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Natural Immunity in Mice to the Envelope Glycoprotein of Endogenous Ecotropic Type C Viruses: Neutralization of Virus Infectivity

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The ability of naturally immune mouse sera to neutralize ecotropic AKR murine leukemia virus (MuLV) was examined by using unfrozen virus preparations harvested for 1 h. In this assay several mouse sera significantly and consistently neutralized MuLV infectivity. The ability of these sera to neutralize was correlated with the presence of antibodies against MuLV detectable in a radioimmune precipitation assay using radioactively labeled intact virions. This neutralization was specific, in that either N- or B-tropic viruses, but not Friend MuLV, were neutralized. In addition, neutralization could be abrogated with purified AKR MuLV gp71 at concentrations that do not interfere with virus infectivity but could not be abrogated with Rauscher MuLV gp71. Neutralizing activity could be removed by absorption with intact AKR MuLV, but not by absorption with Friend MuLV, a BALB/c xenotropic virus, or with NZB xenotropic virus. All the neutralizing activity of (B6C3)F_1 mouse sera was associated with the immunoglobulin G fraction.

Many murine type C viruses are endogenous in mice (4, 8, 25) and are spontaneously expressed at varying rates in a variety of inbred strains of mice (11, 21). One consequence of the expression of endogenous ecotropic type C viruses is the induction of an immune response specific for this class of virus (9, 16, 23, 30). The existence of this immune response has been demonstrated most clearly with a sensitive radioimmune precipitation (RIP) assay using [^{3}H]leucine-labeled intact virions. In this type of assay "natural" antibody titers of 1/2,560 to 1/5,120 are not uncommon. More recently, several studies were directed at characterizing the immune response both in terms of viral specificity and antigenic specificity (10, 12, 13). The results from these studies demonstrated that, in general, a type-specific immune response against the endogenous ecotropic viral gp71 and a more broadly reactive group-specific response against p15(E) can be detected in a variety of inbred strains of mice. These serological specificities apparently reflect the immunogenic properties of the antigens involved rather than the immunological characteristics of the immune response itself (10, 14, 26).

Although the above studies provided considerable information on molecular properties of this immune response, the potential efficacy of the response in vivo was not considered. Several studies suggested the efficacy of this response by demonstrating the ability of natural immune sera to mediate complement-dependent cytotoxicity in vitro against virus-replicating cell lines (20, 24). However, it was not demonstrated whether this reactivity is a function of an immune response against gp71 or p15(E), or against some other virus-related antigen. Another possible efficacious consequence of such an immune response might be the ability of the response to neutralize infectious murine leukemia virus (MuLV) in vivo. Several studies demonstrated that high levels of infectious MuLV are associated with spontaneous lymphomas in AKR mice and in appropriate genetic crosses (18) and that genetic factors which restrict the ability of the virus to infect cells, such as the Fv-1 gene, can significantly reduce virus burden and concomitantly reduce the incidence of spontaneous leukemia (19). Moreover, there is evidence that there exists an H-2-associated genetic restriction (18, 19), which has recently been correlated with an immune response against MuLV (22). Therefore, there is the possibility that neutralization of MuLV by the natural immune response could influence the in vivo expression of virus and subsequent pathogenesis.

To understand the ability of the immune response to regulate virus in vivo, it is necessary

to determine whether such an immune response can mediate virus neutralization. Previous attempts to demonstrate specific neutralization of endogenous viruses have been equivocal. In particular, in one set of experiments, it was suggested that the immune response against intact virus was cross-reactive in RIP assays with ecotropic virus and xenotropic virus; however, such sera neutralized xenotropic virus only (1). Later experiments, however, demonstrated that the inactivation of xenotropic virus was not immunoglobulin mediated and that this inactivation was not correlated with the presence of a humoral immune response against ecotropic viruses (6, 17). In some studies there was no detectable neutralization of ecotropic virus by a variety of normal mouse sera (1, 16), whereas in other cases the neutralization was variable and weak (6, 9, 20). In one of these studies (9) it was suggested that the variable neutralization might be due to the lability of these viruses in tissue culture. Because of the reactivity of natural immune sera with AKR MuLV gp71 and the general association of this type of immune recognition with neutralization, we have now reexamined the question of the ability of natural immune sera to neutralize MuLV. Moreover, since many of the previous studies demonstrated the lability of C-type viruses (5, 28), we used 1-h harvested, unfrozen supernatants as a source of infectious virus. The results of this assay demonstrate that naturally immune sera significantly neutralize MuLV infectivity, that this neutralization correlates with demonstrable antibodies in the RIP assay, and that the neutralization is mediated by an immunoglobulin G (IgG) immune response against AKR MuLV gp71.

