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Monoclonal Antibodies Specific for Wild Mouse Neurotropic Retrovirus: Detection of Comparable Levels of Virus Replication in Mouse Strains Susceptible and Resistant to Paralytic Disease

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We used AKR/J mice to produce monoclonal antibodies specific for a neurotropic ecotropic (WM-E) virus initially isolated from wild mice. The rationale for this approach involved the observation that these mice were immunologically hyporesponsive to endogenous ecotropic virus (Akv) but fully responsive to type-specific determinants of WM-E. Hybridoma cell lines derived from mice immunized with both denatured and viable virus produced antibodies with specificity for three viral membrane-associated polypeptides, gp70, p15(E), and $p15^{gag}$. Epitopes specific for WM-E virus were detected in each of these polypeptides. Cross-reactivity with Friend ecotropic virus (Friend murine leukemia virus) was observed with some gp70- and $p15^{gag}$-specific antibodies, but no reactivity with endogenous Akv ecotropic virus was seen. The majority of these antibodies did not react with either xenotropic or mink cell focus-forming viruses. Two WM-E-specific anti-gp70 antibodies reacting with different determinants had virus-neutralizing activity in the absence of complement, suggesting that the respective epitopes may participate in receptor binding or virus penetration events. We used these monoclonal antibodies in initial studies to examine the replication of WM-E virus in neonatally inoculated AKR/J mice which are fully resistant to the paralytic disease induced by this virus. Since these mice express high levels of endogenous ecotropic virus, standard assays for ecotropic virus cannot be used to study this question. We present evidence that the resistance to disease does not involve a resistance to virus replication, since these mice expressed levels of viremia and virus replication in spleen and lumbar spinal cord comparable to susceptible NFS/N mice at a time when the latter began to manifest clinical signs of lower-motor-neuron pathology.

An ecotropic virus isolated from wild mice (WM-E) induces lower-motor-neuron disease in wild and in some strains of laboratory mice (6, 15). When this virus is inoculated in newborn susceptible mice, it replicates in both hematopoietic organs and the central nervous system and produces a viremia (1). Although virus replication can be detected in the spleen within days after inoculation (1), neurological disease is characteristically not observed until serveral months of age (15). Recombinant mink cell focus-forming viruses have been detected in the spleens of these mice and appear to precede the clinical signs of neurological disease (7, 14). Mink cell focus-forming viruses, however, have not been isolated from the central nervous system (14; personal observation), suggesting that the neuronal pathology is caused by the ecotropic virus alone. The nature of the neuronal pathology is unclear, although there is evidence that a local accumulation of either envelope (12, 22) or *gag* (13) polypeptides may be associated with the disease. Host genes appear to modulate the tempo of disease, and some mouse strains are completely resistant (8).

The nature of both viral and host factors in this disease prompted our efforts to produce antibody reagents with defined viral polypeptide specificities, which would be capable of detecting the WM-E virus exclusive of other endogenous viruses expressed in the same tissues. In this report, the virus and polypeptide specificities of these antibodies are described as well as evidence of replication of this virus in mice that are highly resistant to the neurological disease.

Monoclonal antibodies which we have generated in the past to other mouse retroviruses of diverse origin (3, 4, 18, 19) were initially surveyed for reactivity with WM-E virus. We found that the only antibodies reactive with this virus were those which exhibited broad murine-retrovirus specificity. Our approach to preparing more specific reagents involved the observation that AKR/J mice are immunologically hyporesponsive to endogenous ecotropic virus (Akv) (9). We suspected that these mice, inoculated with WM-E virus, would produce antibody specific for WM-E virus but nonreactive with Akv. To test this hypothesis, AKR/J mice were inoculated intravenously with molecularly cloned WM-E (pBR-NE-8) kindly provided by P. Jolicoeur, Institut de Recherches, Université de Montreal, Montreal, Quebec, Canada (10). NFS/N mice served as controls since they lack endogenous ecotropic viral sequences. The titers of plasma collected weekly was determined by indirect immunofluorescence on live *Mus dunni* cells (11) infected with the ecotropic viruses WM-E, Akv (AKR2a), and Friend murine leukemia virus clone 57 (F-MuLV) (18). The respective titers (Table 1) indicated that AKR/J mice produced antibody specific for WM-E virus and a small amount of antibody cross-reactive with F-MuLV. In contrast, NFS/N mice produced comparable levels of anti-Akv and anti-WM-E antibodies. This virus specificity was maintained in mice inoculated with virus denatured with Nonidet P-40 (NP-40) and ether (5), although the antibody titer was considerably lower (data not shown).

