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Localization of Murine Mammary Tumor Virus Polypeptides on the Surface of Tumor Cells*

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Abstract—*Antisera raised in rabbits against purified components gp52, p28 and p12 of murine mammary tumor virus (MuMTV) were first characterized by means of immunodiffusion, immunoelectrophoresis and radioimmunoassay. No significant cross-reactivity could be detected.*

The three polypeptides were detected by means of these antisera in the cytoplasm of murine mammary tumor cell lines Mm5mt/c1 and C3HMT/cl11, as well as in the murine leukemia cell lines GRSL18 and L1210 by means of fixed cell immunofluorescence. The concentration of gp52 in the cytoplasm seems to be considerable lower than that of the two other polypeptides.

By use of the membrane immunofluorescence assay, only gp52 proved to be localized on the cell surface. Humoral cytotoxicity was only accomplished with anti-gp52 serum. No reaction was found in either fixed-cell or membrane immunofluorescence assay or the cytotoxicity test with the control cell lines EMT-6 and BALB/3T3 infected with Rauscher murine leukemia virus.

INTRODUCTION

OUR LABORATORY is engaged in the development of the murine mammary tumor virus (MuMTV) protein vaccines for prophylaxis of primary mammary tumor development [1]. For such vaccination studies it is important to know which viral polypeptides are expressed on the tumor cell surface. We have so far succeeded in the isolation of three of the major viral polypeptides: a glycoprotein with a mol. wt (MW) of 52,000 (gp52) and two polypeptides with MW of 28,000 (p28) and 12,000 (p12), respectively [2]. Antisera have been raised in rabbits to these purified proteins and were used for the detection of viral constituents on the cell membrane.

MATERIALS AND METHODS

Cells

The following cell lines were used: Mm5mt/c1 [3] and C3HMT/cl11 [4], which are derived from C3H mouse mammary tumors and which produce large quantities of MuMTV-S, EMT-6 [5], a BALB/c mouse mammary tumor line which produces only type C oncoviruses, BALB/3T3 cells chronically infected with Rauscher murine leukemia virus [6], and the murine leukemia lines L1210 [7] and GRSL18 [8] which arose in respectively the DBA/2 and GR strain, and produce only small quantities of MuMTV virions.

The first four cell lines were cultured in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum (FCS). The GRSL18 was grown in RPMI 1640 medium +10% FCS and the L1210 in Fischer's medium +3% FCS +7% horse serum $+6.10^{-5}$ M 2-mercaptoethanol. Penicillin (100i.u./ml) and streptomycin (100 μg/ml) were added to all cultures. Two days after plating 10^{-5} M dexamethasone and insulin (10 μg/ml) were added. All lines were grown in 75 cm^2 plastic flasks (Costar, Cambridge, MA).

Virus

Purified tissue culture derived MuMTV was obtained through the Office of Resources and Logistics, Virus Cancer Program,

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National Cancer Institute, Bethesda, MD, USA. The virus originated from the C3H mammary tumor cell line Mm5mt/c1 [5].

Purification of viral proteins

The major viral proteins gp52, p28 and p12 were purified from murine mammary tumor virus as described previously [2], with some minor modifications. Briefly, pelleted virus was disrupted in phosphate buffered saline (PBS), pH 7.2, containing 1% Nonidet, 1% Sodium deoxycholate (DOC), 0.02% sodium azide. After ultrasonication for 4 × 15 sec in MSE sonicator, medium power, wave length 8 μM in ice and incubation for 10 min at 37°C followed by 90 min at 4°C, the unsolubilized fragments were pelleted by centrifugation for 60 min at 164,000 *g* (Beckman, Sw60Ti, 40,000 rev/min). The supernatant was dialyzed overnight against PBS, containing 0.02% DOC, 0.02% Nonidet, 0.02% sodium azide (buffer A) and applied to Concanavalin A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). After elution of non-glycosylated material with buffer A, the column (0.9 × 15 cm) was washed with buffer A without Nonidet (buffer B). Gp52 was eluted from the column with 0.2 M methyl α-D-mannopyranoside (Calbiochem, San Diego, California) in buffer B. Further purification was performed by gel filtration on Sephadex-G150.

The polypeptides p28 and p12 were isolated from the nonglycosylated fraction by chromatography on phosphocellulose as described previously [2]. All steps were carried out at 4°C.

Preparation of antisera

Rabbits were immunized with 100–150 μg purified protein in 500 μl PBS emulsified with an equal volume of Freund complete adjuvant. Half of the suspension was injected intradermally and the other half intramuscularly at multiple sites. Booster injections with the same amount of protein in Freund incomplete adjuvant were given at 2 and 4 weeks. Rabbits were bled 2 weeks after the last injection. Bleeding was continued every 2 weeks until reactivity against the antigen strongly declined. An anti MuMTV-S serum was raised in a rabbit by inoculation with the dialyzed supernatant of the disrupted virions preparation. An antiserum was raised in a goat to a mixture of MuMTV-S, −O and −P [9] in a similar way.

Antisera were routinely absorbed for activity against normal mouse serum and fetal calf serum by means of affinity chromatography, using CNBr-activated Sepharose 4-B beads to which these antigens were covalently coupled [10] using 2 mg protein per ml of activated beads. Residual active groups were deactivated by treatment of the beads for 6 hr with 0.5 M ethanolamine, pH 9.5.

Immunodiffusion test

Double immunodiffusion was carried out on microscope slides, employing 0.6% agarose plus 0.01% sodium azide in PBS. Wells were punched in solidified agarose with the microdiffusion set of LKB. The wells contained 15 μl of reagent. Slides were incubated for 24 hr in a humidified box at room temperature. Staining was made in Coomassie brilliant blue G250.

Immunoelectrophoresis

Reagents were electrophoresed in 1.5% agarose in Veronal buffer, pH 8.6 (I=0.12), containing 1% Nonidet, 1% DOC, 0.02% sodium azide. Normally, 1–5 μg of purified protein was electrophoresed at 2.5 mA/cm for 2–3 hr. Immunodiffusion against the absorbed antiserum took place for 24 hr, after which staining was performed with Coomassie brilliant blue G250.

