Jpn. J. Cancer Res. 83, 650–655, June 1992

Inhibition of B6RV2 Leukemia Growth by Immunization with Purified Unique Antigen

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Monoclonal antibody (mAb) NU7-99 reacted with only B6RV2 cells, not with 28 other leukemia cell lines, fibroblasts or normal tissues. Biochemical analyses of the unique antigen on B6RV2 cells that reacted with NU7-99 mAb indicated its relationship to xenotropic murine leukemia virus gp70. The antigen that reacted with NU7-99 mAb was extracted from the surface of B6RV2 cells with *n*-butanol and purified by ion-exchange chromatography and affinity chromatography. Growth of B6RV2 tumors in semi-syngeneic mice was inhibited by immunization of the mice with a purified preparation of this unique antigen.

Key words: Murine leukemia — Unique antigen — Murine leukemia virus — Tumor rejection antigen

Some radiation leukemia virus (RadLV[3])-induced leukemias are highly immunogenic, and immunization of semi-syngeneic mice with these tumors induces cross-protection against other RadLV tumors.[1)] This is in sharp contrast to the tumor rejection antigens on methylcholanthrene-induced fibrosarcomas in mice, which are individually distinct.[2–5)] *In vitro* analysis of cell-surface antigen on RadLV-induced leukemias, however, has revealed the presence of a unique antigen that has been defined serologically[6,7)] and by cell-mediated cytotoxicity.[1,6)] Thus, monoclonal antibodies (mAb) NU7-4 and NU7-99 react with only B6RV2 cells, not with 28 other leukemia cell lines, fibroblasts or normal tissues. Biochemical analyses of the unique antigen on B6RV2 cells that reacts with these mAbs indicated its relationship to xenotropic murine leukemia virus (MuLV) gp70.[7)] In this study, we extracted the antigen that reacts with NU7-99 mAb from the surface of B6RV2 cells with *n*-butanol and purified it by affinity chromatography. Then we investigated the effect of immunizing semi-syngeneic mice with this antigen. We found that growth of B6RV2 cells was inhibited in mice immunized with the purified antigen fractions.

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[3] The abbreviations used are: RadLV, radiation leukemia virus; mAb, monoclonal antibody; MuLV, murine leukemia virus; B6, C57BL/6; $CB6F_1$, $(BALB/c \times C57BL/6)F_1$; ELISA, enzyme-linked immunosorbent assay; RSC, relative salt concentration; p-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride; SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Mice C57BL/6 (B6) and $(BALB/c \times C57BL/6)F_1$ ($CB6F_1$) mice were obtained from our breeding colonies or from Japan SLC (Shizuoka). These mice were used at 8 to 12 weeks of age.

Tumor B6RV2 and B6RV4 are leukemias induced in neonatal B6 mice by injection of RadLV.[8)] EL4 is a chemically induced leukemia of C57BL origin.[6)] The tumors have been maintained by serial passage in the strain of origin.

Antibody mAb NU7-99 was produced by fusion of spleen cells from a $CB6F_1$ mouse hyperimmunized with B6RV2.[7)] The immunoglobulin class of the mAb is $\gamma 2a\kappa$, and its titer against B6RV2 is 1:800,000 as assayed by rosette formation using protein A-bound sheep erythrocytes. The antibody was biotinylated by incubating 1 mg of the purified antibody (1 mg/ml) with 100 μl of sulfo-NHS-biotin (1 mg/ml) (Pierce, Rockford, IL) for 4 h at room temperature. The mixture was then dialyzed against phosphate-buffered saline (PBS).

Enzyme-linked immunosorbent assay (ELISA) Samples were diluted with a solution of 0.1 *M* $NaHCO_3$, pH 8.3, and transferred to microwells (Immulon 96-well U plates, Dynatech Lab., Alexandria, VA). After blocking with 5% bovine serum albumin (BSA)-PBS, biotinylated NU7-99 mAb was added to the wells and the plates were incubated for one hour at room temperature. Peroxidase-avidin D conjugate (Vector Lab., Burlingame, CA) and *o*-phenylenediamine as a substrate were then added, and the optical density at 492 nm was measured.

