Mouse Immunoglobulin Antibodies Require Complement for Neutralization of Mouse Retroviruses

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The addition of guinea pig complement was found to enhance the neutralizing capacity of mouse antibodies directed against the endogenous ecotropic murine leukemia viruses. The same immune sera, when tested without complement, had low or negligible neutralizing capacities, regardless of whether freshly harvested, unfrozen virus was used to preserve virus infectivity. Antibodies in high titers were found in sera from NFS congenic mice carrying the mouse leukemia virus inducing locus *Akv-2*. These mouse antibodies were type specific and failed to neutralize either Friend or Moloney leukemia virus. The mouse serum immunoglobulin fraction containing the complement-dependent antibodies was tentatively identified as immunoglobulin M.

The immunological response of mice against endogenous ecotropic type C viruses has been demonstrated by several techniques; most studies have used radioimmunoprecipitation assays with [³H]leucine-labeled intact virions (6, 13, 20, 21). Tissue culture neutralization tests analogous to those used for retroviruses of other species, such as chickens and cats (15, 28, 29), have been of limited usefulness; in particular, antibodies to viral gp70 fail to neutralize virus infectivity satisfactorily (1, 9, 13, 17, 20). Ihle and Lazar (14) have attempted to improve the neutralization test for mouse antibodies against the endogenous ecotropic virus of AKR mice by using freshly harvested, unfrozen virus with a high proportion of intact, infectious virus. By this procedure, they have been able to observe neutralization of 66% of the virus dose used by serum dilutions of up to 1:60.

We report here a new, simple neutralization test which is highly sensitive and reproducible. The method simply involves the addition of guinea pig complement to virus-serum mixtures before incubation. Using conventionally prepared, frozen virus, diluted to contain approximately 100 XC PFU of virus, we were able to detect antibodies in mouse sera diluted to over 1:1,280. Mouse immunoglobulins against mouse retroviruses thus appear to require complement for efficient neutralization of virus in a manner previously found for many enveloped and non-enveloped viruses (2, 5, 7, 8, 12, 19, 22, 27).

MATERIALS AND METHODS

Sera. The studies reported here were done chiefly with sera from uninoculated, adult NFS mice congenic for the *Akv-2* ecotropic virus-inducing locus from

AKR (NFS.*Akv-2*) mice (24, 25) and sera from NFS controls. In addition, we tested sera from NFS congenic mice carrying various other ecotropic murine leukemia virus (MuLV)-inducing loci (24, 25), as well as sera from adult mice of various inbred and hybrid strains, and obtained comparable results.

Blood samples were obtained by bleeding from the retroorbital plexuses. The sera were diluted 1:5 in saline and stored at -20°C. The standard positive serum (pool no. 2) was pooled undiluted sera from mice of a congenic inbred NFS.*Akv-2* subline; these mice showed endogenous-virus titer suppression indicative of endogenous antibody responses. This serum was strongly positive by radioimmunoprecipitation and enzyme-linked immunosorbent assay with AKR ecotropic virus. The standard negative serum was pooled sera from normal NFS mice.

The sera were not heat inactivated before use. Exposure of antibody-positive sera to 56°C for 30 min generally resulted in a lowering of the titers of neutralizing antibodies against viruses (ecotropic and mink cell focus-forming (MCF) viruses) by one or two twofold dilutions, presumably due to the lability of immunoglobulin M (IgM) antibodies. Although caution must be exercised in using unheated sera that might contain residual virus, representative sera that were obtained from mice with high titers of virus in tissue extracts and that were processed and stored in the standard manner did not give XC plaques in SC-1 cultures when tested under the standard neutralization test conditions.

Virus. The AKV strain of endogenous ecotropic MuLV was originally isolated from a pool of tail extracts of NIH Swiss mice partially congenic for the *Akv-1* locus (24, 25). Virus stocks were prepared in NIH 3T3 cell cultures as previously described (4). Stocks of Friend and Moloney leukemia viruses were similarly prepared in SC-1 cultures. Stocks of MCF virus, strain MCF 247 (11), were prepared in mink lung cell (CCL 64) cultures. Virus was assayed by mink cell focus assay (11) in mink lung cell cultures.