Labeling of viral proteins

Polypeptides were iodinated, using the chloramine-T method [11], with some modifications. To 25 μl of 0.5 M phosphate buffer, pH 7.5, we added 15 μl polypeptide solution containing 5–10 μg protein (either purified protein, or complete MuMTV, disrupted in PBS, containing 1% Nonidet), 10 μl ^{125}Iodine (1 mCi/0.010 ml, Amersham) and 10 μl chloramine-T solution (1 mg/ml chloramine-T in 0.1 M phosphate buffer, pH 7.5). The reaction was stopped after an incubation period of 60 sec by adding sodium metabisulphite (5 μl of a 20 mg/ml solution in 0.1 M phosphate buffer, pH 7.5) and 50 μl 0.1 M potassium-iodine. The ^{125}I-labeled protein was separated from free iodine by Sephadex G-50 chromatography.

The labeled material was analysed by SDS-polyacrylamide gel electrophoresis as described below. For the radioimmunoassay procedure, the labeled polypeptide fraction was diluted with 20 mg/ml crystalline BSA in TEN buffer (20 mM Tris–HCl, pH 7.6, 1 mM EDTA, 100 mM NaCl) to yield a 400 ng protein/ml solution.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecylsulphate polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn [12]. Gels contained 10% acrylamide. Samples were heated for 2 min at 100°C prior to electrophoresis, which was carried out at 2.5 mA/gel for approximately 20 hr. Gels were sliced into 1.1 mm fractions using a Bio-rad gel slicer. ^{125}I-radioactivity was determined by using a gamma counter.

Molecular weights were estimated on the basis of the relative electrophoretic mobility of the labeled polypeptides. Standards were bovine serum albumin (69,000) ovalbumin (43,000), carbonic anhydrase (29,000) and lysozyme (14,500).

Immunoprecipitation

Iodinated MuMTV-proteins were brought in 200 µl immunoprecipitation buffer (10 mM Tris–HCl, pH 7.5, containing either 1% Nonidet, 0.5% DOC and 0.1% SDS or 0.02% Nonidet and 1 M NaCl). To the solution a pretitrated amount of anti-serum was added (usually 2 µl serum) after which an incubation period of 2 hr at 37°C was maintained. Thereafter, 50 µl of pig antirabbit IgG serum was added. The reaction mixture was incubated at 4°C for 16 hr.

The precipitate was collected by centrifugation for 30 min at 3000 ***g***, after which the pellet was washed twice with 0.5 ml immunoprecipitation buffer. The precipitates were analysed by SDS-PAGE.

Radioimmunoassay

A double antibody competition immunoassay was performed as described by Strand and August [13] with some modifications. The iodinated polypetide fraction was diluted 1:4 with normal rabbit serum (diluted 1:9 in 2 mg/ml BSA in TEN buffer). To 40 µl of this ^{125}I-labelled polypeptide solution, 50 µl of TEN buffer and 10 µl of antiserum in serial twofold dilutions were added.

The reaction mixture was incubated at 37°C for 4 hr; 30 µl of pig antirabbit IgG serum (Nordic Immunological Laboratories, Tilburg, The Netherlands) was then added. Incubation was afterwards prolonged for 1 hr at 37°C and overnight at 4°C. Cold TEN buffer (0.5 ml) was added and the precipitate was collected by centrifugation for 25 min at 3000 ***g*** at 4°C. The pellet was washed twice and counted in a gamma counter.

Antibody titers were defined as that antiserum dilution at which approximately 50% of the labeled antigen was precipitated.

For the competition assay, a binding site occupation assay, in which the test antigen was first incubated with the antibody before addition of the labeled antigen, was used. Dilutions of test antigen were made in TEN buffer containing 0.2% Triton X-100 and 2 mg BSA per ml. The antiserum dilution at which approximately 50% of the labeled antigen was precipitated, was used in this test.

To 40 µl of TEN buffer, 10 µl of test antigen and 10 µl of the antiserum dilution were added. After an incubation period of 2 hr at 37°C, labeled antigen was added; this was followed by an additional incubation period of 2 hr at 37°C. After addition of pig anti-rabbit IgG serum, the procedure was continued as described above.

Sepharose bead immunofluorescence assay

Sepharose 4B beads were coupled covalently with either disrupted MuMTV, FCS, normal mouse serum (NMS) or ovalbumin (OVA). The beads were stored in PBS with 0.01% merthiolate. For antiserum testing, 50 µl of a bead suspension were incubated for 1 hr at room temperature with 50 µl of various dilutions of an antiserum in flat bottom microtiter plates with continuous agitation. After repeated washing, the beads were incubated with a goat antiserum to rabbit IgG conjugated with fluoresceine isothiocyanate (FITC) (Nordic). After washing, the individual bead fluorescence was measured with a microfluorometer [14]. At least five beads were measured per dilution.

Cytoplasmic immunofluorescence

Mammary tumor cell suspensions were washed after trypsinization and then deposited in wells of microscope slides covered with Teflon. The slides were kept for 20 hr in a humid incubator (5% CO_2) at 37°C. Thereafter, they were incubated 3 × 10 min with PBS at 4°C, then 3 × 5 min in acetone at −28°C and subsequently for 3 × 10 min in PBS. The slides were stored at −28°C. Cells of the leukemia lines GRSL18 and L1210, which grow in suspension, were deposited on microscope slides by means of a cytocentrifuge [15] and fixed immediately in cold acetone and further processed in the same manner as were the other cell lines. For immunofluorescence, the wells were incubated with twofold dilutions of the antisera, beginning at a 20 times dilution. the slides were kept for 45 min at 37°C in a

humid incubator (5% CO_2) and then washed 2 times for 10 min with PBS. Subsequently, the wells were incubated for 45 min with a goat antiserum to rabbit immunoglobulins conjugated with fluoresceine isothyocyanate (FITC) (Nordic) diluted 40 times. After washing, the wells were embedded in Elvanol. Cells incubated with normal rabbit serum absorbed with NMS and FCS, wells incubated with the conjugate only and wells incubated with only PBS, were used as controls.

The slides were examined with a Leitz Orthoplan microscope, using a FL 40x/1.30 objective and GW 4× oculars. Epiillumination was accomplished with a mercury arc CS 100 W/2. The filter combination for excitation was 2×KP 490+1 mm GG 455; the K515 filter was used for emission.

Membrane immunofluorescence

After trypsinization, the target cells were grown in plastic microtiter plates. After one-night incubation at 37°C in a humidified atmosphere, the plates were used for immunofluorescence studies. Wells were incubated with test serum for 45 min, after which the wells were washed three times with medium (RPMI 1640, 100 i.u./ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol). The wells were then incubated for 30 min. with a goat antiserum to rabbit IgG conjugated with FITC, diluted 20× in PBS. After three washings in medium, the cells were examined with the Leitz Orthoplan microscope. The sera were tested in two log dilutions. BALB/3T3 cells infected with RLV were used as control cells. Goat anti-MuMTV serum and normal rabbit serum absorbed with NMS and FCS served as positive and negative controls, respectively. All experiments were carried out in triplicate. The cells were examined independently by two investigators.