MATERIALS AND METHODS

Animals and test sera. NIH Swiss, BALB/c, C57BL/6, (B6C3)F_1, [(C57BL/6x C3H/Anf)F_1] and C3H/Anf mice were obtained from the Central Animal Facility of the Frederick Cancer Research Center, Frederick, Md. 129/J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. All mice were specific pathogen-free.

Blood was collected from mice by cardiac puncture and allowed to clot at room temperature. Sera were separated by centrifugation, portioned, and stored at −70°C until use. To obtain serum pools from antibody-positive and -negative mice, 30 to 50 individual mice of various ages were bled from the tail, and the sera were collected and tested in the RIP assay. Blood from these mice was collected later by cardiac puncture, and the sera of antibody-positive or -negative mice were separated.

Cell lines and viruses. AKR MuLV was obtained from an established line of AKR mouse embryo cells that had spontaneously initiated virus synthesis. Friend MuLV was obtained from the Eveline cell line, which was derived from the STU strain of mice (27). B-tropic virus was obtained from a culture of SC-1, wild mouse cells, infected with a B-tropic virus isolated from a C57BL/6 mouse (kindly provided by Janet Hartley, National Institutes of Health). A cloned murine sarcoma virus-transformed 3T3FL cell derivative line (FG-10), which was sarcoma virus-positive and leukemia virus-negative (S^+L^-), was used for assays of ecotropic viruses and was kindly provided by P. Fischinger of the National Institutes of Health. The derivation and methods of propagation of these cells were described previously (2, 3).

RIP assays for antibody capable of precipitating intact AKR MuLV and radioimmunoassays (RIA) for antibodies against AKR MuLV virion proteins were described previously in detail (15). The procedures for the purification of virion antigens and for the production of monospecific antisera were also described previously (10; J. N. Ihle, D. R. Joseph, and N. H. Pazmiño, J. Exp. Med., in press.). The procedures used for the purification and characterization of mouse 7S and 19S immunoglobulins were reported previously (16). In the present studies, the 7S and 19S fractions were judged free of cross-contamination by immunodiffusion with specific antisera. All sera were heat inactivated (56°C for 20 min) and filtered prior to use.

Neutralization assays. To obtain supernatants for source of infectious virus, the appropriate cells from a single confluent 100-mm plate were dissociated by treatment with 0.02% EDTA in divalent ion-free Hanks balanced salt solution. The cells were subsequently pelleted by centrifugation (250 × *g* for 10 min) and suspended in 0.5 ml of modified McCoy 5A medium containing 10% fetal calf serum. The cells were incubated for 1 h at 37°C with occasional shaking and were pelleted by centrifugation (500 × *g* for 10 min). The supernatant was removed and filtered through a 0.45-μm Millex filter and used immediately for virus infectivity or neutralization assays.

For neutralization assays, approximately 150 focus-inducing units of virus were incubated for 1 h at 37°C in 0.2 ml of medium containing the appropriate serum dilutions. The mixture was subsequently diluted with medium to approximately 0.5 ml and used. Infectivity assays were conducted as described previously (3). Briefly, FG-10 cells were suspended in modified McCoy 5A medium containing 10% fetal calf serum and plated at a concentration of 1×10^5 cells per plate into 60-mm Integrid dishes. After 24 h, the cells were treated with 25 μg of DEAE-dextran per ml in the above media for 30 min prior to the addition of virus. The cells were subsequently infected with virus in 0.2 to 0.5 ml of media for 30 min at 37°C on a rocking platform and then fed complete media. The cells were refed at 5 days and scored at 6 to 7 days for focus formation. The degree of virus neutralization is expressed by the ratio describing the surviving fraction (V_n/V_0).

RESULTS

To examine the ability of normal mouse sera to neutralize AKR MuLV and to correlate this

neutralization with antibodies detectable in the RIP assay, several serum samples were obtained. The characteristics of these sera in the RIP assay and their reactivity with AKR MuLV virion proteins are shown in Table 1. Pooled sera from NIH Swiss, 129/J, and BALB/c mice had no demonstrable titers of antibody against AKR MuLV in an RIP assay against the intact AKR MuLV or any demonstrable antibodies against the virus antigens tested. Two separate pools of sera were obtained from C57BL/6 and (B6C3)F_1 mice: one pool from antibody-positive mice and one pool from antibody-negative mice. The antibody-positive sera from these mice were characterized by precipitating antibodies against intact AKR MuLV and AKR MuLV gp71, but there were no detectable antibodies against the other viral antigens tested. Finally, a pool of C3H sera that reacted with AKR MuLV and AKR MuLV gp71, but did not react with AKR MuLV p15, p30, p12, or p10, was obtained.