Cultures. SC-1 cells (10) were used for neutralization tests with ecotropic viruses. The cells were used between 70 and 88 *in vitro* passages. They were propagated in a medium consisting of Eagle minimal essential medium supplemented with 10% fetal bovine serum (heated to 56°C for 30 min), L-glutamine, and 50 µg of gentamicin per ml. The cells were planted at a concentration of 2.5×10^5 per dish (60-mm diameter, plastic, disposable [Costar]) in 4-ml amounts of medium containing 10% fetal bovine serum. The next day, before the neutralization test, the culture medium was replaced with one containing 5% fetal bovine serum and 16 µg of polybrene per ml.

Complement. Guinea pig complement was obtained from BioQuest Div., BBL Microbiology Systems, Cockeysville, Md., as lyophilized serum. After suspension in sterile distilled water at twice the recommended volume, the 1:2 dilution of complement was immediately filtered through a 0.22-µm membrane filter (Millipore Corp., Bedford, Mass.) and stored in suitable portions at -70°C. Assay of complement with rabbit hemolysin and sheep erythrocytes gave a hemolytic titer of 125 U per ml of undiluted complement. We employed 2.5 U of complement in a 0.2-ml volume of virus-serum mixture.

Neutralization test. Virus was suitably diluted and mixed with requisite amounts of complement and diluent to give a 1:5 dilution of complement and 100 XC PFU of ecotropic virus (or 100 focus-forming units of MCF virus) per 0.1 ml. The virus-complement mixture was incubated at 37°C for 5 min, and 0.1-ml portions were added to 0.1-ml portions of twofold serum dilutions; thus, each virus-serum mixture contained a 1:10 dilution of complement and 100 PFU or focus-forming units of virus per 0.2 ml. Controls for each test included normal NFS mouse serum plus virus and complement (1:20 dilution of serum), positive mouse serum (dilution that gave 4 neutralizing-antibody units) plus virus and complement, diluent plus virus and complement, diluent plus virus without complement, and a titration of virus. The samples were thoroughly mixed and then incubated for 45 min in a humidified CO₂ incubator at 37°C with the caps slightly loose. At approximately 15-min intervals, the rack was shaken three or four times. Subsequently, 0.5 ml of diluent was added to each vial, and all of the 0.7 ml in each vial was inoculated into one tissue culture dish. The cultures were agitated gently several times and then returned to the CO₂ incubator.

The next day, the polybrene-containing medium was removed, and the cultures were subsequently maintained in a medium with 5% fetal bovine serum. The ecotropic virus-infected cultures were exposed to UV light, overlaid with XC cells on day 5, and stained and read on day 8 as previously described (26).

In neutralization tests with MCF virus, the virus-serum mixtures and controls were similarly inoculated into polybrene-treated mink lung cell cultures in petri dishes. The cultures were suitably fed and examined for MCF foci 7 to 9 days after inoculation.

The titer of a serum was taken to be the highest dilution which caused a $\geq 80\%$ reduction of plaques (or MCF foci), as compared with the control culture inoculated with the mixture of virus, complement, and normal mouse serum (1:20 dilution).

Determination of the nature of immunoglobulins responsible for complement-dependent virus neutraliza-

tion. High-titer sera from three NFS.Akv-2 mice were pooled to give 1 ml. This pooled sample was subjected to affinity chromatography on a column of staphylococcus protein A-Sepharose, which specifically binds IgG. The IgG absorbed on the column was eluted with 0.1 M acetate buffer, pH 3.0. After dialysis, the eluate and wash were concentrated to 1 ml each by filtration (Diaflo membrane; Amicon Corp., Lexington, Mass.) under positive pressure. The samples were filtered through 0.22-µm filters (Swinney; Millipore Corp.) and tested for neutralizing activity against AKV virus as described above.