Cytotoxcity test

Antisera were first heat-inactivated (at 56°C for 30 min) and then absorbed with packed BALB/3T3 cells infected with RLV for 1 hr at 37°C. Cells were plated in plastic microtiter plates: 10^4 per well. On the next day, the cells were incubated with ^{51}Cr (2 μCi per well) for 2 hr. Thereafter , the cells were washed three times and then taken up in 20 μl ice-cold RPMI1640 medium containing 10% FCS and twofold dilutions of the antiserum. Rabbit complement, absorbed with agarose for removing natural anti-mouse reactivity, was added (dilution 24 ×) and the cells were incubated for 1 hr at 37°C. After this, 200 μl ice-cold medium was added. Radioactivity was measured in a gamma counter. Maximum ^{51}Cr release was estimated after adding saponin to labeled cells. All experiments were carried out in triplicate.

Possible anticomplementarity of antisera was determined by incubating labeled cells with heat-inactivated antiserum, washing twice with 200 μl medium per sample and then adding 20 μl rabbit complement per 30 μl sample.

Indirect cytotoxicity testing was performed by incubating labeled cells with antiserum and complement for 1 hr at 37°C. A goat antiserum to rabbit Ig (Nordic) diluted 80 × was then added and cells were incubated again for 1 hr at 37°C.

RESULTS

The gp52 preparation obtained with a slightly modified isolation procedure is, in contrast to our earlier report [2], not contaminated with gp69, as demonstrated by PAGE analysis (Fig. 1A). The two other polypeptide preparations also show a degree of purity of near 100% (Fig. 1B, C).

All antisera against the isolated proteins gave a single precipitation line with detergent disrupted virus, in double immunodiffusion, while an antiserum raised against disrupted virus particles produced three lines (Fig. 2). The line produced by anti-p12 is weakly discernible. The lines produced by either antiserum to a single protein do not fuse, indicating antigenic unrelatedness. Since these lines fuse with lines produced by the rabbit antiMuMTV-S serum, it can be concluded that this antiserum recognizes at least gp52, p28 and p12.

In immunoelectrophoresis, the antiserum to MuMTV-S gives rise to at least five precipitation lines with disrupted virus. The antisera to gp52 and p12 produce single lines at different sites, whereas anti-p28 gives two lines, which fuse (Fig. 3). The addition of the detergents Nonidet and DOC to the system is required for the production of clearly separated precipitation lines.

The MuMTV polypeptides gp52, p28 and p12 were iodinated at specific activities ranging from $8–15 \times 10^4$ counts/min/ng viral protein. More than 90% was TCA precipitable. The iodinated gp52 showed a molecular weight of only 49,000 in SDS-PAGE analysis, while the molecular weight of iodinated p28

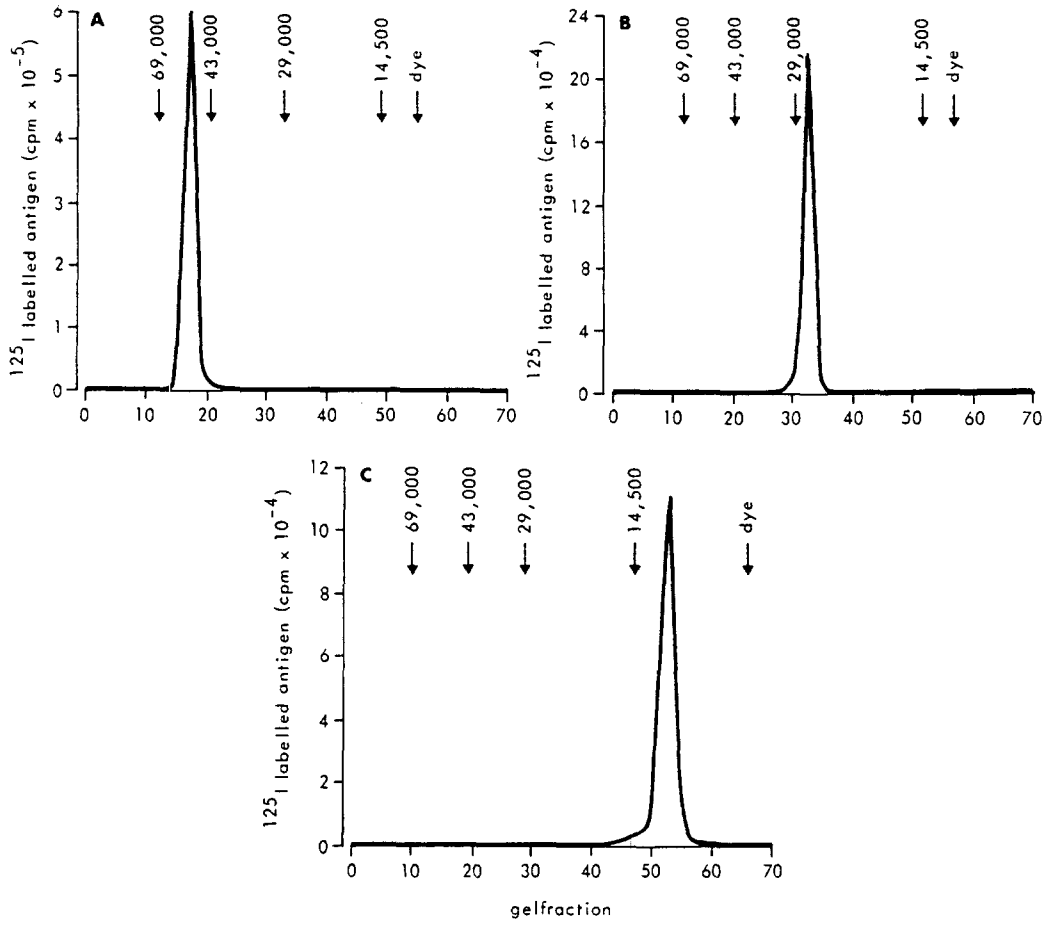

Fig. 1. SDS-polyacrylamide gel electrophoresis of iodinated purified MuMTV-polypeptides in 10% acrylamide gels (A) gp52; (B) p28 and (C) p12.

and p12 still is estimated as 28,000 and 12,000, respectively.

