MCF-Specific Murine Monoclonal Antibodies Made Against AKR-247 MCF Virus Recognize a Unique Determinant Associated with the gp70-p15(E) Complex

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Hybridomas obtained from (NFS × AKR)F₁ mice immunized with syngeneic cells infected with AKR-247 MCF virus produced antibodies specific for only AKR-247 or closely related MCF viruses which share a previously defined MCF antigen (MCFA-3). These monoclonal antibodies recognized a new type of viral antigenic determinant which appeared to be a conformational determinant associated with the env precursor polyprotein (pr8000) or its disulfide-linked gp70-p15(E) complex (gp80) but not with free gp70 or p15(E) or any other virion or virus-induced protein.

Within the genome of the mouse are gene sequences coding for, or related to, a variety of RNA type C viruses (3, 14, 21). In the infectious form, these endogenous murine leukemia viruses (MuLV) are generally classified as ecotropic (viruses capable of infecting mouse cells but usually not cells from other animal species), xenotropic (viruses capable of infecting cells of heterologous species but not mouse cells), amphotropic (viruses from wild mice capable of infecting mouse cells and cells of a few other species), or MCF or dual-tropic viruses (recombinant viruses capable of infecting mouse cells and cells of several species). The MCF class of endogenous MuLV appears to arise during the lifetime of the mouse by recombination between endogenous ecotropic virus and MCF proviral gene sequences closely related to xenotropic viruses (13; S. K. Chattopadhyay et al., submitted for publication). Biochemical studies have revealed striking heterogeneity among MCF isolates but they all appear to have non-ecotropic sequences within part or all of the env gene region (2, 9, 20). Biologically they exhibit new properties in comparison to ecotropic or xenotropic viruses, including, for some MCFs, the capacity to induce lymphomas in certain strains of mice (7, 18).

Serological characterization of MCF viruses has been accomplished by using heterologous (rabbit) (6, 12) and homologous (mouse) (24) antisera. The studies with rabbit antisera showed that MCF viruses possess ecotropic-, xenotropic-, and several MCF-specific antigens, whereas murine antibodies to MCF virus appear to be more restricted. The Gₐ(AKSL₂) MCF-specific antigen defined by natural murine antiserum (24), in fact, appeared to be different from any of the specificities defined by rabbit antibodies.

A somewhat different and, in some cases, a more detailed analysis of antigenic specificity can be accomplished by characterizing monoclonal antibodies obtained from immunized animals. This approach was used in this study to analyze antibodies obtained from (NFS × AKR)F₁ mice immunized against AKR-247 MCF virus. These mice were previously shown to mount a strong humoral immune response to AKR-247 MCF virus, but this response appeared to be ineffective in preventing the induction of lymphoma (8). It was therefore of interest to further study these antibodies.

At 2 to 5 days of age, (NFS/N × AKR/J)F₁ mice, bred at the Rocky Mountain Laboratories, were injected in the region of the thymus with approximately 10³⁴ mink cell focus-forming units of thymotropic AKR-247 MCF virus. After 6 weeks, when MCF virus replication in the thymus had reached a high level, thymuses were removed, the cells were dissociated in medium without serum, and 5 × 10⁷ cells were injected intravenously into 6-week-old syngeneic F₁ recipients. After 2 weeks, spleens from recipients were removed and fused with HAT-sensitive NS1 myeloma cells by the technique of Kohler and Milstein (15) as described in detail elsewhere (4).

Hybridoma clones obtained were screened for the production of antiviral antibodies by reacting culture supernatants in indirect membrane immunofluorescence on cells infected with ecotropic, xenotropic, amphotropic, and MCF viruses as described previously (5, 6). Of the 36 clones producing detectable antiviral antibodies, only
antibodies reactive with MCF virus were observed. Six hybridoma clones, derived from separate fusion wells and which maintained continued growth and antibody secretion, were eventually selected for study. Clones Hy-7, Hy-34, and Hy-49 were obtained from one mouse and clones Hy-13, Hy-40, and Hy-58 from another mouse. Two clones (Hy-7, Hy-13) produced immunoglobulin (Ig)M antibodies, one secreted IgG2a antibodies (Hy-40), and three produced IgG3 (Hy-34, Hy-49, and Hy-58), as determined by gel diffusion with class-specific antiserum (obtained from Litton Bionetics, Kensington, Md.).

