JOURNAL OF VIROLOGY, Oct. 1974, p. 773-781
Vol. 14, No. 4

Printed in U.S.A.

Autogenous Immunity to Endogenous RNA Tumor Virus: Differential Reactivities of Immunoglobulins M and G to Virus Envelope Antigens

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Received for publication 3 May 1974

The autogenous humoral immune response of mice to their endogenous leukemia virus has been examined in terms of the reactivities of individual classes of antibody present in normal B6C3F$_1$ serum. Whole serum and the immunoglobulin (Ig) M and IgG fractions of serum from animals of different age groups were compared by radioimmune precipitation assays and viral infectivity neutralization assays. Both IgM and IgG fractions were able to precipitate virus, although not as effectively as whole serum. Virus-specific antibody levels, as well as total antibody concentrations in whole serum, appeared to increase with age. Sodium dodecyl sulfate gel electrophoresis analysis was performed with immune precipitates obtained when whole serum or 19 or 7*S* fractions from animals of different age groups were reacted with disrupted virus. The 19*S* antibody fraction reacted with three antigenic determinants on the viral envelope. These antigens have apparent molecular weights of 17,000, 43,000, and 68,000. The last two appear to be glycoproteins and may correspond to the M_2 and M_1 antigens. In contrast, the 7*S* component reacted only with the 17,000-molecular-weight protein. Neutralization assays against BALB:virus-2, a xenotropic endogenous mouse type C virus, revealed that 19*S* and whole serum but not the 7*S* fraction possessed neutralizing activity. These findings indicate that there are differential reactivities of IgM and IgG antibodies in normal serum of B6C3F$_1$ mice, with respect to both recognition of viral envelope antigens and neutralization of endogenous MuLV. These results are consistent with the hypothesis that the autogenous humoral immune response is a systemic host function that may be important in the regulation of endogenous type C virus expression in vivo.

Evidence has progressively accumulated that the genetic information of murine leukemia virus (MuLV) is naturally present in mouse cells (7, 8), and that the virus is etiologically linked to leukemias in mice (9, 14). The incidence of lymphoid neoplasia among mouse strains, however, is highly variable. Intrinsic cellular controls of the viruses are currently under investigation in a number of laboratories. Although this approach is important in the understanding of virus-cell interactions, it has become increasingly clear that systemic host functions may be involved in the control of viral carcinogenesis. In the last few years evidence has accumulated that, rather than immunologic tolerance to endogenous MuLV-associated antigens, a chronic autogenous immune response to a variety of viruses and virus-induced surface antigens exists in some mouse strains. Earlier studies of naturally acquired immunity to endogenous MuLV-related antigens indicated that the major detectable autogenous immune response is directed at virus-induced cell surface antigens (4, 5) rather than virus envelope antigens. More recently, however, the widespread occurrence of antibodies with specificity for virus envelope antigens has been demonstrated (3, 6, 12, 15, 16; R. C. Nowinski and L. Kaehler, Science, in press).

Certain strains of mice characterized by a low natural incidence of lymphoma have been shown to produce high levels of natural antibody to endogenous MuLV envelope antigens (16). The natural antibody levels, as quantitated by a radioimmune precipitation assay, have been demonstrated to be age dependent. Virus-precipitating antibody titers increase as the animals develop immunologic competence and decrease as a function of senescence. Furthermore, in these strains of mice there is an

age-associated incidence of glomerulonephritis (6, 12). This lesion has been shown in part to be a consequence of the deposition of virus envelope antigen immune complexes. The specificity of antibody for virus envelope antigens from the kidneys as well as the serum has been demonstrated by immunoelectron microscopy (6). More precisely, analysis of immune precipitates of disrupted MuLV by sodium dodecyl sulfate (SDS) gel electrophoresis techniques clearly indicates that the primary antigenic determinants of the virus envelope are glycoproteins, with molecular weights of approximately 68,000 and 43,000, and a smaller protein of approximately 17,000 molecular weight. (Recent evidence demonstrates that the 17,000-molecular-weight polypeptide described here corresponds to the p15 component of MuLV as determined by guanidine hydrochloride analysis; J. N. Ihle et al., Virology, in press.) Recently, the biologic activity of this natural antibody has been demonstrated by its ability to neutralize a class of endogenous mouse type C virus (3).