Extraction of NU7-99 mAb-reactive antigen from the surface of B6RV2 cells A sample of 5×10^{10} B6RV2 cells was suspended in 2.5% *n*-butanol PBS (250 ml) contain-

ing 5 m*M* EDTA and 0.2 m*M* (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (p-APMSF) and incubated for 7.5 min at room temperature. The cells and debris were then removed by centrifugation at 2,000*g* for 10 min and at 20,000*g* for 20 min, respectively, and the supernatant was used for further purification of the antigen.

Purification of the antigen by chromatography The NU7-99 mAb-reactive antigen was purified by anion-exchange chromatography on Q-Sepharose (Pharmacia, Uppsala, Sweden) and NU7-99 mAb-fixed Sepharose 4B columns. The supernatant of the *n*-butanol extract was applied to a Q-Sepharose column (1.7×25 cm) after reduction of the *n*-butanol concentration to 1.0% and the relative salt concentration (RSC) to 0.02 *M*. The column was washed with 6 bed volumes of 8 m*M* phosphate buffer containing 0.1% Chaps, 5 m*M* EDTA, 0.2 m*M* p-APMSF and 5 m*M* 2-mercaptoethanol and then materials bound to the column were eluted with a linear gradient of 0.02 to 1.0 *M* NaCl at a flow rate of 50 ml/h. Fractions of 5 ml of eluate were collected and 75 μl of each fraction was tested for reactivity with NU7-99 mAb by Western blotting. Reactive fractions were pooled, concentrated with an Amicon ultrafilter YM30 (cut-off size, 30,000 mol.wt., Amicon Co., Danvers, MA) and applied to an NU7-99 mAb-fixed Sepharose 4B column. NU7-99 mAb-fixed Sepharose 4B was prepared by coupling 118 mg of purified NU7-99 mAb to 5 g of CNBr-activated Sepharose 4B (Pharmacia). After extensive washing of the column, bound materials were eluted with 50 m*M* diethylamine.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Aliquots of fractionated samples or the concentrated eluate (75 μl) were dissolved in 25 μl of sample buffer (8.0% SDS in 0.25 *M* Tris-HCl, pH 6.8), boiled for 5 min, and analyzed in 7.5% acrylamide gel by the method of Laemmli.[9] The gel was stained with silver using a Bio-Rad kit (Bio-Rad Lab, Richmond, CA).

Western blotting For Western blotting, the proteins were separated by SDS-PAGE and then blotted onto a nitrocellulose membrane. After blocking by immersion in 5% BSA-PBS, the membrane was washed with 0.05% Tween 20-PBS, overlaid with biotinylated NU7-99 mAb (10 μg/ml) and incubated for one hour at room temperature. The membrane was then washed extensively and treated with horseradish peroxidase-avidin D (Vector Lab.). Color was developed with 4-chloro-1-naphthol as the substrate.

Determination of protein concentration Protein concentrations of preparations were estimated with an ISS Protein Gold assay kit from ISS-Enprotech (Hyde Park, MA) with BSA as a standard.

Assay of tumor rejection activity $CB6F_1$ mice were immunized subcutaneously with the dialyzed preparation three times at 10-day intervals and challenged intradermally with tumor cells 10 days after the last immunization. Tumor widths at right angles were measured with calipers.

RESULTS

Extraction of NU7-99 mAb-reactive B6RV2 unique antigen *n*-Butanol was used to extract NU7-99 mAb-reactive antigen from the surface of B6RV2 cells. To minimize contamination of the protein with cytosolic components, we used extraction conditions that did not damage the cells appreciably. After incubation of B6RV2 cells with 1.0, 2.5, 3.5 and 4.0% *n*-butanol in PBS for 7 min at room temperature the cell viabilities were 94.5, 93.2, 75.0 and 6.3%, respectively. The cell viabilities and protein yields on incubation with 2.5% *n*-butanol for various times are shown in Fig. 1. In the first 7.5 min, cell viability remained constant, while the yield of protein increased. The amount of B6RV2 unique antigen detected by ELISA with biotinylated NU7-99 mAb reached a plateau after incubation for 10 min. Based on these results, for large-scale extraction we chose a concentration of 2.5% *n*-butanol and incubated the cells for 7.5 min at room temperature. Under these conditions, we obtained about 300 mg of protein from 5×10^{10} B6RV2 cells.