In the immunoprecipitation test, using iodinated detergent-disrupted virus particles, two different immunoprecipitation-buffers were used (see Materials and Methods). With the antisera to gp52 and p28 satisfactory results were obtained using Tris buffer, containing 1% Nonidet, 0.5% DOC and 0.1% SDS as immunoprecipitation buffer. The antiserum to gp52 precipitated only gp52, whereas the antiserum to p28 precipitated besides p28 also small amounts of a protein with a mol. wt. of 23,000 (Fig. 4). The antiserum to p12 precipitated, besides p12, small amounts of some other MuMTV-proteins. These results (which are not shown) were probably due to aggregation. Considerably better results were obtained by using Tris buffer, containing 0.02% Nonidet and 1 M NaCl as immunoprecipitation buffer (Fig. 4). Apparently under circumstances of high ionic strength aggregation of iodinated p12 with the other proteins is prevented.

In a double antibody radioimmunoassay, the titers of the antisera proved to be 200 (anti-p12), 250 (anti-p28) and 500 (anti-gp52), respectively. As shown in Fig. 5, gp52, p28 and p12 competed to approximately 95% in the respective homologous assays. Addition of 1 ng of gp52, 1 ng of p12 and 1.4 ng of p28 caused 50% blocking. Competition in each of the assays with the other two viral components showed that only a 100-fold excess or more resulted in some displacement of the labelled input counts. Similar aspecific inhibition at excess of unrelated protein has been found in radioimmunoassays of structural proteins of mammalian type-C oncoviruses [16]. It can be concluded, that the three MuMTV components are immunologically unrelated.

Immunofluorescence

By means of the Sepharose bead immunofluorescence assay, it was found that all three antisera showed strong reactivity with fetal calf serum and normal mouse serum (Fig. 6). Absorption removed these unwanted reactivities to a large extent. Unabsorbed antisera reacted strongly with all cell lines in the fixed cell immunofluorescence assay, irrespective of

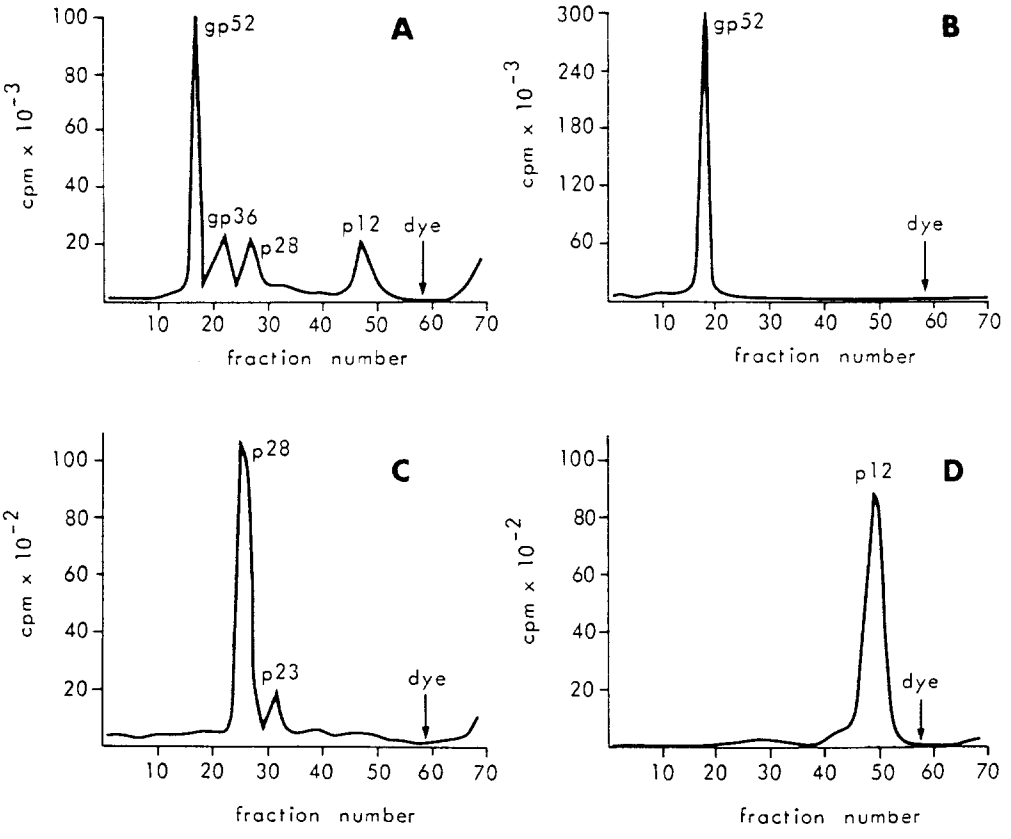

Fig. 4. SDS-polyacrylamide gel electrophoresis of detergent disrupted iodinated MuMTV (A) and of the immunoprecipitates from MuMTV obtained with anti-gp52 (B), anti-p28 (C) and anti-p12 (D).

