivo efficacy of the response.

The immunological reactivity in terms of the viral antigens recognized appears qualitatively consistent in all the mouse strains examined. All the natural sera examined react with the 68,000, 43,000, and 17,000 mol wt species. Antibodies that precipitate the 19,000 and 15,000 mol wt species are somewhat more variable; in the present studies, all natural sera except the B6C3F1 appeared to react with these proteins. Of considerable interest is the observation that C57BL/6 anti-K36 antiserum, which enhances the antivirus titer in the radioimmune precipitation assay, reacts with the same viral components as sera from control animals, except for an additional reactivity at a mol wt of 35,000. This observation suggests that hyperimmunization only slightly modifies the existing immune response qualitatively but can significantly alter the response quantitatively.

The observations reported here have been limited to the autogenous immune response to a single type of virus, a spontaneously activated MuLV from AKR cells. This virus represents an N-tropic Gross-type MuLV. We are presently examining the autogenous immune reaction with regard to other viral isolates. In particular, it is necessary to determine whether the serological differences...
recognized by heterologous antisera correspond to differences in reactivities in the autogenous immune reactions.

**SUMMARY**

The viral antigenic determinants recognized in an autogenous immune response in mice against their endogenous C-type virus have been identified by SDS-polyacrylamide gel electrophoresis of immune precipitates between various sera and H3-labeled intact or disrupted AKR leukemia virus. Normal B6C3F1 [(C57BL/6 × C3H/Anf)F1] serum reacts with viral envelope antigens having mol wt of approximately 68,000, 43,000, and 17,000. In addition, minor reactions with viral antigens having mol wts of approximately 19,000 and 15,000 are demonstrable. The 68,000 and 43,000 mol wt antigens can be labeled with [3H]glucosamine and may correspond to the major viral envelope antigens M2 and M3, respectively.

The antigens recognized by autogenous immune sera do not differ with respect to age of the animal, nor are they significantly different in sera from various strains of mice (BALB/c, C57BL/6, and C3H/Anf). These results suggest that the age-associated and strain variations in the autogenous immune response, as determined by radioimmune precipitation assays against intact virus, are due to quantitative and qualitative alterations of antibody levels against common antigens.

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