Purification of NU7-99 mAb-reactive B6RV2 unique antigen by chromatography The supernatant of the *n*-butanol extract was first applied to a Q-Sepharose

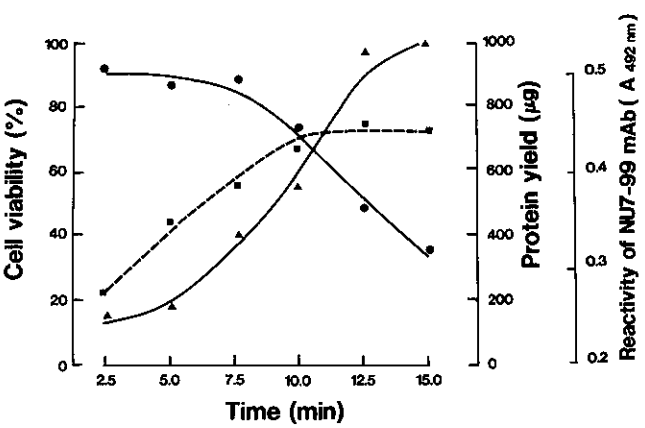

Fig. 1. *n*-Butanol extraction. B6RV2 cells (10^8 cells) were incubated for the indicated times in 2.5% *n*-butanol in PBS (1.0 ml). Cell viability (●) was estimated by trypan blue exclusion. The amount of protein extracted (▲) was determined with a protein assay kit (see "Materials and Methods"). Reactivity of NU7-99 mAb with the extract (■) was examined in 96-well plastic plates coated with 5 μg of protein/well by ELISA with biotinylated NU7-99 mAb.

column after reducing the butanol concentration to 1.0% and RSC to 0.02 *M*. The column was washed with 8 m*M* phosphate buffer containing 0.1% Chaps and then bound material was eluted with a linear gradient of 0.02–1.0 *M* NaCl. Fig. 2 shows the resulting chromatogram. The reactivity of each fraction with NU7-99 mAb was examined by Western blotting (Fig. 3). The reactivity with NU7-99 mAb was detected mainly in the fractions eluted with 0.04–0.13 *M* and 0.19–0.25 *M* RSC. These fractions were pooled, concentrated, and applied to an NU7-99 mAb-fixed Sepharose 4B affinity column. The column was washed extensively, and then bound materials were eluted with 50 m*M* diethylamine. This chromatographic procedure was repeated once more. Materials of 85 kd and 83 kd were identified by silver staining (Fig. 4A). On Western blotting on a nitrocellulose membrane, biotinylated NU7-99 mAb reacted with both molecules (Fig. 4B). The total protein content of the eluate was 1.3–2.0 μg (n=5).

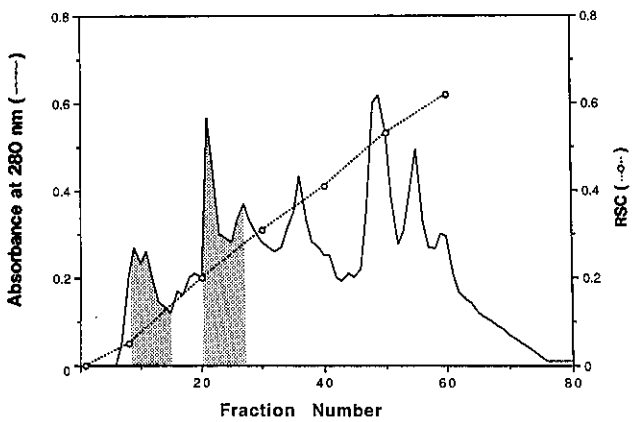

Fig. 2. Anion-exchange chromatography of the *n*-butanol extract. The *n*-butanol extract from (5×10^{10}) B6RV2 cells was applied to a Q-Sepharose column and the bound materials were eluted with a linear gradient of 0.02–1.0 *M* NaCl (···○···). Shaded fractions were pooled.