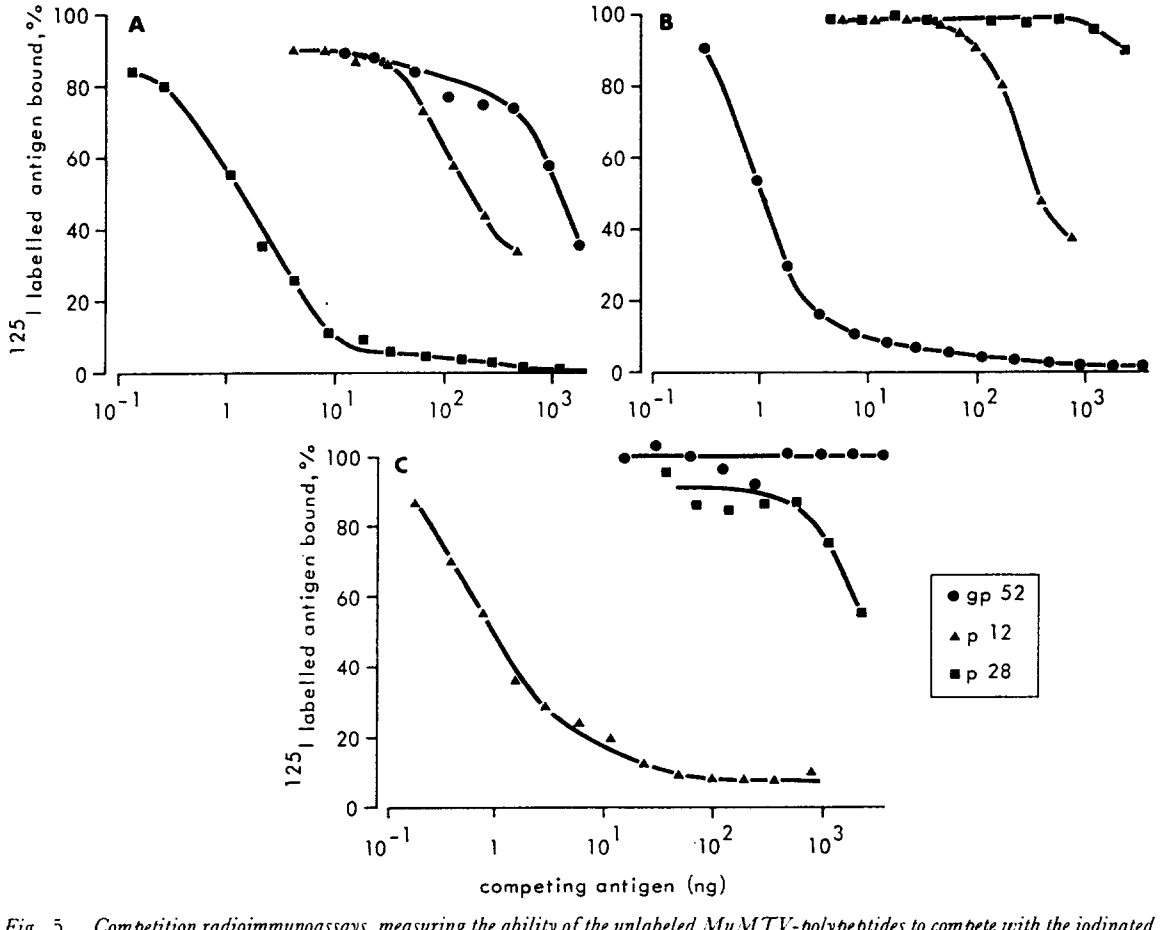

Fig. 5. Competition radioimmunoassays, measuring the ability of the unlabeled MuMTV-polypeptides to compete with the iodinated MuMTV-polypeptides in their respective homologous assays: (A) ^{125}I-p28 vs anti-p28; (B) ^{125}I-g52 vs anti-gp52; (C) ^{125}I-p12 vs anti-p12. The unlabeled antigens were tested at serial twofold dilutions.

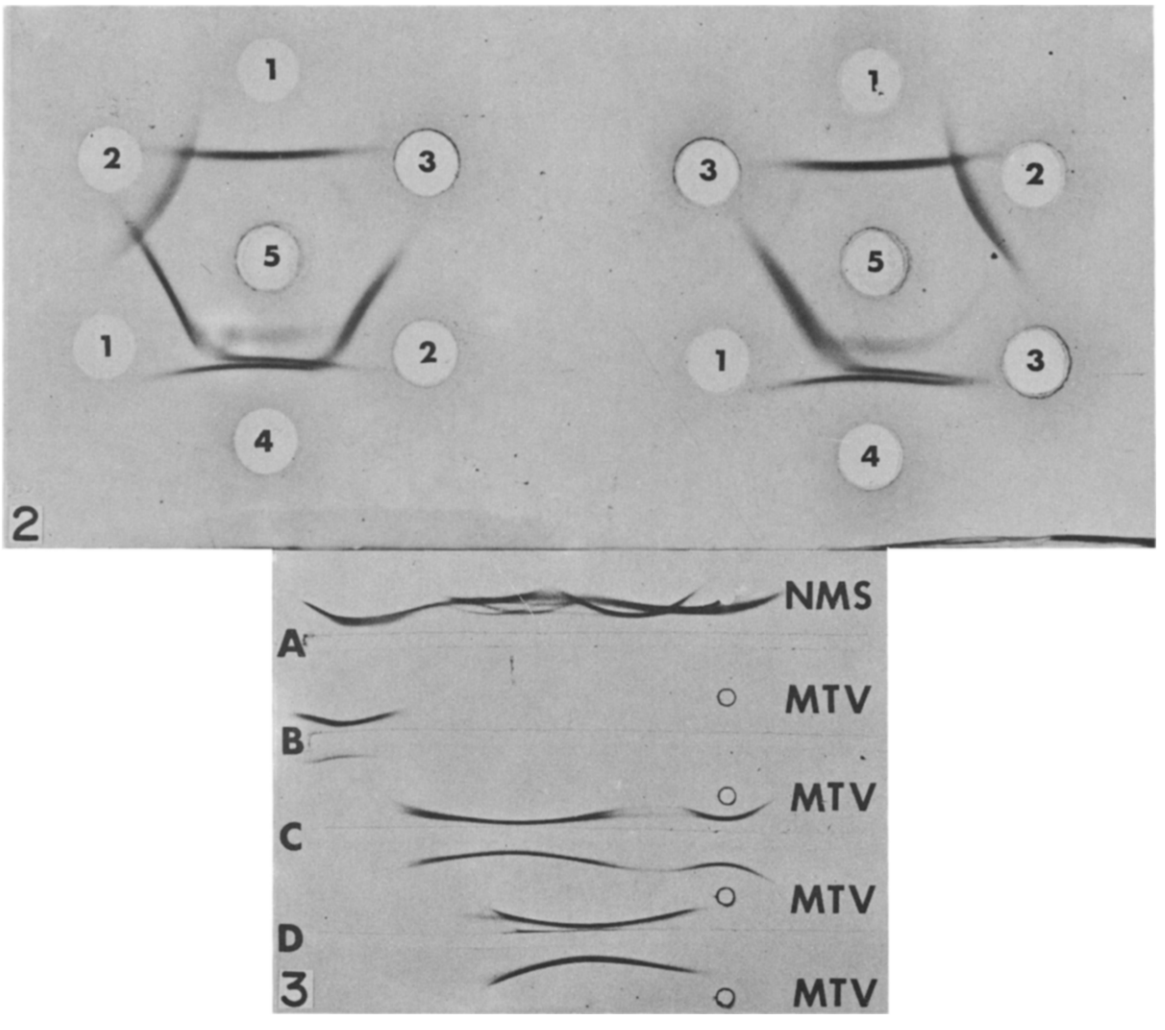

Fig. 2. Double immunodiffusion of antisera to the purified MuMTV polypeptides, against nonidet-distupted MuMTV virions. (1) *rabbit anti-gp52;* (2) *rabbit anti-p28;* (3) *rabbit anti-p12;* (4) *rabbit anti-MuMTV;* (5) *nonidet-disrupted MuMTV.*