The purpose of the present studies was to characterize more thoroughly the biologic nature of autogenous immunity to MuLV. It is highly probable that among the major factors contributing to age-associated changes in autogenous immunity to MuLV antigens are qualitative and quantitative changes in classes of reacting antibody. Therefore, we separated the 19 and 7*S* natural antibody-containing fractions from sera of B6C3F$_1$ mice—a strain known to have high titers of antibody to MuLV—of various ages. We tested these 19 and 7*S* fractions for specificity to the various virus envelope antigens and for their ability to neutralize endogenous C type virus.

MATERIALS AND METHODS

Animals. Male B6C3F$_1$ (C57BL/6 ♀ × C3H/Anf ♂) mice were used, ranging in age from 11 weeks to 30 months. All mice were specific-pathogen-free (SPF). B6C3F$_1$ mice have a mean survival time of approximately 884 days and a low (<5%) natural incidence of thymic lymphoma; however, beyond 2 years of age 46% of these mice develop a type A reticulum cell sarcoma (11).

Test sera. Blood was collected from mice of various ages by orbital bleeding or cardiac puncture, allowed to clot at room temperature for 1 h, and left at 4 C overnight. Serum was separated by centrifugation at 1,200 × *g* for 15 min. Age-matched sera were pooled and stored at −70 C prior to use.

Serum fractionation. Pooled normal sera were subjected to 50% ammonium sulfate salt precipitations. The precipitates, containing mainly γ-globulins, were dissolved in distilled water and reprecipitated twice with 35% ammonium sulfate. The final precipitates were dialyzed extensively against 0.015 M Tris-hydrochloride (pH 8.0) buffer. The immunoglobulin (Ig) M and IgG fractions of the γ-globulin-enriched preparations were separated through DEAE-cellulose ion exchange and/or Sephadex G-200 gel filtration techniques. DEAE-cellulose columns were equilibrated with 0.015 M Tris-hydrochloride (pH 8.0), and immunoglobulins were eluted stepwise with 0, 0.0175, 0.15, and 0.3 M NaCl in 0.015 M Tris-hydrochloride (pH 8.0). IgM- and IgG-containing fractions were separately pooled, concentrated, and applied to a Sephadex G-200 column (100 by 2.5 cm) for final separation of IgM and IgG. A typical elution profile of IgM and IgG from a Sephadex G-200 column is shown in Fig. 1.

The serum fractions were concentrated and tested for antigenic purity by the Ouchterlony immunodiffusion technique against corresponding test antisera. Radioimmunoprecipitation assays were used to determine their respective reactivities. The two major purified antibody components were adjusted to protein levels approximating that of whole serum.

Quantitation of B6C3F$_1$ serum of various age groups. Quantitative immunodiffusion plates (Meloy Laboratories, Springfield, Va.) for determination of mouse serum protein concentrations were used for quantitation of the major classes of immunoglobulins according to Mancini et al. (18). Diameters of diffused rings were measured and compared to known standards. Concentration of a particular class of antibody was expressed as milligrams per 100 l.

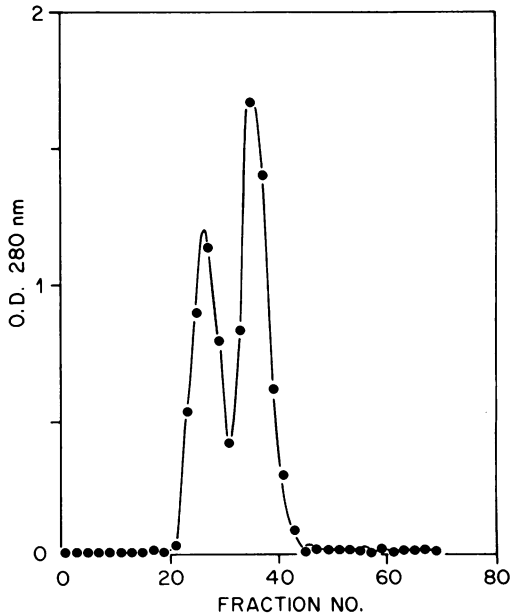

FIG. 1. *Sephadex G-200 gel filtration elution profile of normal B6C3F$_1$ serum after salt precipitation and DEAE chromatography. The protein peaks were pooled, concentrated, and tested for antibody activity.*

Preparation of radioactively labeled virus. [^{3}H]leucine-labeled virus was prepared as previously described (16). [^{3}H]glucosamine-labeled virus was prepared similarly, using Eagle minimal essential medium supplemented with 2 mM glutamine and 10% fetal calf serum, containing 50 μCi of D-glucosamine [6-^{3}H]hydrochloride per ml (12.6 Ci/mmol; Amersham/Searle, Arlington Heights, Ill.).