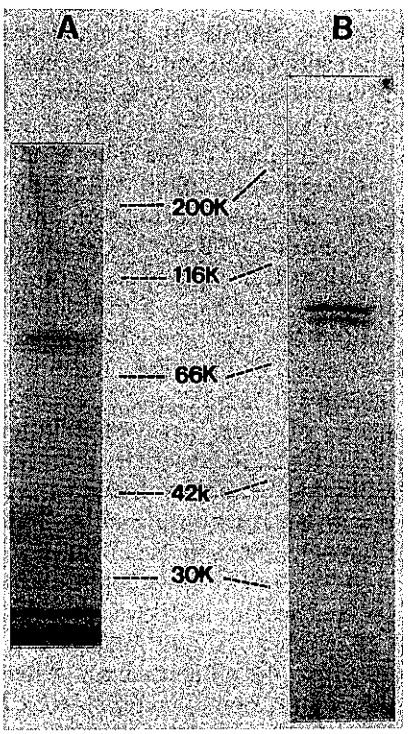

Fig. 4. SDS-PAGE and Western blotting. The active fraction (75 μl) from the NU7-99 mAb-Sepharose 4B column was separated by SDS-PAGE in 7.5% acrylamide gel under non-reducing conditions and stained with silver (A). In a separate experiment, the separated proteins were transferred to a nitrocellulose membrane and probed with biotinylated NU7-99 mAb (B).

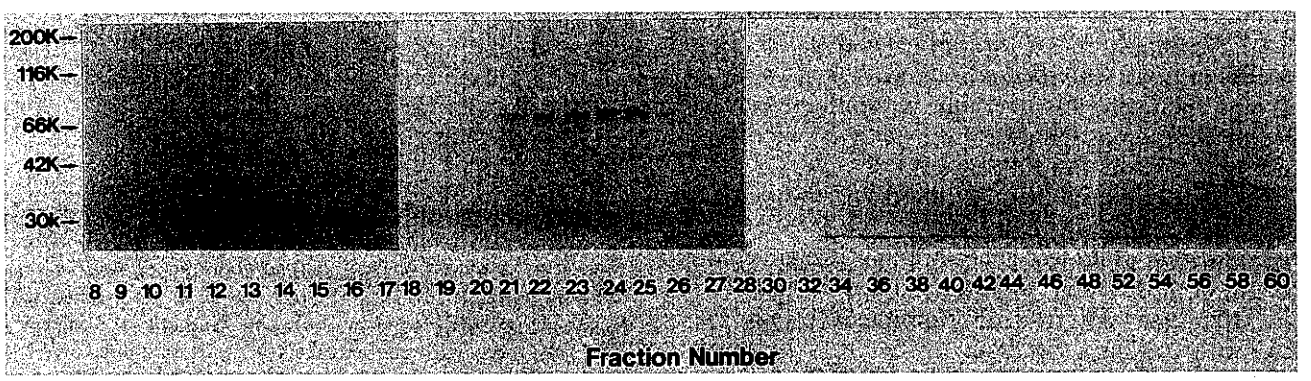

Fig. 3. Western blotting of the fractions shown in Fig. 2 with biotinylated NU7-99 mAb.

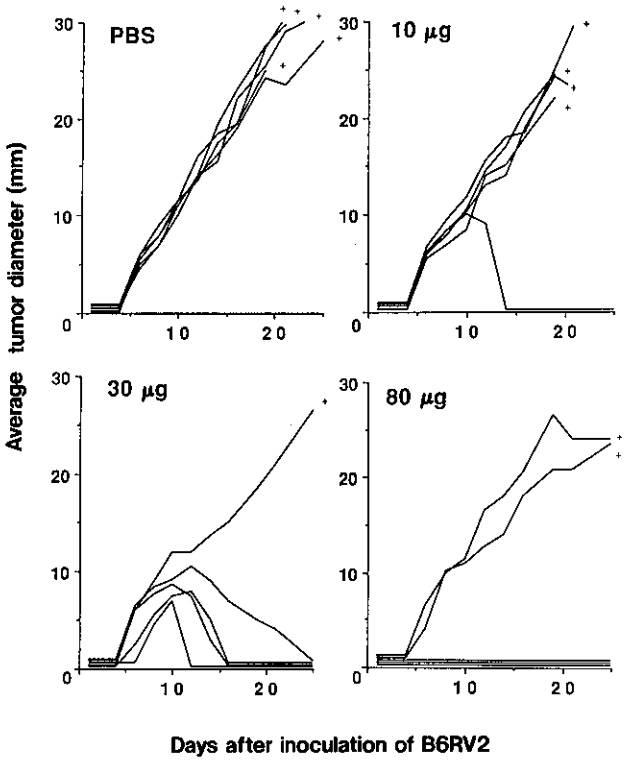

Fig. 5. Effect of immunization with the crude butanol extract of B6RV2 cells on B6RV2 growth. Male $CB6F_1$ mice were immunized 3 times at 10-day intervals with the indicated amounts of extract and challenged with 1×10^6 B6RV2 cells 10 days after the last immunization. Each group consisted of 5 mice.