RESULTS

In our experience, the neutralization test with fresh unfrozen virus (14) was cumbersome and insensitive. With this test, our positive mouse serum pool, which had a titer of 1:1,280 by the enzyme-linked immunosorbent assay, failed to neutralize more than 70% of the AKV virus, even at the lowest dilution (1:10); the titer of this serum, determined according to the criterion of 66% virus neutralization (14), was erratic, unpredictable, and no higher than 1:80. On the other hand, we found, to our surprise, that regardless of the kind of virus preparation used (fresh or frozen), the addition of complement to virus-serum mixtures markedly enhanced neutralization by this seemingly low-titer serum. Thus, in the presence of complement, we observed complete neutralization of the virus dose used by serum dilutions of up to 1:320 and a $\geq 80\%$ reduction in plaques at the 1:640 dilution (Fig. 1). Cultures inoculated with virus-serum mixtures without complement showed little or no reduction in XC plaques (Fig. 1). Control cultures inoculated with virus plus complement (without antibody) showed no reduction in plaques. Repeated tests gave the same results and the same titration endpoint for this positive serum pool. Judged by the criterion of $\geq 80\%$ virus neutralization used for computing titration endpoint, this serum scored $< 1:10$ without complement and 1:640 with complement.

Results obtained for sera from eight additional AKV antibody-positive mice are shown in Fig. 2. Without complement, all of the sera scored $< 1:20$. However, in the presence of complement, three sera completely neutralized the virus at all serum dilutions tested, up to and including 1:640. Two scored 1:320, and one scored 1:80.

Heating complement to 56°C for 30 min completely destroyed its enhancing effect on the observed neutralization. Also, reducing the complement concentration in the virus-serum mixtures to below 5% resulted in a parallel lowering of the titer of the serum (Table 1).

Failure of AKV-neutralizing mouse serum to neutralize Friend and Moloney leukemia viruses.

In the presence of complement, positive mouse serum pool no. 2, which had antibody against AKR at a titer of 1:640, failed to neutralize either Friend or Moloney leukemia virus, even at the lowest dilution (Fig. 3).

Complement-dependent neutralizing antibody against MCF virus in AKV-neutralizing mouse serum. Positive mouse serum pool no. 2 neutralized MCF virus strain MCF 247 in all dilutions, up to and including 1:1,280. Positive serum-virus mixtures without complement or negative serum-virus mixtures with or without complement showed little or no evidence of virus neutralization (titers, <1:20). Guinea pig complement often inactivated up to 50% of MCF virus, so the test must be carefully standardized.

Absorption of positive mouse serum pool no. 2 with MCF virus (4×10^{11} particles per ml from infected mink lung cell cultures) for 18 h at 4°C resulted in the lowering of titers of neutralizing antibody against MCF but not AKV virus. Thus, a comparison between absorbed heated (56°C for 30 min to eliminate infectious MCF virus) serum and parallel control heated serum pool no. 2 showed a drop in neutralizing-antibody titer from 1:320 (control unabsorbed serum) to 1:20 (absorbed serum) and no detectable differ-

FIG. 1. Failure of an antibody-positive mouse serum to cause satisfactory neutralization of freshly harvested or frozen AKV and acquisition of neutralization ability after addition of guinea pig complement to virus-serum mixtures. Complete neutralization of virus is shown as <0.01 (fraction of surviving virus).

FIG. 2. Parallel assays for neutralizing antibody against AKV in eight antibody-positive mouse sera without complement (C-) at 1:20, 1:40, and 1:80 and with complement at 1:80, 1:160, 1:320 and 1:640. Each panel shows the results obtained for two sera. Complete neutralization of virus is shown as <0.01 (fraction of surviving virus).

ence in the titers of neutralizing antibody against AKV (titers, 1:320).