Radioimmune precipitation assays against intact virus. The radioimmune precipitation assay against intact radioactively labeled AKR virus has been described in detail elsewhere (15). In brief, 0.1 ml of the test serum, or serum fraction, was serially diluted twofold in TNE buffer (0.05 M Tris-hydrochloride, pH 7.5, 0.1 M NaCl, 1 mM EDTA), 0.1 ml (6,000 counts/min) of labeled virus was added, and the mixture was incubated 1 h at 37 C to allow the formation of immune complexes. Subsequently, a volume of 0.1 ml of anti-γ-globulin (Cappel anti-mouse γ-globulin), diluted 1:2 in TNE was added, and the mixture was incubated again at 37 C for 1 h and finally at 4 C for 2 h. The precipitates were collected by centrifugation at 1,200 $\times$ *g* for 15 min, and the supernatant was removed for determination of radioactivity. The precipitates were further washed three times with TNE, resuspended in 0.4 ml of TNE, and prepared for counting. All samples were counted in 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) in a Packard Tricarb scintillation counter. The percent of precipitation was expressed as the percentage of counts in the precipitate relative to the combined counts in the precipitate and in the first supernatant. A number of parameters of the radioimmune precipitation assay were found to affect the results, and these have been described in a previous report (16).

To prepare immune precipitates for polyacrylamide gel electrophoresis, 25 to 50 μliters of serum was allowed to react with 2 $\times$ 10^5 counts/min of virus and subsequently precipitated with anti-γ-globulin as described above. The precipitates were washed four times with TNE and then sedimented through a cushion of 25% sucrose containing TNE and 0.5% deoxycholate. The precipitates were resuspended in TNE and pelleted at 1,200 $\times$ *g* for 20 min. The pellets were carefully drained dry and resuspended in 0.05 ml of 1% SDS, 1% β-mercaptoethanol in 0.01 M sodium phosphate buffer at pH 7.4, and incubated at 60 C for 1 h and then at 37 C overnight to dissolve.

SDS polyacrylamide gel electrophoresis of immune precipitates. SDS polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (25). Bromophenol blue was used as a reference standard to determine relative mobilities and was generally allowed to migrate 8 cm. Standard protein samples were used to calibrate the system as described (15). The gels were sectioned into 1-mm slices, which were dissolved in 30% hydrogen peroxide at 75 C overnight. Radioactivity of each gel slice fraction was determined in a liquid scintillation counter.

Neutralization assays. Normal rat kidney (NRK) cells were grown in Dulbecco's modified Eagle medium containing 10% calf serum, in plastic petri dishes as previously described (2). Viruses used in the tests were BALB:virus-1 and BALB:virus-2, biologically distinguishable viruses of BALB/c mouse cells, which were propagated in NRK cells (2). A rat type C virus induced from NRK cells (1) was also used. Normal B6C3F$_1$ sera and serum fractions were heat-inactivated at 56 C for 30 min and filtered through membrane filters (0.45 μm pore size; Millipore Corp.) before use. Neutralization tests were performed by a focus reduction method. Approximately 100 focus-forming units (FFU) of each helper virus pseudotype of the Kirsten strain of murine sarcoma virus (KiMSV) were incubated with serially diluted test serum samples for 30 min at 37 C. The surviving virus fraction was assayed on NRK cells treated with polybrene (2 μg/ml). The number of KiMSV foci was scored at 7 days. Neutralizing antibody titer was presented as the reciprocal of the highest serum dilution that gave 67% or greater reduction in the number of KiMSV foci.

RESULTS

Quantitation of immunoglobulin classes in whole serum as a function of age. Serum samples from B6C3F$_1$ mice of various ages were evaluated by radial immunodiffusion for age-associated differences in IgM and IgG levels. Table 1 shows the relative concentration (in milligrams per 100 ml) of each serum antibody component. There was increase in total immunoglobulin concentration in mice from 11 to 12 weeks to 1.5 years of age. Both IgM and IgG increased in quantity in 1.5-year-old animals as compared to that of 11-to 12-week-old mice. The concentrations of IgG_1 and IgG_2 in sera of 11- to 12-week-old B6C3F$_1$ mice were approximately of equal proportion.