Spleen cells from AKR/J mice inoculated with live and denatured viruses were fused with P3-NS1/1-Ag4-1 myeloma cells as previously described (19), and the antibody produced by the hybridomas was screened initially in a virion-

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TABLE 1. Plasma antibody titers of two mouse strains inoculated with WM-E virus

Mouse strain[a]	Antibody titer[b]		
	Akv	F-MuLV	WM-E
AKR/J	<1:10	1:20	1:320
NFS/N	1:640	NT[c]	1:320

[a] WM-E virus (4×10^5 focus-forming units) was inoculated intravenously into 6-week-old mice, and plasma was collected at 7, 14, and 21 days.

[b] Titers of plasma were determined by indirect membrane immunofluorescence on live *M. dunni* cells chronically infected with ecotropic viruses Akv, F-MuLV, and WM-E. Values are peak antibody titers on day 14 expressed as the averages of two mice per group.

[c] NT, Not tested.

binding radioimmunoassay with virus-coated plastic plates (18) and by an indirect membrane immunofluorescence assay with live-virus-infected cells (19). All wells that were found positive in either assay were cloned twice by limiting dilution.

NP-40 lysates of SC-1 cells acutely infected with WM-E virus and metabolically labeled with [^{35}S]methionine (19) were immunoprecipitated with tissue culture supernatants of the hybridoma cell lines. Resolution of the immunoprecipitates by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis revealed two general polypeptide specificities (Fig. 1A). One group of antibodies represented by the prototype 710 immunoprecipitated the viral envelope precursor pr85^{env} and the two envelope polypeptides gp70 and p15(E). The second group of antibodies represented by antibody 716 precipitated the *gag* gene product pr65 (Fig. 1A).

Further resolution of the *gag*-specific antibodies was accomplished by immunoprecipitation analysis of a ^{125}I-labeled lysate of purified WM-E virions (4). All of the antibodies which immunoprecipitated [^{35}S]methionine-labeled pr65^{gag} reacted with ^{125}I-labeled p15^{gag} (Fig. 1B, antibody 716). An incidental finding was that all of the anti-p15^{gag} monoclonal antibodies coprecipitated gp70 (Fig. 1B). This finding is consistent with the suggestion that p15^{gag} is inserted in the viral membrane (16) and is associated with one or both of the viral envelope proteins (20). We detected no antibodies reactive with *gag* polypeptides present in the viral core. This apparent skewing of the antibody response was likely because the virus-binding radioimmunoassay and membrane immunofluorescence assay used to initially screen the hybridomas would be expected to detect only determinants exposed at the surface of virions or at the plasma membrane of the cells, respectively.

Those antibodies which immunoprecipitated [^{35}S] methionine-labeled pr85^{env} in cell lysates were further examined by Western blot analysis (18) to determine their reactivity with isolated virion envelope polypeptides (Fig. 1C). Antibody 710 represented a group of gp70-specific antibodies, whereas antibody 682 reacted with p12(E) and p15(E). In contrast to other monoclonal anti-gp70 antibodies which we have analyzed by this technique (3, 18), no preferential binding to the