Immunoglobulin fraction causing complement-dependent neutralization. Affinity chromatography of an antibody-positive serum on a staphylococcus protein A-Sepharose column resulted in the separation of virus-neutralizing activity from

TABLE 1. Titers of mouse serum pool no. 2 neutralizing antibody against AKV at various concentrations of complement

Complement concn (%)	Titer ^a
10	1:640
5	1:640
2.5	1:320
1.25	1:160

^a Highest serum dilution giving $\geq 80\%$ reduction of 40 PFU of virus.

FIG. 3. Failure of mouse serum pool no. 2 with virus-neutralizing antibody against AKV to neutralize antigenically distinct ecotropic MuLVs.

the IgG fraction. The wash containing IgM, IgA, IgE, and albumin had antibody against AKV at a titer of 1:80, whereas the eluate containing the IgG fraction had antibody at a titer of less than 1:2.

DISCUSSION

Although most virus neutralization tests are not affected by the presence or absence of complement, there are a number of instances when complement is required; this has been seen with both enveloped and nonenveloped RNA and DNA viruses (2, 5, 7, 8, 12, 16, 19, 22, 27). We found this to be true for mouse antibodies against certain mouse retroviruses. As observed in most of the aforementioned studies with RNA and DNA viruses, our preliminary studies also suggested that the serum IgM, rather than the IgG fraction, was responsible for complement-dependent neutralization. Mouse serum IgG also participates in the neutralization of AKR endogenous virus (without complement), as shown by the studies of Ihle and Lazar (14).

The ability of naturally immune sera to specifically neutralize AKR MuLV and not Friend or Moloney MuLV shows that the neutralizing antibody detected with AKV is a consequence

of a type-specific immune response, presumably against AKV gp70. The complement-dependent virus neutralization test was useful in determining the immune response of mice against their endogenous ecotropic viruses and should be useful in determining type-specific similarities and differences in viral envelope gp70 antigens in various isolates of murine ecotropic viruses. In preliminary studies, we have found that mouse antisera against Friend and Moloney leukemia viruses show the same dependency on complement for virus neutralization.

The presence of MCF virus-neutralizing antibody in positive mouse serum pool no. 2 was conceivably due to immunization of the mice (NFS.Akv-2) with MCF-type viruses spontaneously arising in these mice (4, 11). The fact that in the absence of complement no neutralization of MCF virus occurred rules out the possibility of neutralization by a non-immunoglobulin type of mouse serum factor which neutralizes mouse xenotropic viruses (18). Also, NFS mouse serum generally lacks this factor.

Although human and primate sera are known to cause lysis of RNA tumor viruses (30, 32), we found that, as reported by others (32), guinea pig complement does not cause a noticeable adverse effect on the infectivity of ecotropic MuLV unless specific antibody present.

Possible modes of virus neutralization in the presence of complement and antibody include virolysis (3, 22, 23, 32), formation of large aggregates of virus-antibody complexes resulting in the physical blocking of virus by large complement molecules, and prevention of virus attachment to cells (5, 19, 22, 31). The exact mechanism remains to be clarified.

The ability of complement to enhance the neutralization during the early phases of antibody response is conceivably aimed at helping to strengthen the early feeble antibody response and appears to be one of the clearly defined biological functions of complement.

The neutralization test we describe is highly reproducible and simple to perform. Its sensitivity far exceeds that obtained by a method employing fresh virus without complement (14). The usefulness of the test was further established by comparable results obtained in additional tests (data not shown) with sera from NFS congenic mice carrying various other MuLV-inducing loci (24, 25), as well as sera from adult mice of various inbred and hybrid strains. The specificity of the test was established by the generally parallel results we obtained in the complement-dependent neutralization test, the enzyme-linked immunosorbent assay, and the radioimmunoprecipitation assay (data not shown) and by the type specificity of the neutralization reaction observed.

The role of humoral antibody responses to endogenous MuLVs in determining *in vivo* virus expression and leukemogenicity has not been adequately evaluated. The test described here should greatly facilitate such studies.

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