Precipitation of intact labeled virus by sera and serum fractions of mice from various age groups. Radioimmune precipitation assays were performed on normal whole serum and serum fractions of B6C3F$_1$ mice at various ages. Three age groups of normal sera, as well as the serum fractions from 11-week-, 1.5-year-, and 2.5-year-old mice, were studied (Fig. 2). Serum

TABLE 1. *Concentrations of immunoglobulin in B6C3F$_1$ serum and serum fractions measured by radial immunodiffusion assay*

Age of B6C3F$_1$	Sample tested	Concn (mg/100 ml)		
		IgG_1	IgG_2	IgM
11–12 weeks	Whole serum	90	90	10.8
	IgM	0	0	5.8
	IgG	45	45	0
1.5 years	Whole serum	320	820	81
	IgM	0	0	43
	IgG	160	440	0
2.5 years	Whole serum	280	680	81
	IgM	0	0	49
	IgG	150	360	0

from 11-week-old mice exhibited a low precipitating titer (50% precipitation at 1:256) (Fig. 2A). As reported previously, a high precipitating antibody activity was observed in the normal sera from 1.5-year-old and 2.5-year-old B6C3F$_1$ mice (50% precipitation was attained at 1:2048 and 1:1024 dilution, respectively). The decrease in titer of the 2.5-year serum is significant and may reflect a decrease in immune competence or an increase in virus burden with age.

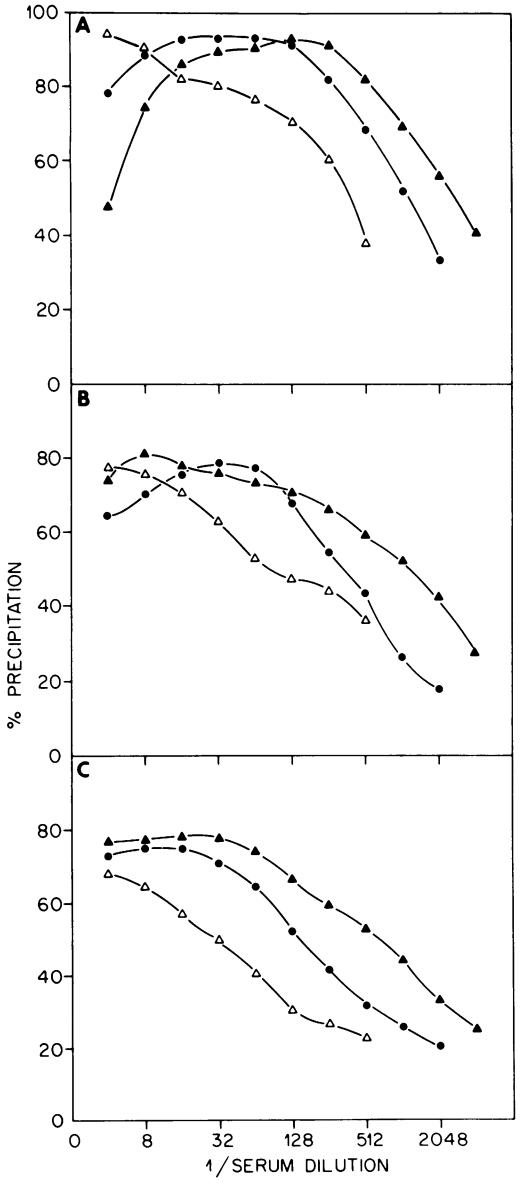

FIG. 2. *Titration curves of (A) whole sera and (B) 19S and (C) 7S fractions of B6C3F$_1$ mice of various ages by the radioimmune precipitation assay. Symbols: △, 11 to 12 weeks old; ▲, 1.5 years old; ●, 2.5 years old.*

When the precipitation curves of serum fractions of isolated IgM and IgG were compared among the various age groups, both classes of antibodies precipitated virus to a similar extent, although the 7*S* antibody titer at 50% precipitation was generally half that of 19*S* (Fig. 2B and C). Total virus precipitation by 19 and 7*S* was not cumulative as compared to that of the respective whole serum. The fact that the maximum virus precipitation of each individual purified antibody component was unequivocally lower than that of the whole serum suggests a loss of activity as a consequence of the fractionation procedures.