The viral specificity of these antibodies was demonstrated by indirect immunofluorescence on uninfected cells and cells infected with various MuLVs (Table 1). None of the antibodies reacted with uninfected mink or NIH/3T3 cells, but each reacted strongly with those cells infected with AKR-247 MCF virus. They were also highly reactive with cells infected with the closely related Akv-1-C36 MCF virus and to lesser degrees with cells infected with C58L1 and CB208 MCF viruses. Hy-34 and Hy-49 displayed very weak staining for cells infected with C58L1 and CB208 MCF viruses. None of the hybridoma antibodies reacted with cells infected with AKR-13, Akv-2-C34, Friend, or HIX (Mooney) MCF strains or with other classes of MuLV (ecotropic, xenotropic, or wild mouse amphotropic). Thus, these monoclonal antibodies recognized only MCF-specific determinant(s) which were induced by a subclass of MCF virus.

The antigens recognized by these monoclonal antibodies were identified in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis of immunoprecipitates. None of the antibodies, except Hy-40, were able to immunoprecipitate under standard conditions (Fig. 1). Hy-40 precipitated under nonreducing conditions an 80-kilodalton protein from Nonidet P-40 (NP-40) lysates of 125I-labeled AKR-247 MCF virus or labeled surface proteins of infected cells. This protein was also precipitated by antibodies to p15(E) and gp70 (Fig. 1, left panel). It has been shown that this 80-kilodalton protein is a complex of two envelope proteins, gp70 and p15(E), which exists either as an uncleaved precursor molecule, pr80o, or as a cleaved but disulfide-linked complex of gp70 and p15(E) (gp80) (10, 17, 19). Work by Pinter et al. has further indicated that gp70 and p15(E) do not naturally exist in the virion membranes as the disulfide-linked complex (gp80), but rather they spontaneously form this complex after the disruption of the virion by nonionic detergents (19). Although Hy-40 reacted with the 80-kilodalton protein, it did not precipitate free gp70, even though the presence of gp70 was demonstrated by precipitation with goat antiserum to gp70 or an anti-gp70 hybridoma. Free p15(E) was not demonstrated, since it is not labeled by iodination (16); the monoclonal antibody specific for p15(E) precipitated the pr80o or gp80 polypro-
reacting with p15(E) determinants, NP-40 lysates of metabolically labeled AKR-247 MCF-infected cells were used to demonstrate p15(E) precipitation (Fig. 2). A monoclonal antibody specific for p15(E) immunoprecipitated large amounts of free p15(E) from [3S]methionine-labeled AKR-247 MCF mink cells and the precursor polypeptide pr80env (track E). gp70-specific antiserum or monoclonal antibody precipitated free gp70, pr80env, and small

FIG. 1. SDS-polyacrylamide gel electrophoresis analysis of immunoprecipitates from NP-40 lysates of 125I-labeled AKR-247 MCF virus or mink cells infected with this virus. Details of the procedures involved have been reported elsewhere (4). Briefly, precleared lysates were incubated with the antibodies overnight at 4°C, followed by incubation with 50 μl of a 10% mixture of Formalin-fixed Staphylococcus aureus Cowan I strain for 30 min and centrifugation at 8,000 × g for 1 min. Precipitates bound to S. aureus Cowan I strain were washed with lysing buffer alone or lysing buffer plus 0.1% SDS and 0.5% deoxycholate and were eluted from S. aureus Cowan I strain by boiling in Tris buffer containing 2% SDS, 10% glycerol, and, where appropriate, 5% 2-mercaptoethanol (2-ME). They were electrophoresed in 10% polyacrylamide slab gels in the presence of SDS at 25 mA per gel for 3 h. The gels were stained with Coomassie brilliant blue, fixed, destained, and dried. Autoradiography was carried out with Kodak X-Omat R film at −70°C, employing intensifying screens. The following antibodies used to precipitate were: (A) anti-p15(E) hybridoma (372), 0.5 ml (4); (B) anti-247 hybridoma (Hy-40), 0.1 ml; (C) anti-247 hybridoma (Hy-40), 0.5 ml; (D) anti-gp70 hybridoma (18-1), 0.5 ml (manuscript in preparation); and (E) goat anti-R MuLV gp70, 5 μl (4).