Effects of immunization with the crude butanol extract and the purified NU7-99 mAb-reactive antigen on tumor growth Male $CB6F_1$ mice were immunized subcutaneously with the crude B6RV2 extract and affinity-purified NU7-99 mAb-reactive antigen three times at 10-day intervals and then were challenged with 1×10^6 B6RV2 cells. As shown in Fig. 5, immunization with the crude butanol extract at protein concentrations of 30 and 80 μg, but not 10 μg significantly inhibited B6RV2 tumor growth. Immunization with the affinity-purified antigen caused significant inhibition of B6RV2 growth at 20 ng, less significant inhibition at 5 ng, and enhancement rather than inhibition of tumor growth at 80 ng (Fig. 6A). Immunization with 20 ng of affinity-purified NU7-99 mAb-reactive antigen had no effect on growth of either B6RV4 or EL4 (Fig. 6B).

DISCUSSION

B6RV2 was immunogenic to syngeneic mice and its immunogenicity was greater and demonstrated consistently in semi-syngeneic $CB6F_1$ mice.[6,7] In this study, we showed that immunization of male $CB6F_1$ mice with serologically defined B6RV2 unique antigen induced an anti-tumor response *in vivo* against challenge with B6RV2 leukemia. We extracted the antigen from the surface of B6RV2 cells with *n*-butanol and purified it by anion exchange and NU7-99 mAb-fixed Sepharose 4B affinity chromatographies. Tumor growth was inhibited significantly by 30 or 80 μg, but not by 10 μg of the crude butanol extract. The increased inhibition of tumor growth by the affinity-purified fraction suggests that the antigenic molecule detected by NU7-99 mAb has a determinant that induces an *in vivo* anti-tumor response in recipient mice. The yield of the unique antigen on NU7-99 mAb-affinity chromatography was 0.02–0.03% of the total protein applied. This low yield may be due to low affinity of NU7-99 mAb for its ligand.

Unique antigen was originally found as a tumor-specific transplantation antigen on murine methylcholanthrene-induced fibrosarcoma[2-5] and thereafter on many types of tumors of rodents. But *in vitro* detection of unique antigen has long been unsuccessful because of its low antigenicity and/or the low sensitivity of assays for its detection, and so its molecular nature has not been determined. DeLeo *et al.*, however, raised a specific antibody against it by prolonged immunization of syngeneic BALB/c mice with Meth A sarcoma[10] and suggested its relationship with transplantation antigen. Attempts to purify tumor rejection antigen have also been made by monitoring its activity in tumor transplantation assay. In this way, Srivastava *et al.*,[11,12] Dubois *et al.*,[13,14] and LeGrue *et al.*[15,16] obtained highly purified, active tumor rejection antigens from Meth A sarcoma, although they did not clearly define the relationships of these antigens to serologically defined Meth A antigen.

In contrast to the antigens of chemically induced fibrosarcomas, only shared tumor rejection antigens have been found on leukemias in mice. Morishita *et al.*[1] investigated the specificities of rejections of BALBRVB or BALBRVD in $CB6F_1$ mice, and observed cross-protection against challenge with 4 other RadLV-induced leukemias and a radiation-induced leukemia of BALB/c origin. These results were consistent with previous reports that immunization against MuLV gp70 led to cross-protection against other syngeneic tumors.[17] *In vitro* studies, however, demonstrated unique antigens on murine leukemias. These unique antigens on B6RV2,[6] BALBRVB and BALBRVD,[1] and BALBRL♂1,[18,19] have been defined by *in vitro* cell-mediated cytotoxicity, and that on B6RV2 has been defined serologically.[6,7] Biochemical analyses revealed that the serologically defined unique antigen on B6RV2 cells was related to the xenotropic MuLV gp70 molecule. Thus, the diversity of individually distinct antigens on murine leukemias