Fig. 3. Immunoelectrophoretic characterization of the antisera to the MuMTV-polypeptides. Trench (*A*) *rabbit anti-normal-mouse serum* (*Nordic*); (*B*) *rabbit anti-p12;* (*C*) *rabbit anti-p28;* (*D*) *rabbit anti-gp52. The upper well contained normal mouse serum, the others nonidet-disrupted MuMTV virions.*

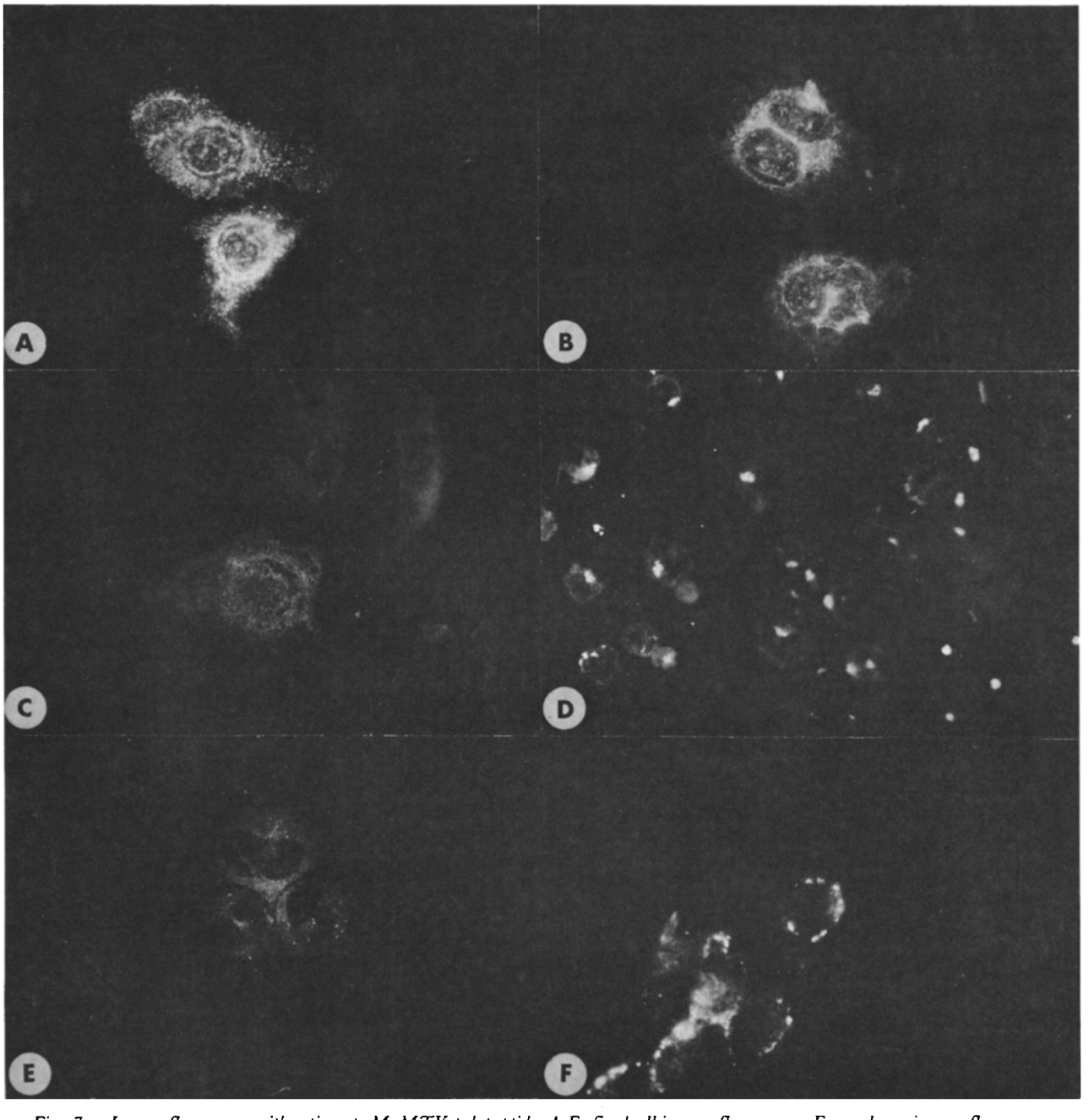

Fig. 7. Immunofluorescence with antisera to MuMTV-polypeptides A-E: fixed cell immunofluorescence; F: membrane immunofluorescence. (A) C3HMT/cl11 with anti-p12; (B) C3HMT/cl11 with anti-p28; (C) C3HMT/cl11 with anti-gp52; (D) L1210 with anti-p28; (E) GRSL18 with anti-gp52; (F) Mm5ml/cI with anti-gp52.