SDS acrylamide gel electrophoresis of immune precipitates formed by normal sera or serum fractions with disrupted virus. To determine which viral antigens were recognized and precipitated by normal whole serum and different classes of antibodies from B6C3F$_1$ mice of various ages, immune precipitates were examined by SDS polyacrylamide gel electrophoresis. The gel profiles of immune precipitates obtained when disrupted [^{3}H]leucine-labeled AKR virus was reacted in repeated experiments using either whole serum or 7 or 19*S* fractions are presented in Fig. 3. A typical gel profile of disrupted virus alone is shown for comparison (Fig. 3A). When normal serum from 1.5-year-old mice was reacted with disrupted labeled AKR virus, the results shown in Fig. 3B were obtained. Three distinct viral protein peaks were detected with whole sera, with mobilities relative to bromophenol blue that corresponded to molecular weights of approximately 68,000, 43,000, and 17,000. When this was compared to the gel profile of disrupted viral protein alone, only a small proportion of these viral polypeptides was recognized by whole serum from B6C3F$_1$ mice. When the 19*S* fraction was reacted under similar conditions, the results shown in Fig. 3C were obtained. All antigenic determinants recognized by whole serum were also precipitated by the 19*S* fraction. In contrast, the 7*S* fraction reacted only with the 17,000-molecular-weight protein (Fig. 3D). Similar results were obtained when serum or 19 or 7*S* fractions from 11- to 12-week-old and 2.5-year-old mice were tested. In some but not all experiments, a peak corresponding to 30,000 molecular weight, the region designated as the gs antigen (23) was detected with 2.5-year-old B6C3F$_1$ serum.

It has been previously demonstrated that

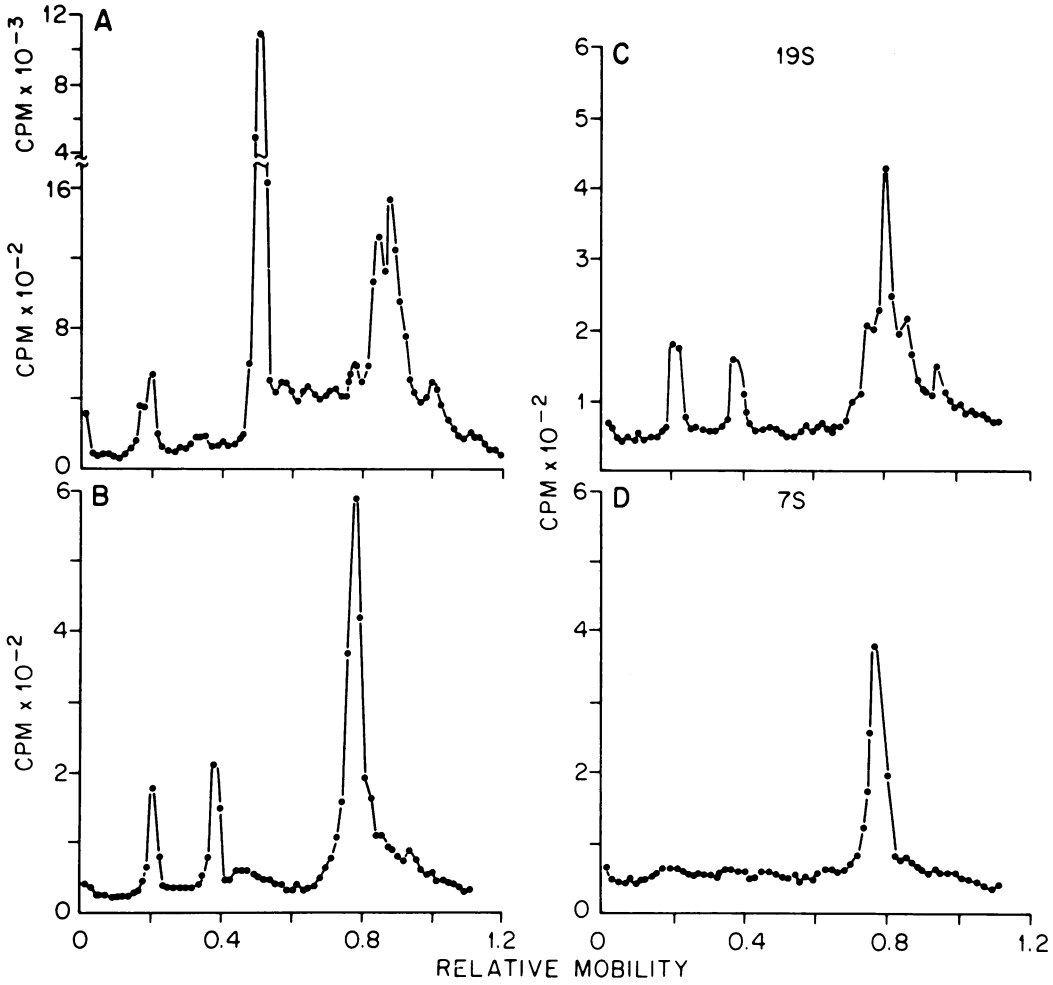

FIG. 3. *SDS polyacrylamide gel electrophoresis profiles of (A) Triton-disrupted [³H]leucine-labeled AKR virus, (B) an immune precipitate of 1.5-year B6C3F₁ whole serum with Triton-disrupted AKR virus, (C) an immune precipitate of purified 19S fraction of 1.5-year B6C3F₁ serum with Triton-disrupted AKR virus, and (D) an immune precipitate of purified 7S fraction of 1.5-year B6C3F₁ serum with Triton-disrupted AKR virus.*