FIG. 2. SDS-polyacrylamide gel electrophoresis analysis of immunoprecipitates from NP-40 lysates of [3S]methionine-labeled mink cells chronically infected with AKR-247 MCF virus. Antigen-antibody complexes were coprecipitated with sheep anti-mouse immunoglobulin and the complexes were washed with buffer without detergents. The precipitates were dissolved in eluting buffer containing 2-mercaptoethanol before application on the gel, and the gel was processed for autoradiography by the procedure of Bonner and Laskey (1). The following antibodies used to precipitate were: (A) goat anti-R-MuLV (Tween ether disrupted), 5 μl; (B) goat anti-R-MuLV gp70, 5 μl; (C) goat anti-R-MuLV p30, 5 μl; (D) anti-gp70 hybridoma (18-1), 0.5 ml; (E) anti-p15(E) hybridoma (372), 0.5 ml; (F) anti-247 hybridoma (Hy-58), 0.5 ml; (G) anti-247 hybridoma (Hy-49), 0.5 ml; (H) anti-247 hybridoma (Hy-34), 0.5 ml; (I) anti-247 hybridoma (Hy-40), 0.5 ml; (J) anti-247 hybridoma (Hy-13), 0.5 ml; (K) anti-247 hybridoma (Hy-7), 0.5 ml; and (L) no antibody (control).

tein owing to their p15(E) components. Treatment of the immunoprecipitates with 2-mercaptoethanol to reduce disulfide linkages (Fig. 1, right panel) converted the immunoprecipitated polypeptide to gp70, revealing that most of the 80-kilodalton polypeptide immunoprecipitated from extracts of both iodinated virus or cell surfaces was gp70-p15(E) complex (gp80) and not pr80env.

Since Hy-40 could have precipitated gp80 by
amounts of P15(E) (tracks B and D). Hy-40, however, only precipitated pr80env and not free p15(E) or gp70 (track I). Immunoprecipitation by the other anti-247 MCF monoclonal antibodies was also successful in this case when the antibodies were used to precipitate NP-40 lysates of [35S]methionine-labeled cells and coprecipitation was done with sheep anti-mouse immunoglobulin followed by washes in buffer without detergents (Fig. 2). Under these conditions, all of these monoclonal antibodies precipitated only pr80env. Reduction of immunoprecipitates with 2-mercaptoethanol did not appreciably convert the 80-kilodalton polypeptide to gp70 (data not shown), demonstrating that little [35S]methionine-labeled gp80, but large amounts of labeled precursor pr80env, was present in these lysates. Thus, from immunoprecipitation analysis, the monoclonal antibodies specific for AKR-247 MCF virus did not recognize either gp70 or p15(E) but did bind to pr80env and gp80.

There could be several reasons why an antigenic determinant may exist on a precursor molecule but not on its known cleaved and separated products. One possibility is that the determinants in question may be conformational, determined by secondary or tertiary structure unique to the precursor molecule or complexed gp70-p15(E). A similar phenomenon has been seen with antibodies to staphylococcal nuclease which recognized an antigenic determinant formed by the combination and folding of separate polypeptide fragments (23). Another possibility may be that an additional polypeptide covalently bound to the envelope precursor is being recognized. Such a polypeptide, designated as the R protein, has been identified in some MuLV systems, and antibodies to it immunoprecipitate both the envelope precursor and the free R protein (25). The MCF-specific monoclonal antibodies probably do not recognize such a protein, since (i) they recognize, in addition to pr80env, the cleaved but complexed gp80, and (ii) they do not immunoprecipitate a small R protein. The lack of R-protein precipitation was also confirmed by using [3H]leucine-labeled infected cells (data not shown). A third possibility concerning the nature of these determinants could be that they may be associated with carbohydrate moieties. Although we cannot totally exclude this possibility, Hy-40 was used to immunoprecipitate NP-40 lysates of MCF-infected cells treated with tunicamycin. This drug inhibits the formation of lipid-linked N-acetylglucosamine compounds, which serve as carbohydrate donors in the glycosylation process (26); this can result in total inhibition of glycosylation. Hy-40 was able to precipitate a deglycosylated precursor polypeptide of around 70 kilodaltons (data not shown), suggesting that carbohydrate was not needed to maintain this determinant.