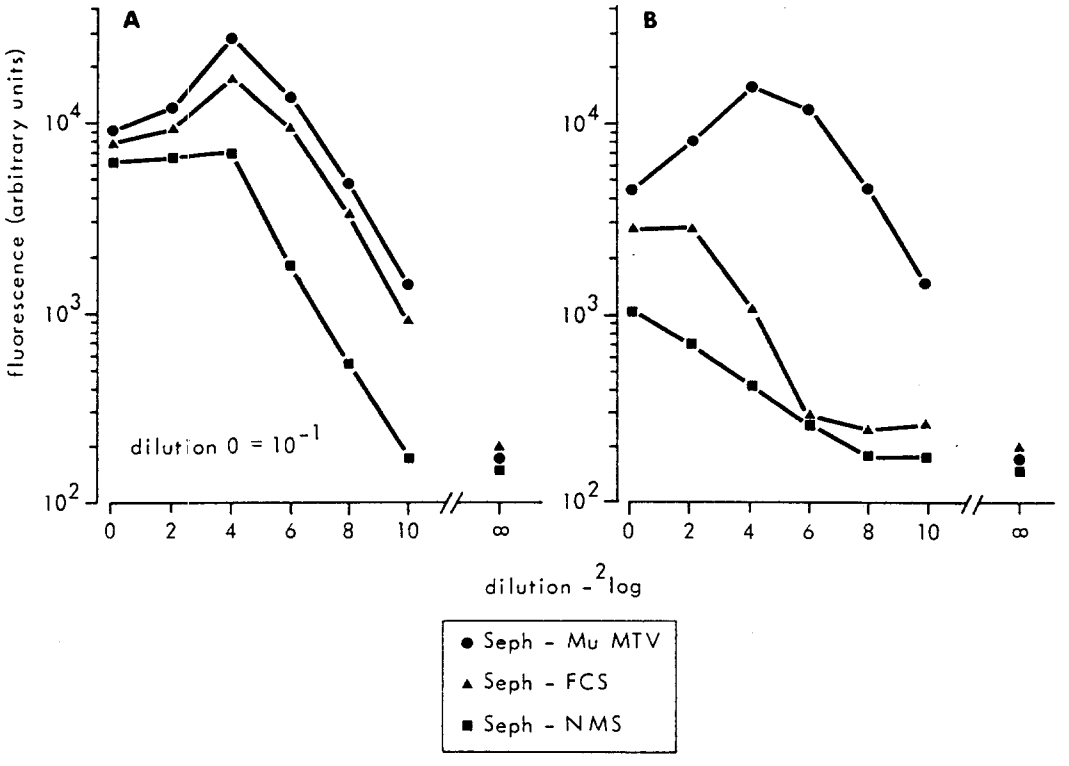

Fig. 6. Sepharose bead immunofluorescence assay. Rabbit anti-gp52 was tested against beads coupled with, respectively, disrupted Mu.MTV, fetal calf serum (FCS) and normal mouse serum (NMS): (A) before absorption with FCS and NMS; (B) after absorption.

the virological status of the cell. Virus specific cytoplasmic immunofluorescence was repeatedly found with all three absorbed antisera in the two mammary tumor cell lines, Mm5mt/c1 and C3HMT/cl11 (Fig. 7 A–C). The reactions were found at dilutions of the antiserum where no activity with FCS was evident in the sepharose bead immunofluorescence assay. No virus specific fluorescence was observed in the mammary tumor line EMT-6 (which does not release MuMTV) or in BALB/3T3 cells infected with MuLV-R. The leukemia lines L1210 and GRSL18, which are known to produce MuMTV antigens, also proved to be positive with all three antisera (Fig. 7 D, E). Remarkably, in these two leukemia lines, immunofluorescence with anti-p28 was mainly confined to a few large bright dots inside the cell (Fig. 7D). The end-point titers for all three antisera on the two leukemia cell lines were considerably lower as compared to the mammary tumor lines (Table 1). In all MuMTV-positive cell lines the antiserum to gp52, which in the radioimmunoassay has the highest titer, gives the weakest fluorescence (Fig. 7C) and the lowest end-point titer (Table 1).

In the membrane immunofluorescence test on all four MuMTV-producing cell lines, a very faint reaction was found with anti-p28 in less than 20% of the cells. A negative reaction was found with anti-p12 and a strong positive one with anti-gp52 (Fig. 7F). The latter antiserum reacted with more than 90% of the cells, when these have been stimulated with dexamethasone. No reaction was found with any of the antisera on the MuMTV-free cell lines EMT-6 and BALB/3T3-RLV.

Cytotoxicity test

The absorbed antisera to p12 and p28 showed no cytotoxic reaction to the MuMTV-positive cell lines Mm5mt/Cl, GRSL18 or L1210. This negative reaction does not seem to be due to anticomplementary activity of the sera, since prior incubation of cells with antisera, subsequent washing and addition of complement did not induce cytotoxicity. Also the indirect cytotoxicity test did not yield positive reactions with either antiserum.

Only the antiserum to gp52 gave positive reactions with Mm5mt/Cl, L1210 and GRSL18 (Fig. 8 A, B, D). The serum did not react with BALB/3T3 cells infected with MuLV-R. When GRSL18 was passaged *in vivo*, a considerably higher cytotoxic reaction

Table 1. Cytoplasmic immunofluorescence with antisera to constituents of murine mammary tumor virus

Cell lines	Immunofluorescence reactivity as expressed in endpoint titers*					
	Mm5mt/C1	C3HMT cl11	GRSL18	L1210	EMT-6	BALB 3T3 +MuLV-R
Antiserum to:						
disrupted virions	5120	10240	640	320	—	—
p12	640	1280	40	40	—	—
p28	320	1280	40	40	—	—
gp52	160	160	20	40	—	—

*No reaction at dilution 20 x.

was found than when cells were cultured *in vitro* (Fig. 8 C, D).

DISCUSSION

The three MuMTV polypeptides p12, p28 and gp52 apparently have retained their antigenic nativity during isolation, since denaturation of virus-containing samples was not needed in various immunoassays using antisera raised against these polypeptides. These antisera showed no substantial cross reactivity in the different immunological tests, indicating the antigenic uniqueness of these three MuMTV constituents. Yagi and Compans [17] suggested that MuMTV virions would contain fragments of proteolytically cleaved major polypeptides. Apparently, p12 and p28 are not breakdown products of gp52, and p12 not of p28.

Immunoelectrophoresis yielded better resolution than did the double immunodiffusion test, since the antiserum to disrupted virions yielded several more precipitation lines in the first technique. The antisera to p12 and gp52 also produced only single precipitation lines in immunoelectrophoresis, but the antiserum to p28 produced two lines when tested with disrupted virus. Since these two lines fuse, it can be concluded that they originate from antigenically related entities. When the antiserum to p28 is tested against purified p28, only one line is formed (not shown). This suggests that the second line at the site of the application well represents either a putative precursor protein of the core proteins or an aggregate of p28 which has lost its electrophoretic mobility under the chosen conditions. A third possibility is that the second precipitation line originates from a degradation product of p28. The results obtained with the immunoprecipitation technique show that p23 in this case is the main candidate. Whether there is a precursor-product relationship between p28 and p23 is not clear at this moment.

All three proteins proved to be present in the cytoplasm of the four tested MuMTV-producing cell lines, as indicated by fixed-cell immunofluorescence with the antisera to the individual proteins. The endpoint titers of the antiserum to gp52 in the fixed cell immunofluorescence assay are consistently low (Table 1), whereas in the radioimmunoassay the titer of this antiserum is the highest. The concentration of gp52, or possibly its precursor [18] in the cytoplasm of infected cells seems therefore to be lower than that of p28 and p12 or their precursor. The differences in endpoint titers in fixed-cell immunofluorescence between the two virus-producing mammary tumor cell lines as compared to the two leukemias are probably due to a lesser content of MuMTV antigens in the leukemias and may be also to antigenic differences among the milkborne standard strain of MuMTV and the endogenous viruses produced by the leukemias of the GR and DBAf mouse strains [19–21]. The large fluorescent dots in the GRSL18 and L1210 lines, produced by anti-p28, are probably due to the large clusters of A-type particles, which are present in these cell lines [22, 23]. It is known that such A-type particles contain antigenic determinants cross-reacting with p28 [24].