The ability of these sera to neutralize AKR MuLV infectivity is shown in Fig. 1. For these assays, unfrozen AKR virus harvested for 1 h was used. All sera were heat inactivated prior to use for neutralization assays. As demonstrated by the results, the RIP-positive sera from C57BL/6, (B6C3)F_1, and C3H mice significantly neutralized AKR MuLV infectivity. The titers of these sera, defined as the serum dilution giving 66% neutralization of virus infectivity, are approximately 1/60. In contrast, sera from 129/J, NIH Swiss, BALB/c, and the RIP-negative C57BL/6 and (B6C3)F_1 mice did not significantly neutralize AKR MuLV infectivity. These results therefore demonstrate that autogenous immune sera from mice can neutralize AKR MuLV infectivity and suggest that there is a correlation between this neutralization and antibodies detectable against AKR MuLV in the RIP assay or AKR MuLV gp71 in RIA.

The immunological specificity of the neutral-

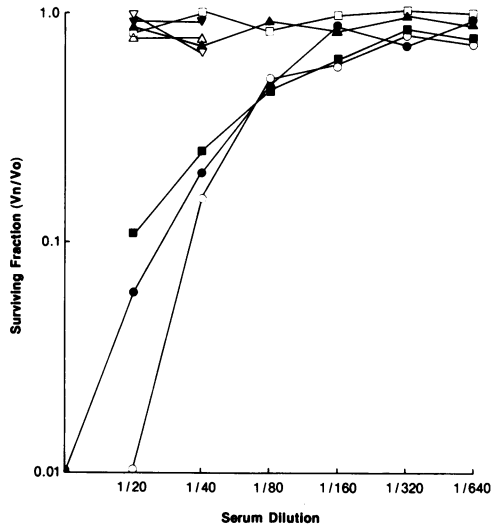

FIG. 1. *Neutralization of AKR MuLV infectivity by naturally immune mouse sera. The sera described in Table 1 were assayed for their ability to neutralize AKR MuLV. Heat-inactivated sera were incubated with virus at 37°C for 1 h, and the surviving virus was determined by the focus assay on FG-10 cells as described in Materials and Methods. The results are plotted as the surviving fraction (V_n/V_o) of virus relative to untreated virus versus serum dilution. The sera include RIP-negative sera from BALB/c (▼), NIH Swiss (▽), 129/J (△), C57BL/6 (□) and (B6C3)F_1 (▲) mice and sera that were RIP positive from C57BL/6 (■), C3H/Anf (●) and (B6C3)F_1 (○) mice.*

TABLE 1. *Reactivity of sera from various mouse strains with intact AKR MuLV and with AKR MuLV virion proteins*

Strain	RIP titer against AKR MuLV[a]	RIA titer against:[b]				
		gp71	p30	p15	p12	p10
129/J	<1/5[c]	<1/5	<1/5	<1/5	<1/5	<1/5
NIH Swiss	<1/5	<1/5	<1/5	<1/5	<1/5	<1/5
BALB/c	<1/5	<1/5	<1/5	<1/5	<1/5	<1/5
C57BL/6	1/1,280	1/80	<1/5	<1/5	<1/5	<1/5
C57BL/6	<1/5	<1/5	<1/5	<1/5	<1/5	<1/5
(B6C3)F_1	1/2,560	1/160	<1/5	<1/5	<1/5	<1/5
(B6C3)F_1	<1/5	<1/5	<1/5	<1/5	<1/5	<1/5
C3H/Anf	1/640	1/80	<1/5	<1/5	<1/5	<1/5

[a] Serum titers against AKR MuLV were determined with an RIP assay as described in Materials and Methods. Titers are the serum dilution giving 50% precipitation of the labeled virus.

[b] Serum titers against iodinated viral antigens were determined as described in Materials and Methods. Titers are the serum dilution giving 25% precipitation of labeled antigen.