both M_2 (68,000 molecular weight) and M_1 (43,000 molecular weight) proteins are glycoproteins (15, 21, 24). To further identify the viral components recognized in this autogenous immune reaction, [³H]glucosamine-labeled virus was used. The advantage of employing [³H]glucosamine-labeled virus is that the glycoprotein moieties on the viral envelope are preferentially labeled. Two antigenic reactivities were observed when the immune precipitates were analyzed by SDS acrylamide gel electrophoresis. The gel profiles of representative immune precipitates are presented in Fig. 4. As expected with serum or serum fraction from each age group (11 to 12 weeks, 1.5 years, and 2.5 years), only 19S fractions and whole serum reacted with [³H]glucosamine-labeled M_2 and M_1 viral envelope antigens. The M_1 glycoprotein precipitated was not clearly discernible, due to weak reactivity of antibody against this antigen and the lower specific activity of the [³H]glucosamine label. This further demonstrates the differential reactivities of the two classes of antibodies with regard to which virus envelope antigens they recognize.

Neutralizing activities of B6C3F₁ sera and purified components. Serum samples and purified antibody fractions from 11- to 12-week-old B6C3F₁ mice were tested for their neutralizing activity against BALB:virus-2, a representative xenotropic endogenous mouse type C virus (1). In sharp contrast to previous findings with the