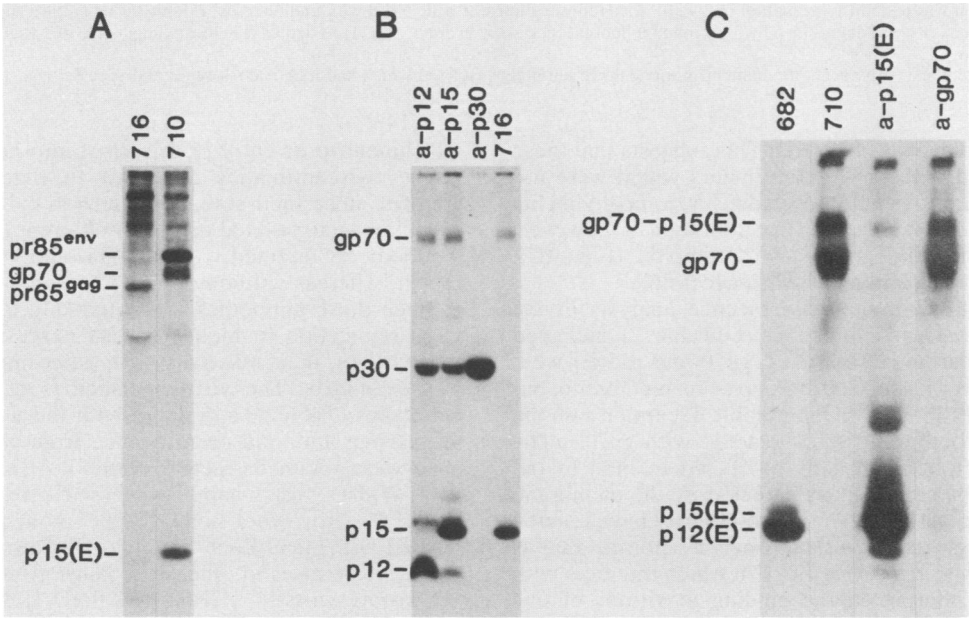

FIG. 1. (A) SC-1 cells infected with WM-E virus were labeled with [^{35}S]methionine, lysed with 0.5% NP-40, and immunoprecipitated with monoclonal antibodies 716 and 710. Precipitates were resolved in 10% SDS-polyacrylamide gels, the autoradiograms of which are shown. Two polypeptide specificities illustrated are pr65^{gag} and pr85^{env}. (B) The pr65^{gag}-specific monoclonal antibodies were further analyzed by immunoprecipitation of NP-40 virus lysates labeled with ^{125}I. Viral *gag* proteins were detected with goat antisera to Rauscher MuLV p12, p15, and p30 (obtained from the Biological Carcinogenesis Branch of the National Cancer Institute). Immunoprecipitates were resolved in 12% SDS gels, the autoradiograms of which are shown. Monoclonal antibodies represented by 716 reacted specifically with p15. (C) The pr85^{env}-reactive monoclonal antibodies (represented by 682 and 710) were analyzed by Western blot. Purified WM-E virions were lysed directly in 2% SDS without reducing agent, and the viral proteins were separated in 10% SDS gels. After being electroblotted onto nitrocellulose paper, tracks were incubated separately in monoclonal antibody or rabbit antisera to gp70 and p15(E) (supplied by G. Hunsmann, Freiburg, Federal Republic of Germany). Antibody binding was revealed by autoradiography with ^{125}I-protein A after enhancement with rabbit anti-mouse immunoglobulin serum.

TABLE 2. Reactivities of monoclonal antibodies to WM-E virus

Immunogen[a] and antibody no.	Isotype[b]	SA binding[c]	Polypeptide specificity[d]	Virus specificity[e]	Virion binding[f]	Immunofluorescence[g]		Neutralization[h]
						Membrane	Cytoplasmic	
Denatured virus								
709	IgG2b	+	gp70	W + F	+	+	+	−
710	IgG1	+	gp70	W + F	+	+	+	−
704	IgG1	+	gp70	W + F	+	+	+	−
693(9)[i]	IgG1	+	gp70	W	+	+	+	−
697	IgG1	+	gp70	W	+	+	+	−
689(2)	IgG1	+	gp70	W	+	−	+	−
703	IgM	−	p15	W + F	+	−	+	−
691(2)	IgG2a	+	p15	W + F	+	−	+	−
692(2)	IgG2b	−	p15	W + F	+	−	+	−
712(3)	IgG2b	−	p15	W + F	+	−	+	−
690	IgG2a	+	p15	W + F	+	−	+	−
705(3)	IgG1	+	p15	W + F	+	−	+	−
Live virus								
668(2)	IgG2a	+	gp70	W	+	+	−	−
667	IgG2a	+	gp70	W	+	+	+	+
672	IgM	−	gp70	W	+	+	−	+
678(2)	IgG1	+	gp70	W	+	+	+	−
682	IgM	−	p15(E)	W	+	+	+	−
685	Unknown	+	p15	W	+	−	+	−

[a] AKR/S mice (6 weeks old) were inoculated intraperitoneally with 50 μg of NP-40–ether-denatured WM-E virus (5) in complete Freund adjuvant and were given a booster inoculation with the same preparation in incomplete Freund adjuvant on day 21. Spleen cells were fused on day 24. Mice which received live virus (see Table 1) were sacrificed on day 14.