[c] <1/5 indicates lack of any detectable antibody in these assays and represents an approximate limit of detection.

ization by (B6C3)F_1 serum was examined by comparing the results of neutralization assays with other ecotropic viruses prepared under comparable conditions. As illustrated in Fig. 2, this serum neutralized both N- and B-tropic isolates of ecotropic viruses but did not neutralize Friend MuLV. The serological specificity of this neutralization was also examined by absorption of (B6C3)F_1 serum with various viruses. As illustrated in Table 2, absorption with either Friend MuLV, BALB/c xenotropic virus, or NZB xenotropic virus failed to remove the reactivity of this serum against intact AKR MuLV in the RIP assay and did not abrogate the ability of this serum to neutralize AKR MuLV infectivity. In contrast, absorption of this serum with comparable amounts of AKR MuLV significantly removed detectable antibodies against AKR MuLV in the RIP assay and significantly abrogated the ability of this serum to neutralize AKR MuLV infectivity. These results, therefore, demonstrate that the ability of (B6C3)F_1 serum to neutralize AKR MuLV is highly type specific for the endogenous ecotropic viruses.

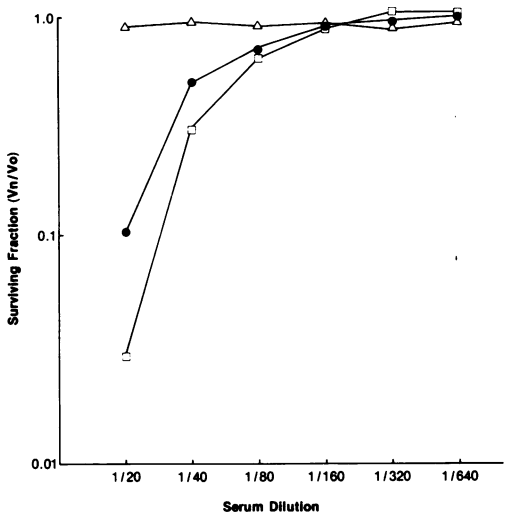

FIG. 2. *Neutralization of various ecotropic MuLV's by (B6C3)F_1 serum. (B6C3)F_1 serum was assayed for the ability to neutralize a B-tropic MuLV (●) and Friend MuLV (△) compared to AKR MuLV (□). The assays were as described in Materials and Methods and in the legend to Fig. 1.*

TABLE 2. *Absorption of neutralizing activity of (B6C3)F_1 serum with intact viruses*

Serum[a] absorbed with:	RIP[b] titer	Surviving fraction (V_n/V_0) at serum dilution:	
		1/20	1/40
	1/2,560	0.07	0.17
AKR MuLV	1/10	0.47	0.80
Friend MuLV	1/2,560	0.07	0.20
BALB:X	1/2,560	0.07	0.28
NZB:X	1/2,560	0.07	0.25

[a] (B6C3)F_1 serum was absorbed with the indicated viruses as follows. To serum samples (0.1 ml), 1 mg of the appropriate virus in 0.1 ml of TNE was added. The mixtures were incubated for 45 min at 37°C and for 45 min at 4°C, and the virus was removed by centrifugation (30,000 rpm for 1 h). The procedure was repeated once, and the sera were stored at −70°C. Control sera were treated with virus buffer and handled identically to the absorbed sera.

[b] The absorbed sera were titered against AKR MuLV in the RIP assay as described in Materials and Methods.

Previous studies suggested that an immune response against the major virion envelope glycoprotein gp71 is associated with neutralization of virus infectivity of Rauscher or Friend MuLV (7, 29). Similar experiments were performed with AKR MuLV using rabbit antisera prepared against a variety of AKR MuLV virion proteins. As illustrated in Table 3, antisera against AKR MuLV gp71 significantly neutralized AKR MuLV infectivity but did not neutralize Friend MuLV. Similarly, an antiserum prepared against Rauscher MuLV gp71 significantly inhibited Friend MuLV, but only weakly neutralized AKR MuLV. In contrast, antisera prepared against AKR MuLV p30, p15, p12, or p10 did not neutralize either AKR MuLV or Friend MuLV infectivity. These results suggest that the primary immune reactivity associated with neutralization of AKR MuLV infectivity is against the major glycoprotein gp71.

To determine whether the neutralization of AKR MuLV by autogenous immune sera was similarly associated with an immune response against gp71, we examined the ability of purified AKR MuLV gp71 to abrogate neutralization. As illustrated in Table 4, increasing concentrations of AKR MuLV gp71 abrogated the ability of (B6C3)F_1 serum to neutralize AKR MuLV infectivity. In contrast, purified Rauscher MuLV gp71 did not block neutralization. These results demonstrate that neutralization of AKR MuLV infectivity by (B6C3)F_1 serum is probably associated with an immune reactivity with gp71, and they further demonstrate the type-specific characteristics of this reactivity.