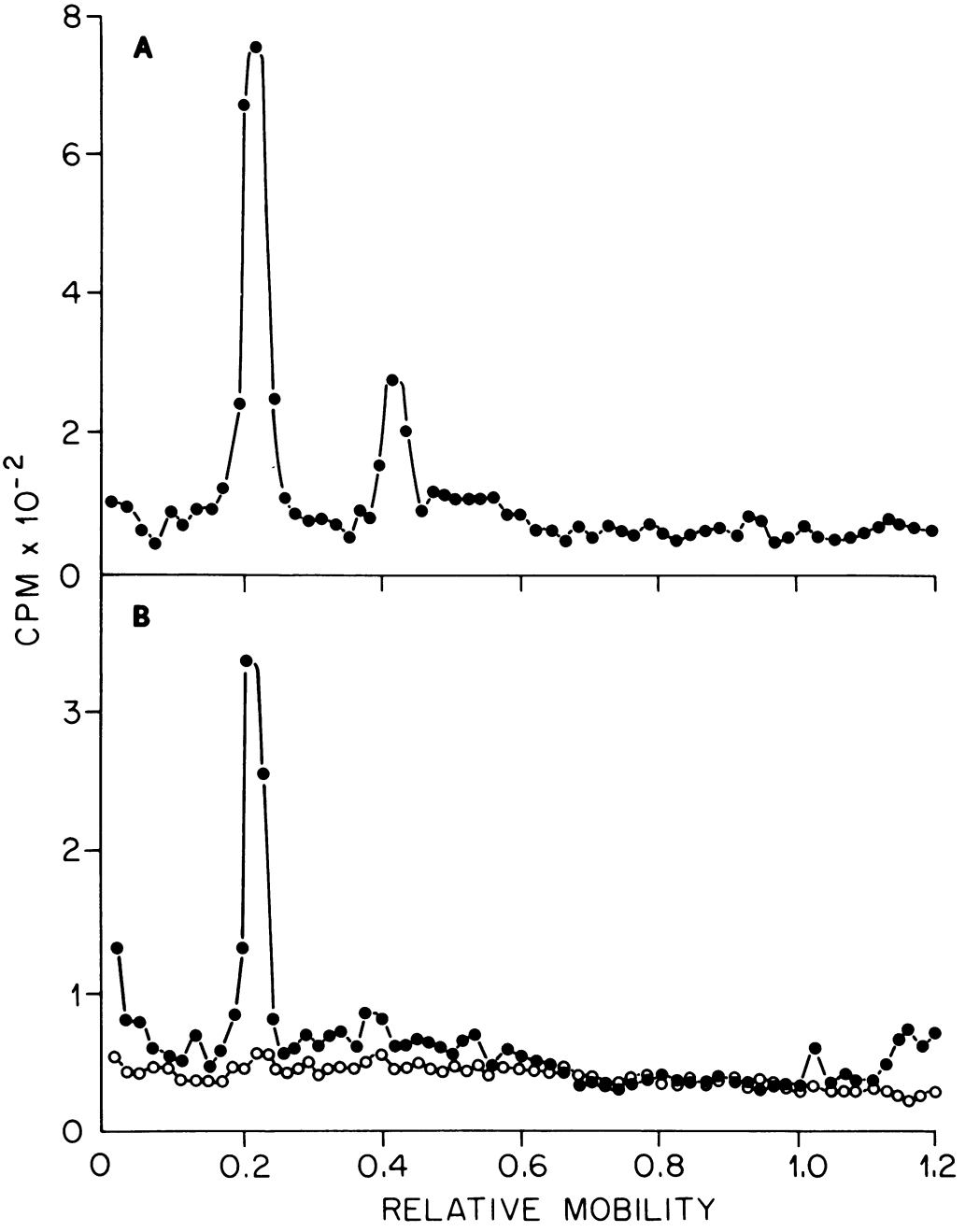

FIG. 4. *SDS-polyacrylamide gel electrophoresis profiles of (A) an immune precipitate of 1.5-year B6C3F$_1$ whole serum reacted with Triton-disrupted [^3H]glucosamine-labeled AKR virus and (B) immune precipitates of serum fraction of 1.5-year B6C3F$_1$ reacted with Triton-disrupted [^3H]glucosamine-labeled AKR virus. Symbols: ●, 19S; ○, 7S.*

XC neutralization test in AKR or Moloney virus (6), where low and inconsistent neutralization titers were obtained, the neutralization titers of B6C3F$_1$ sera against BALB:virus-2 reached levels higher than 1:2000.

When purified serum antibody components were tested for neutralization activity, IgM was shown to neutralize the virus at a titer of 1:200. In contrast, IgG had no detectable neutralizing activity even at a dilution as low as 1:2. The

specificity of the reactivity of the IgM fraction was determined by its lack of neutralizing activity against another BALB/c endogenous virus, BALB:virus-1, or a rat type C virus induced from NKR cells, the cells in which each mouse type C virus had been propagated. Since comparable protein concentrations were used for the 7 and 19S fractions (Table 2), the present results indicate that biologic activity resides in the 19S fraction. However, the lack of complete recovery of neutralizing activity in this fraction indicates some loss during purification.

TABLE 2. *Neutralization activities of different serum fractions from B6C3F$_1$ mice*

Serum or serum fraction	Neutralization titer against[a]		
	BALB: virus-1	BALB: virus-2	Rat type C virus
Whole serum	<20	2,000	<20
IgM	<2	200	<2
IgG	<2	<2	<2

[a] Neutralization tests were performed by the focus reduction method. Around 100 FFU of each virus were incubated with whole serum or the appropriate serum components from 11- to 12-week-old B6C3F$_1$ mice for 30 min at 37 C. BALB:virus-2 and rat type C virus pseudotypes of KiMSV were assayed on NRK cells treated with polybrene (2 μg/ml). The BALB:virus-1 pseudotype of KiMSV was assayed on NIH/3T3 cells (2). The number of MSV foci was scored at 7 days. Results are expressed as the reciprocal of the highest serum dilution giving 67% or greater reduction in the number of MSV foci and are the results of at least three separate experiments.

DISCUSSION

The concept that mice are immunologically tolerant to their endogenous C type RNA viruses was first accepted on the basis of a known chronic virus infection and the inability to detect serum antibody specific for MuLV (5, 14). It was subsequently demonstrated that natural antibody to virus-associated antigens, such as the virus-induced cell surface antigens, could be detected by immunoelectron microscopy (4). Recently, however, we have demonstrated by direct assays that several strains of mice have an age-associated humoral immune response to endogenous RNA C type virus (6, 12, 16). The specificity of this natural antibody to virus envelope antigens has been shown by immunoelectron microscopy (6) and by radioimmune precipitations of intact MuLV (16).

As demonstrated previously in our laboratory (16) and recently verified by R. C. Nowinski and S. L. Kaehler (Science, in press), there is a great variation among mouse strains with regard to antibody titers to AKR virus as measured by the radioimmune precipitation assays. Although we have recognized quantitative differences in antibody titers among different mouse strains, the most interesting correlation is that the antibody level is inversely associated with spontaneous incidence of leukemia in these animals (12, 16).