[b] Isotypes were determined by gel diffusion with immunoglobulin class-specific reagents from Litton Bionetics, Kensington, Md.

[c] SA, Staphylococcus A Cowan I strain.

[d] Viral polypeptide specificity was determined by a combination of immunoprecipitation and Western blot analysis (see text).

[e] Virus specificity was determined by virus-binding radioimmunoassay and by indirect membrane and cytoplasmic fluorescence. W, WM-E; F, F-MuLV.

[f] Virion binding was studied with a radioimmunoassay by using purified WM-E and F-MuLV viruses as antigens as described previously (18).

[g] Indirect membrane and cytoplasmic immunofluorescence was carried out on live and acetone-fixed virus-infected cells, respectively. Differences in intensity were observed but were not quantified.

[h] Virus neutralization was tested by incubation of 2×10^3 focus-forming units of pBR-NE-8 with undiluted hybridoma tissue culture supernatants at 37°C for 30 min, after which infectivity was determined by focal immunofluorescence assay in duplicate (21). A decrease in foci per dish of ≥50% was considered significant neutralization.

[i] The number in parenthesis represents the number of monoclonal antibodies isolated which had identical istotypes and specificities.

gp70-p15(E) complex was observed. This suggests that these antibodies reacted with gp70 determinants which were not affected by the conformational change accompanying its interaction with p15(E). The p15(E) antibody 682, however, did not react with the gp70-p15(E) complex (Fig. 1C), indicating that this determinant was inaccessible.

Indirect membrane immunofluorescence analysis of the antibodies on live-virus-infected cells (Table 2) indicated that, like other murine retroviruses, gp70 and p15(E) were exposed at the external surface of the plasma membrane, but $p15^{gag}$ was inaccessible (20). One notable exception was the gp70-specific antibody 689 which reacted with gp70 in the cytoplasm of acetone-fixed cells but failed to bind to the surface of virus-infected cells as detected by the membrane immunofluorescence assay. This antibody may have reacted with a domain associated with the cell membrane that is located perhaps within a region of gp70 which interacts with p15(E) (17). We interpreted the binding to virions of this antibody and to all of the $p15^{gag}$-specific antibodies (Table 2) to have resulted from damage to the viral envelope which often accompanies purification, resulting in exposure of determinants which might otherwise be inaccessible.

Virus neutralization was observed with only 2 (667 and 672) of the 22 *env*-specific antibodies isolated in this study (Table 2). Both antibodies were recovered from mice immunized with live virus, and both were highly virus type specific. Neutralization was seen without the addition of exogenous complement, suggesting that the determinants with which these antibodies reacted may be involved in attachment to or entry of this virus into host cells or both. These two antibodies appeared to react with different epitopes since their staining pattern in cytoplasmic fluorescence on acetone-fixed cells was different (data not shown). Antibody 667 reacted with gp70 primarily in a paranuclear region, whereas antibody 672 stained only the cell surface.

Since these antibodies are potentially useful in studying virus replication in mouse strains expressing endogenous retroviruses, their reactivity with other murine retroviruses was examined. The virus specificities of the monoclonal antibodies (Table 2) correlated with the virus specificity of the plasma antibody seen in mice from which the hybridomas were obtained (Table 1; AKR/J). All antibodies reacted with WM-E virus; some expressed cross-reactivity with F-MuLV. However, none of the monoclonal antibodies reacted with Akv. Each antibody was also tested for reactivity with viruses in other host range groups, including the xenotropic viruses AKR6, BALB-IU-1, NZBQ-IU-3, and NIH AT124 and the mink cell focus-forming viruses AKR247, Akv-2-C34, C58L1, and F-MCF-1 (19). Only antibody 704 reacted with xenotropic and dualtropic viruses. All other antibodies were specific for WM-E or WM-E and F-MuLV. The high degree of WM-E virus specificity exhibited by these antibodies may reflect the amount of divergence of this virus in the wild mouse population from other ecotropic viruses maintained in the laboratory (2). It was of interest that all of the $p15^{gag}$-specific antibodies except 685 and 703 cross-reacted with the amphotropic viruses 1504A and 4070A (data not shown) (3). This suggests that the $p15^{gag}$