To examine the immunological characteristics of this neutralization, IgG and IgM fractions were purified from (B6C3)F_1 serum and examined for their ability to neutralize AKR MuLV infectivity and to react with purified AKR MuLV gp71. In RIA with iodinated AKR MuLV gp71, both immunoglobulin fractions reacted with AKR MuLV gp71, although the titer of the IgM fraction was considerably lower than the titer of the IgG fraction (IgG = 1/160, IgM = 1/40; data not shown). In neutralization assays,

TABLE 3. *Ability of xenogeneic antisera against various MuLV proteins to neutralize MuLV infectivity*

Antiserum against:[a]	Surviving fraction (V_n/V_0)					
	AKR MuLV			Friend MuLV		
	1/10[b]	1/100	1/1,000	1/10	1/100	1/1,000
AKR MuLV gp71	0.01	0.01	0.65	1.04	0.96	0.89
Rauscher MuLV gp71	0.01	1.09	0.99	0.01	0.01	0.52
AKR MuLV p30	1.0	1.05	0.97	1.01	1.14	1.0
AKR MuLV p15	1.01	1.14	1.0	0.85	0.79	0.90
AKR MuLV p12	0.87	0.86	0.98	0.92	0.81	0.95
AKR MuLV p10	0.92	0.94	0.97	0.94	0.82	0.97

[a] Rabbit antisera were prepared against the viral antigens indicated. Antigens were purified as described by Ihle et al. (in press) and were homogeneous by polyacrylamide gel electrophoresis. The titers of each antisera against its homologous iodinated antigen were AKR gp71, 1/2,560; Rauscher gp71, 1/1,280; AKR p30, 1/320; AKR p15, 1/1,280; AKR p12, 1/2,560; AKR p10, 1/320; and represented the serum dilution required for precipitation of 50% of the labeled antigen. The sera against AKR MuLV proteins had no detectable titers against the nonhomologous virion proteins.

[b] Serum dilution.

TABLE 4. *Ability of AKR MuLV gp71 to abrogate neutralization of AKR MuLV by (B6C3)F_1 serum*

Serum[a]	Additions	Surviving fraction (V_n/V_0) (μg of MuLV gp71)			
		0	0.012	0.12	1.2
None	Rauscher MuLV gp71	ND[b]	0.86	0.89	0.29
None	AKR MuLV gp71	1.0	1.1	1.2	1.0
(B6C3)F_1	AKR MuLV gp71	0.07	0.32	ND	0.70
(B6C3)F_1	Rauscher MuLV gp71	0.07	0.11	0.11	0.05

[a] Virus samples were incubated in a 0.5-ml final volume with Rauscher MuLV or AKR MuLV gp71 at the indicated concentrations alone or in the presence of a 1/20 final dilution of (B6C3)F_1 serum. The surviving virus fraction was determined by the focus assay on FG-10 cells as described in Materials and Methods.

[b] ND, Not determined.

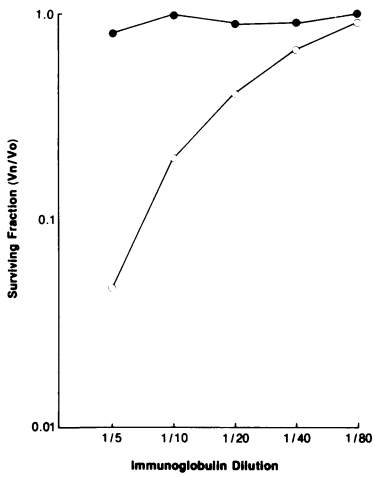

FIG. 3. *Ability of purified IgG and IgM fractions from (B6C3)F_1 serum to neutralize AKR MuLV infectivity. IgG (○) and IgM (●) were purified and judged homogeneous as described in Materials and Methods. The concentration of each fraction was approximately 1/4 the concentration found in normal serum. Assays for neutralization were performed as described in Materials and Methods, and the surviving virus fraction was determined with the focus assay using FG-10 cells.*

however, all the neutralizing reactivity was associated with the IgG fraction (Fig. 3). These results suggest that the neutralization of AKR MuLV by (B6C3)F_1 serum is associated with an IgG immune response against AKR MuLV gp71.