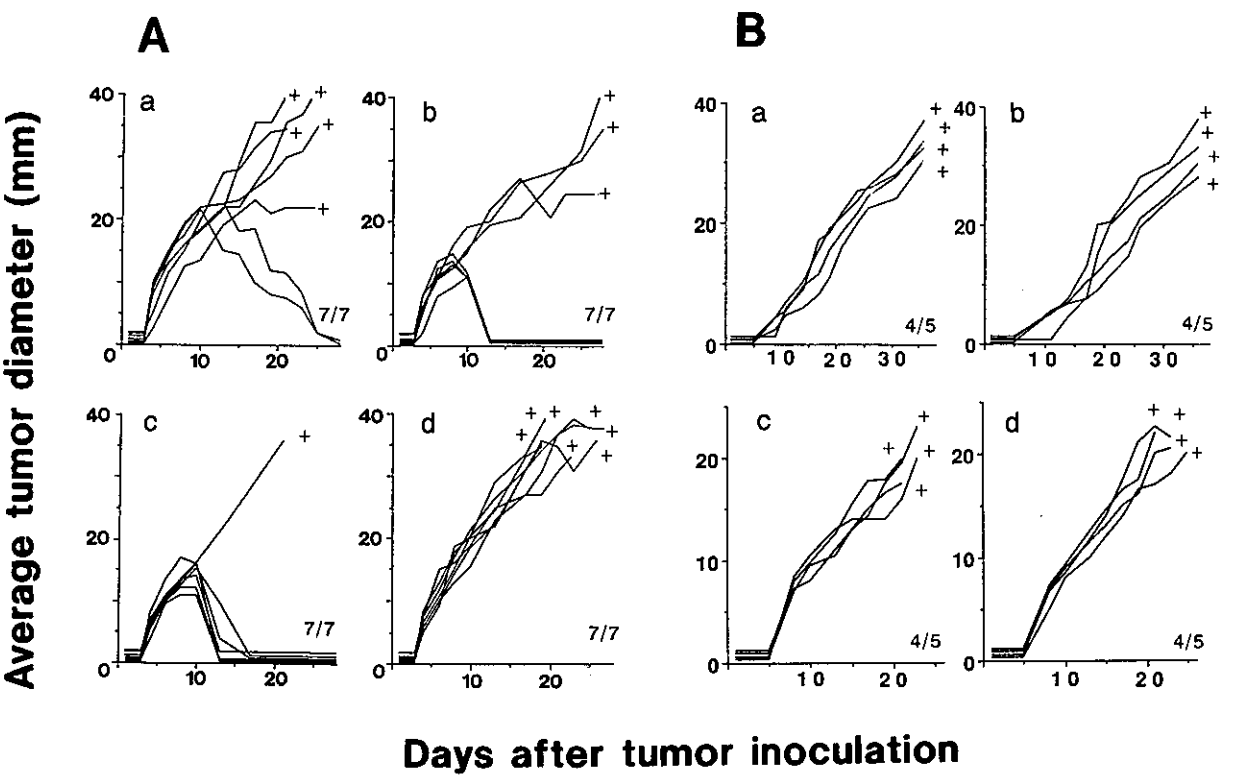

Fig. 6. Effect of immunization with affinity-purified B6RV2 unique antigen on tumor growth. In A, male $CB6F_1$ mice were immunized with 5 (b), 20 (c) or 80 (d) ng of eluate from an NU7-99 mAb-Sepharose 4B column or PBS (a) (control) 3 times at 10-day intervals and challenged with 1×10^6 B6RV2 cells 10 days after the last immunization. In B, male $CB6F_1$ mice were immunized with 20 ng of eluate from an NU7-99 mAb-Sepharose 4B column (b and d) or PBS (a and c) (control) 3 times at 10-day intervals and challenged with 5×10^5 B6RV4 cells (a and b) or 5×10^5 EL4 cells (c and d) 10 days after the last immunization. The ratio of the number of mice in which a tumor was recognized macroscopically to the number of mice inoculated with tumor cells is shown.

appears to be due to recombination of the MuLV *env* gene. In the present study, we found that a serologically defined B6RV2 unique antigen inhibited leukemia growth.

Tumor-enhancing activity was observed with 80 ng of affinity-purified antigen. Similar enhancing activity with a fractionated cytosol preparation of Meth A sarcoma has been reported.[11] The reason for this tumor-enhancing activity is unknown, but our findings suggest that it is due to a dose effect, rather than a tumor-enhancing antigen in the preparation.

ACKNOWLEDGMENTS

We thank Dr. T. Fujita for assistance in preparation of the affinity column.

(Received January 6, 1992/Accepted March 25, 1992)

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