TABLE 3. Replication of WM-E virus in AKR/J and NFS/N mice

Mouse strain[a]	Neurological disease[b]	Splenic infectious centers (FFU/10^6 cells)[c]		Viremia FFU/ml of serum[d]		Lumbar cord infectious centers (FFU/g)[e]	
		WM-E[f]	Akv	WM-E	Akv	WM-E	Akv
NFS/N	+	9.0×10^3	NT[g]	5.8×10^3	NT	2.7×10^4	NT
AKR/J	−	2.6×10^3	1.9×10^2	1.1×10^3	3.6×10^2	1.3×10^4	8.2×10^3

[a] Mice were inoculated with 6.3×10^3 focus-forming units of WM-E virus (pBR-NE-8) intraperitoneally at <24 h of age.
[b] The pattern of susceptibility and resistance to neurological disease has been reported by Hoffman and Morse (8) and has been observed by us.
[c] Infectious center assay on dissociated spleen cells was done with *M. dunni* cells (11) as indicator cells. Foci of infection were detected on day 4 by focal immunofluorescence assay with monoclonal antibodies. Results are the means of 4 to 10 mice. FFU, Focus-forming units.
[d] Viremia was assayed by focal immunofluorescence assay with undiluted serum.
[e] Lumbar cords were dissociated in 2.5 ml of 0.25% trypsin–0.25% collagenase in phosphate-buffered saline without Ca^{2+} or Mg^{2+} for 30 min at 37°C, followed by gentle aspiration 10 times with a Pasteur pipette. The enzymes were neutralized with 7.5 ml of RPMI 1640 contained 10% heat-inactivated fetal calf serum, and infectious center assays were done with *M. dunni* cells as indicator cells.
[f] Monoclonal antibodies used in the focal immunofluorescence assays were 667 for WM-E and 24-8 (19) for Akv.
[g] NT, Not tested. NFS/N mice do not express Akv since they lack endogenous Akv sequences.

protein of WM-E virus is related to that of the amphotropic viruses which, like WM-E, were also isolated from wild mice.

AKR/J mice are fully resistant to the neurological disease induced by WM-E (8). Since AKR/J mice express endogenous ecotropic virus and WM-E and Akv are in the same viral interference group (data not shown), one might expect that this resistance may be a manifestation of viral interference. Quantitation of WM-E virus replication in AKR/J mice has not been possible because the standard assay for ecotropic viruses, the XC assay, would not distinguish the endogenous Akv ecotropic virus expressed normally in this strain. We have used one of the WM-E gp70-specific monoclonal antibodies (667) and an Akv-specific antibody (19) in a focal immunofluorescence assay (21) to compare the expression of the respective viruses in AKR/J (resistant) and NFS/N (susceptible) mice. Infectious center assay of spleen cells from 6-week-old mice inoculated neonatally revealed no difference in the frequency of WM-E virus-producing cells between the two strains (Table 3). At this age, NFS/N mice characteristically began to manifest neurological signs. In addition, no significant differences were observed in the titers of WM-E virus in the serum of mice from two strains. The frequency of Akv infectious centers in uninoculated AKR mice 6 weeks of age (L. Evans, personal communication) was no different than the frequency of Akv infectious centers in WM-E virus-inoculated mice (Table 3). Thus, WM-E virus had no apparent effect on the expression of endogenous ecotropic virus.

To compare virus replication in the target organ, lumbar cords were aseptically removed from NFS/N and AKR/J mice 6 to 8 weeks after WM-E virus inoculation. Cord segments were minced and dissociated in trypsin-collagenase. This treatment was shown to effectively inactivate extracellular virus by $>2 \times 10^3$ (data not shown). Infectious center assays of these preparations indicated comparable levels of WM-E virus replication in the cords of both susceptible and resistant mice (Table 3). Of interest was the finding that cells from the lumbar cord of AKR/J mice expressed relatively high levels of infectious Akv (Table 3). It was clear that expression of Akv per se did not limit replication of WM-E, even in the target organ of AKR/J mice. Thus, if the resistance mechanism of this strain involves viral interference, it must operate on a limited number of critical target cells, infection of which results in motor neuron dysfunction.

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