DISCUSSION

The results presented here demonstrate, in contrast to previous studies (1, 9, 16), the ability of naturally immune mouse sera to neutralize AKR MuLV infectivity. We were able to consistently demonstrate this neutralization by using as a source of infectious virus unfrozen supernatants harvested for 1 h. This approach was predicated on the observation that the yield of infectious virus versus age of the culture supernatant increased linearly for 2 to 3 h but was followed by a leveling off and then an actual decline in the yield of infectious virus after 8 h. This effect is presumably due to the relatively short half-life for virus infectivity in tissue culture conditions and subsequent interference between noninfectious virus particles and infectious virus. In general, the half-life of type C virus infectivity with both avian and mammalian viruses has been shown to be relatively short in tissue culture (3 to 4 h), in terms

of both infectivity and the chemical stability of the viral genome (5, 28). With the 1-h harvested virus, natural immune sera had titers of approximately 1/60; this is in contrast to previous results we obtained with standard assays in which the highest titers, at best, were less than 1/10. Nevertheless, our results demonstrate that immune mouse sera can neutralize MuLV infectivity in vitro and, therefore, they might be expected to also neutralize virus in vivo.

The ability of naturally immune mouse sera to neutralize AKR MuLV is apparently a consequence of a specific immune response against AKR MuLV gp71, as demonstrated by the abiity of gp71 to abrogate neutralization at concentrations that do not interfere with virus infectivity. This result, however, was not unexpected since antisera against other virion components have generally not mediated neutralization. In particular, our results demonstrate that antisera against AKR MuLV p30, p15, p12, or p10 do not neutralize AKR MuLV, and previous studies (7) demonstrated that antisera against p15(E) only weakly neutralized infectivity, but required complement. These results, therefore, are consistent with extensive studies of Friend MuLV, which demonstrated that only immune reactivities against gp71 mediated efficient complement-independent neutralization (7).

The immune response of naturally immune mouse sera has been shown to be primarily against gp71 and p15(E). Although we have not specifically examined the influence of immune reactivity against p15(E) on neutralization, the data suggest that it neither enhances nor interferes with neutralization by the immune response to gp71. In particular, previous studies demonstrated that p15(E) is serologically identical in both Friend and AKR MuLV (14, 26). Moreover, the ability of mouse sera to react with labeled Friend MuLV in the RIP assay was shown to be mediated by the reaction against p15(E), and absorption with Friend MuLV specifically removes this reactivity (12). The results demonstrated here show that (a) although immune mouse sera react with Friend MuLV in RIP assays, this reaction does not neutralize Friend MuLV infectivity, and (b) absorption of neutralizing sera with Friend MuLV neither enhances nor interferes with the ability of such sera to neutralize AKR MuLV. Therefore, the consequences of an immune response against p15(E) are not presently known.

Our results also demonstrate that the neutralization of AKR MuLV is specifically mediated by an IgG reaction, in contrast to the inactivation of xenotropic viruses by normal mouse sera, which is not mediated by immunoglobulins (6, 17). In particular, purified 7S immunoglobulins retained all the neutralizing activity present in complete serum. These results therefore complement and extend previous studies by Fischinger et al. (6), which demonstrated the ability of specific antisera to mouse IgG_1 and/or IgG_2 to abrogate the neutralization of AKR MuLV by $(B6C3)F_1$ serum without influencing the ability of such sera to inactivate xenotropic virus.

Although the IgG response to gp71 could neutralize AKR MuLV, the IgM fraction had no activity in the neutralization assays but was capable of reacting with intact virus in RIP assays and with iodinated AKR MuLV gp71. However, for both reactivities, the IgM fraction was lower in titer relative to serum concentrations than was the IgG fraction. Moreover, combining the IgG and IgM fraction had no influence on the ability of the IgG fraction to neutralize virus (data not shown). The basis for this difference is not known, but it may be related to either the lower titer of the IgM fraction or the possibility that IgM may have a lower affinity constant for the antigen, or both. Experiments are currently in progress to further examine this difference.

With the results presented here and elsewhere, it now has become clear that in a variety of strains of mice there exists an intact immune response against endogenous ecotropic viruses. The humoral immune response with its preferential specificity for gp71 and p15(E) can mediate both neutralization of infectivity of MuLV and complement-dependent cytotoxicity of virus-replicating cells (20, 24). Recent data have also demonstrated cell-mediated immune reactivity in vitro with immunological specificity for gp71, as detected both by cytotoxicity and blastogenesis (J. C. Lee and J. N. Ihle, in preparation). Now, with an understanding of the in vitro ability of this immune response, we can begin to approach the questions of the significance of this response in vivo on the growth of tumors of both viral and nonviral etiology, but expressing viral antigens.

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