The antigenic determinants of MuLV virus envelope recognized by natural serum antibody are the M_1 and M_2 glycoproteins (molecular weights of 43,000 and 68,000, respectively) and a smaller protein (approximately 17,000 molecular weight). In this report we further characterize the qualitative differences in autogenous immune response to endogenous MuLV in B6C3F$_1$ mice. As analyzed by SDS gel electrophoresis, IgM reacts with all three natural antigenic determinants of the virus envelope, but the 7S fraction reacts only with the 17,000-molecular-weight protein of the virus envelope.

The broader spectrum of reactivity of the 19S natural antibody to MuLV envelope antigens clarifies several points. First, it has previously been reported that natural antibody reactivity to the virus envelope by immunoelectron microscopy can be detected only if ferritin-conjugated rabbit anti-mouse IgM is used in the assay (6). No reactivity can be demonstrated with ferritin-conjugated rabbit anti-mouse IgG. It should be noted that the resolution of immunoelectron microscopy depends on the number of reactive sites and the number of ferritin molecules detectable in the electron microscope. Thus the broader spectrum of reactivity of the 19S fraction may be a requirement for detectable reactivity in immunoelectron microscopy. It is clearly shown, however, that by radioimmune precipitation assays the single specific reactivity of the 7S fraction is adequate for anti-globulin precipitation of intact viruses. Thus the radioimmune precipitation assay is quantitatively more sensitive, as well as more diagnostic, than immunoelectron microscopy for detecting the natural array of antibody specificities to MuLV envelope antigens.

A search of the literature pertaining to the differences in degree of specificity between two major classes of antibody fractionated from sera from actively immunized animals indicates that IgM antibody is more precise than IgG for differentiating minor antigenic differences between virus strains (13, 20). Those results agree with our findings on the broad spectrum of reactivity of autogenous IgM to antigens of C

type MuLV. Functionally, the broader reactivity of the 19*S* fraction might account for its biologic activity against an endogenous MuLV, as shown by neutralization. This may indicate that antibody reactivity as determined by MuLV neutralization depends on interaction with specific antigenic sites, and our results suggest that reactivity with M_2 and/or M_1 glycoproteins is essential for virus neutralization. Such a finding has been described previously for serum from actively immunized animals (22). The correlation of antigens recognized by natural antibody and neutralization of MuLV is still unknown. No conclusion regarding the effective neutralization of BALB:virus-2 by sera of normal B6C3F_1 mice can be reached until the natural antigenic determinants of this virus are characterized. Such experiments are presently underway in this laboratory. An interesting observation is that the level of virus-neutralizing activity of purified IgM is not comparable to neutralization achieved with whole serum. This may indicate that optimum neutralization of intact viruses as a natural host response requires the involvement of antibody and other serum components.

So far, we have concentrated on B6C3F_1 mice, which are F_1 offspring of C57BL/6 ♀ × C3H/Anf ♂ and are known to have a long mean survival time and a low natural incidence of thymic lymphoma (10). A similar hybrid from a reciprocal mating [(C3Hf/Bi ♀ × C57BL ♂)F_1] has been reported to have a much lower susceptibility to Gross passage A virus than C3H (17). One of the genetic factors, other than *Fv-1*, that has been associated with this finding is a weak association of tumorigenesis with *H-2* in C3H × C57BL crosses. Similar findings were observed in the studies of endogenous resistance of certain strains of mice to leukemia-related cell surface antigens where F_1 and reciprocal hybrids were more responsive to an as yet unknown antigen on MuLV and leukemia cells (23). The *Rgv-1* locus, causing resistance to leukemogenesis induced by Gross passage A virus in neonatal mice, has been reported to be located proximal to or within the *K* region of the *H-2* locus, as is the *Ir-1* gene, which determines immune responsiveness to certain antigens (14). Thus the *Ir-1* gene and the *Rgv-1* genes may be identical, functioning in immune control of MuLV antigens (17, 19). Therefore, studies that would further elucidate the relationship between autogenous immunity and susceptibility to Gross virus leukemogenesis in various mouse strains are warranted and are currently in progress.

ACKNOWLEDGMENTS

This research was supported jointly by the Virus Cancer Program of the National Cancer Institute and by the U.S. Atomic Energy Commission under contract with Union Carbide Corporation. J.C.L. is supported by a United Order True Sisters, Inc., Postdoctoral Fellowship in Cancer Immunology.

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