By means of membrane immunofluorescence, only gp52 was shown to be present on the cell surface. Lactoperoxidase radioiodination of cultured mammary tumor cells, as performed by Yang *et al.* [25], did also reveal the presence of gp52 on the cell surface, as

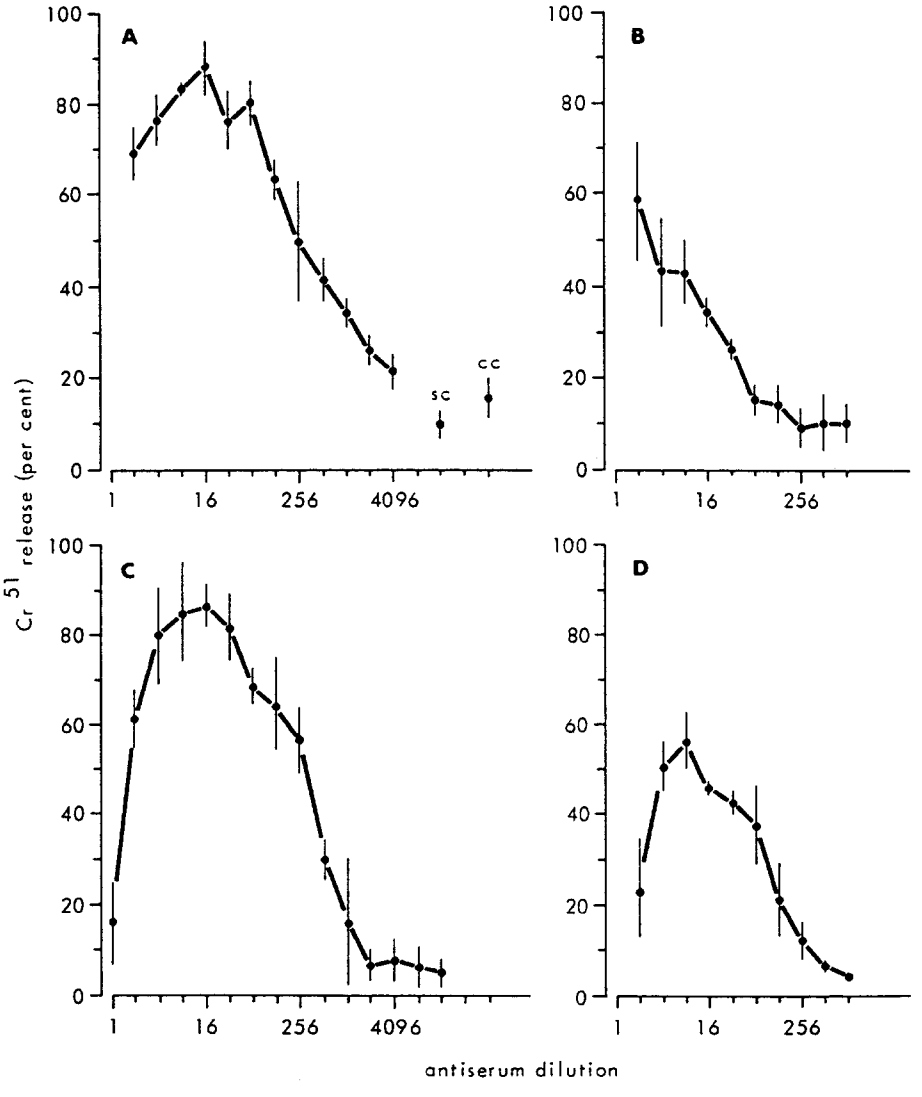

Fig. 8. *Cytotoxicity tests with antiserum to MuMTV-gp*52. (*A*) *Mm5mt/cl;* (*B*) *L*1210; (*C*) *GRSL*18 *cultured* in vivo; (*D*) *GRSL*18 *passaged* in vitro.

determined by immune precipitation and SDS-PAGE analysis; no distinct peaks of smaller MuMTV components were observed. The relative low concentration of gp52-related antigens in the cytoplasm, as compared to the prevalence of this antigen over p12 and p28 on the cell surface, indicates that after synthesis the viral envelope constituents are rapidly transported to the cell surface.

Most likely, the p12 in our studies is comparable to a protein with moll wt of 10,000 in the report by Teramoto *et al.* [26]. In their studies, this p10 is not present in the core, but also not in the envelope projections of the MuMTV virions. We obtained some indications that our p12, like gp52, is not present in the viral core, in contrast to p28. However, p12 as well as gp52 can be found in a preparation of virus membrane fragments. It is hypothesized that p12 is a constituent of the viral envelope, but is covered by the viral glycoproteins.

P12 is not present on the cell-surface of the MuMTV-producing mammary tumor cells as was shown in the membrane immunofluorescence test. The negative reaction with anti-p12 serum in the cytotoxicity test was not due to anticomplementary activity of the serum nor to a sparse distribution of this antigen on the cell surface, as the indirect cytotoxicity test was negative as well.

The faint reaction with anti-p28 in the membrane immunofluorescence test could be interpreted as p28 being an integral part of the cell surface or that a precursor of the core proteins would be localized in the cellular membrane as has been reported for the C-type oncoviruses [27]. It is also possible that

p28 is released by degraded B-type particles and sticks to the cell surface. Yoshiki *et al.* [28] claim, however, that, in analogous leukemia virus systems, the presence of the major core protein p30 is not due to merely passive absorption of free proteins.

An interesting observation is the considerable lesser kill of GRSL18 cells passaged *in vitro* as compared to the ascitic leukemia cells. Obviously, despite the presence of dexamethasone, the culture conditions lead to a lesser expression of gp52 on these cells. The prevalence of gp52 on the cell surface as determined by membrane immunofluorescence, suggest this antigen to be the best candidate for our vaccination studies. This idea is reinforced by the positive cytotoxic reactions with rabbit-anti-gp52 in contrast to the negative results obtained with antisera to the two other viral polypeptides. It remains to be established, however, whether mice are also capable of producing antibodies to gp52 which are cytotoxic to MuMTV-producing cells.

In our laboratory, extensive vaccination experiments in mice have been carried out with a crude MuMTV-protein fraction which was enriched for gp52 [1, 29]. This fraction could elicit antibodies, which react specifically with MuMTV-producing cells in the membrane immunofluorescence test. Some indications were obtained that these antibodies may retard the growth of transplanted MuMTV-positive leukemia cells [1]. However, cellular immunity seems to play the most important role in the growth of transplanted tumors, particularly of mammary neoplasms. Antibodies might even play an unfavorable role by producing factors, which may block cellular immune reaction, by complexing with free viral antigen [30].

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