The most likely explanation for the nature of this dominant MCF-specific antigenic determinant, therefore, is that it is conformationally determined. Further evidence for this was obtained when we found that the determinant could be destroyed when gp70 and p15(E) were dissociated in SDS. It has been shown that although gp70 and p15(E) spontaneously form disulfide-linked complex after dissociation in NP-40, this complex will not form if 0.1% SDS is used for lysis (19). These data indicated that gp70 and p15(E) were not covalently attached to each other in the virus but appeared to be just physically associated. Following this example, we prepared lysates of [35S]methionine-labeled MCF virus or infected cells by using 0.1% SDS to dissociate gp70 from p15(E). We then added 0.5% NP-40 and 0.5% deoxycholate to facilitate immunoprecipitation. Under these conditions, gp80 was not formed, and anti-gp70 antisera or hybridoma precipitated only gp70. Hy-40 precipitated nothing from these lysates (data not shown), further showing that it does not recognize free gp70 in solution. Although the antigenic determinants recognized by the monoclonal antibodies were expressed on cells, it was not known whether they were expressed on virions. Their presence in NP-40 lysates of virus may not represent the natural state of these determinants. Therefore, neutralization and direct virus-binding studies (polyvinyl tray assay) were performed (Table 2). Concordant results were obtained in these assays; only the two IgM monoclonals were reactive with virions, whereas the IgG monoclonals were not. Neutralization of virus by the IgG hybridomas was unsuccessful even with concentrated (∗5) supernatants and complement (data not shown). Virions, therefore, appeared to express some of these determinants, but not to the same extent as infected cells do. The fluorescence data showed that all of the antibodies bound to cell surfaces. It appears that the determinants recognized by the IgG monoclonals were either not exposed or greatly reduced quantitatively on virions in contrast to their expression on cells. Alternatively, the fact that the IgM antibodies were efficient binders to virions may be a function of the multivalent attachment possible with these antibodies. Nevertheless, such findings could have implications as to the in vivo biology of some MCF antibodies, and further studies on this are in progress.

Various degrees of exposure of the antigenic determinants could also explain the fluorescence intensity differences observed on cells infected with different MCF viruses. In particular, Hy-34 and Hy-49 hybridoma antibodies barely bound
to cells infected with C58L1 and CB208 MCF viruses, whereas the other monoclonals extensively bound to these cells. This may indicate that different determinants of the gp70-p15(E) complex were being recognized by different monoclonal antibodies.

Direct presentation of the gp70-p15(E) determinant(s) to spleen cells appeared to be required to subsequently obtain hybridomas. The hybridomas reported herein were only obtained after intravenous injection of MCF-infected cells, some of which end up in the spleen. Four attempts to make hybridomas from spleens of mice that were immunized with MCF virus alone were unsuccessful. It is likely that infected cells express large amounts of gp80 and would be very efficient for immunization. In addition, this virus is specifically thymotropic and does not infect spleen cells (7), so injecting this virus into mice may be expected to be a poor way to stimulate splenic B lymphocytes.

The monoclonal antibodies described in this report appear similar to the majority of mouse serum antibodies detected after immunization with AKR-247 MCF virus (manuscript in preparation). NFS antisera predominantly contain MCF-specific antibodies with very minor specificities for ecotropic virus. The MCF-specific determinant(s) recognized by the murine monoclonal antibodies is expressed by the same MCF viruses that express the previously described MCFA-3 antigen (6). This antigen was defined by rabbit antisera, and preliminary evidence indicates that rabbit MCFA-3 specific antibody, similarly to the mouse monoclonals, preferentially immunoprecipitates pr80"av and gp80, but not gp70 or p15(E) (unpublished data).

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